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Interactions of Bupivacaine with Ionic Channels of the Nicotinic Receptor

Analysis of Single-Channel Currents

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SUMMARY

Bupivacaine and its quaternary derivative, bupivacaine methiodide, were studied on acetylcholine (ACh)-activated single-channel currents recorded in myoballs from neonatal rat muscles using the patch-clamp technique. Under control conditions, the ACh-induced channels had three conductance states, 10, 20, and 33 pS, at a temperature of 10°. The intermediate conductance state (20 pS) was the most prevalent. Moreover, an excessive number of very short events was observed which contributed to a deviation of the channel open-time distribution from a single-exponential function. At 20°, the amplitude of these currents was increased (Q_{10} = 1.4), and the mean channel open time was decreased (Q_{10} = 3). Bupivacaine and its quaternary derivative (5–50 μM), when inside the patch micropipette with ACh, caused shortening of the channel open time, but the single-channel conductance remained unchanged at all concentrations studied. In the presence of bupivacaine, there was a loss of voltage dependence of the mean channel open time seen under control conditions; i.e., the shortening of the channel open time was more pronounced at more negative potentials. The plot of the reciprocal of mean channel open time versus bupivacaine concentration was linear. Similar effects were observed when bupivacaine was added to the bathing medium in both cell-attached and inside-out patch conditions, but in this case the onset of the drug action occurred at a later time and its potency was lower. Application of bupivacaine methiodide via the bathing medium after the establishment of the gigaohm seals, however, had no effect on the kinetics of ACh-activated single channels under both patch conditions (cell-attached and inside-out). The patch-clamp results indicated that the charged form of bupivacaine blocks the open state of ACh-activated ionic channels interacting with sites at the extracellular segment of the ACh receptor-ionic channel complex and creating a species with little or no conductance. A sequential model can be used to explain the interactions of these noncompetitive antagonists of the ACh receptor-ionic channel complex with the open channel. This interpretation of the action of bupivacaine and its quaternary analogue as open channel blockers also was reached based on an analysis of macroscopic events in nicotinic synapses of frog muscle.

INTRODUCTION

In the preceding paper (1) we provided evidence that the local anesthetic bupivacaine blocks the ionic channel of the AChR.[4] It is known that local anesthetics alter the kinetics of ACh-induced conductance changes, thereby producing accelerated and multiphasic EPC decays (2, 3). In contrast, bupivacaine markedly shortened the decay time constant of the EPC and the MEPC without changing the single-exponential nature of the decay phase. Bupivacaine decreased the peak amplitude of EPCs and MEPCs but had negligible effect upon the linearity of the current-voltage relationship. Accordingly, the analysis of the ACh-induced fluctuations in the pres-

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[4] The abbreviations used are: ACh, acetylcholine; AChR, acetylcholine receptor-ionic channel complex; EPC, end-plate current; MEPC, miniature end-plate current; TTX, tetrodotoxin.

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ence of bupivacaine yielded single-component spectra which showed no change in channel conductance but a significant decrease in the mean channel lifetime. Apparently, the effects of bupivacaine on the macroscopic events are consistent with a sequential model in which the drug molecule binds to the activated AChR, creating a blocked state (1–7).

The objective of the present paper was to study the action of this rather novel local anesthetic, routinely used for spinal anesthesia in humans, on the properties of ACh-activated single-channel currents. The direct observation of these currents would allow us to clarify whether the decrease in the peak amplitude and shortening of the decay time constant seen in the macroscopic events could be discerned at the microscopic level. Therefore, we carried out single-channel current studies on myoballs cultured from neonatal rat muscles, using the patch-clamp technique. We also investigated the interaction of the quaternary derivative of bupivacaine to identify the active form of the drug and to determine whether or not there are sites for the local anesthetic binding at the intracellular segment of the AChR macromolecule.

MATERIALS AND METHODS

Cell culture. The procedure for myoball culturing was adapted from that reported by Giller *et al.* (8) for mouse cells and described in detail by Akaike *et al.* (9). Briefly stated, the cells were cultured from hind limb muscles of 1- to 2-day-old neonatal rats [DUB (SD) Dominion Laboratories], and the studies were performed with 1- to 2-week-old cultures. Immediately upon removal of culture dishes from the incubator, the nutrient medium was replaced by a modified Hanks' balanced salt solution with the following composition (millimolar): NaCl, 137; KCl, 5.4; $NaHCO_3$, 4.2; $CaCl_2$, 1.3; $MgSO_4$, 0.81; Na_2HPO_4, 0.34; KH_2PO_4, 0.44; D-glucose, 5.5; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10. The pH of the solution was 7.2, and the osmolarity was adjusted to 340 mOsM with sucrose. TTX, 0.3 μM, was added to this solution to decrease the spontaneous cell contractions.

Single-channel current recordings. ACh-activated channel currents were recorded from myoballs using the improved patch-clamp technique developed by Hamill *et al.* (10). Experiments were carried out at 10° and at room temperature (20–22°). The micropipettes were pulled in two stages from microhematocrit capillary tubes (length = 75 mm; inner diameter = 1.1–1.2 mm) using a vertical electrode puller (David Kopf Instruments). The micropipette tips were heat-polished using a microforge developed in our laboratory, and their shanks were coated with Sylgard. The inner diameter of the micropipette tip was about 1 μm, and the resistance ranged from 2 to 6 Mohm when filled with Hanks' solution. ACh solutions at concentrations varying from 0.02 to 0.3 μM, either alone or combined with bupivacaine (5–50 μM), were used to fill the patch micropipettes. Gigaohm seals (5–20 gigaohm) were achieved by pressing the micropipette against the cell surface and by applying a gentle suction through the micropipette. Patch-clamp recordings were performed either in cell-attached or in cell-free (inside-out) patches. An LM-EPC-5-patch clamp system (List-Electronics, West Germany) was used to measure the single-channel currents. The potential inside the patch micropipette (i.e., exterior of the cell) was clamped to a desired holding potential, which was expressed as an intracellular potential. The single-channel currents were low pass-filtered to 1–3 KHz (second-order Bessel), and the data were stored on FM magnetic tape (Racal, 15 ips, d.c.-5 KHz) for computer analysis. An automated analysis of patch-clamp data developed in our laboratory was performed on a PDP 11/40 (Digital Equipment Corporation, Maynard, Mass.) minicomputer with 28 K words of core memory. The data were sent to the computer through a fourth-order Butterworth (low-pass) filter (1–3 KHz) to improve the signal-to-noise ratio and digitized at 2 KHz by an LPS-11 (Digital Equipment Corporation) 12-bit analogue-to-digital converter. The analysis provided the histograms of the current amplitudes and the channel open times. The method for obtaining open times is described in detail in an earlier publication (9). Briefly stated, each file was divided into records of 2048 points, and the baseline was determined by finding the first local maximum in the number of zero crossings. A channel was considered open when a datum point exceeded a set number of standard deviations from the baseline. Points in the record then were scanned until the signal returned to within a given number of standard deviations from the baseline. The number of standard deviations was chosen to represent about 50% of the unitary conductance. This was considered a channel closure. It is

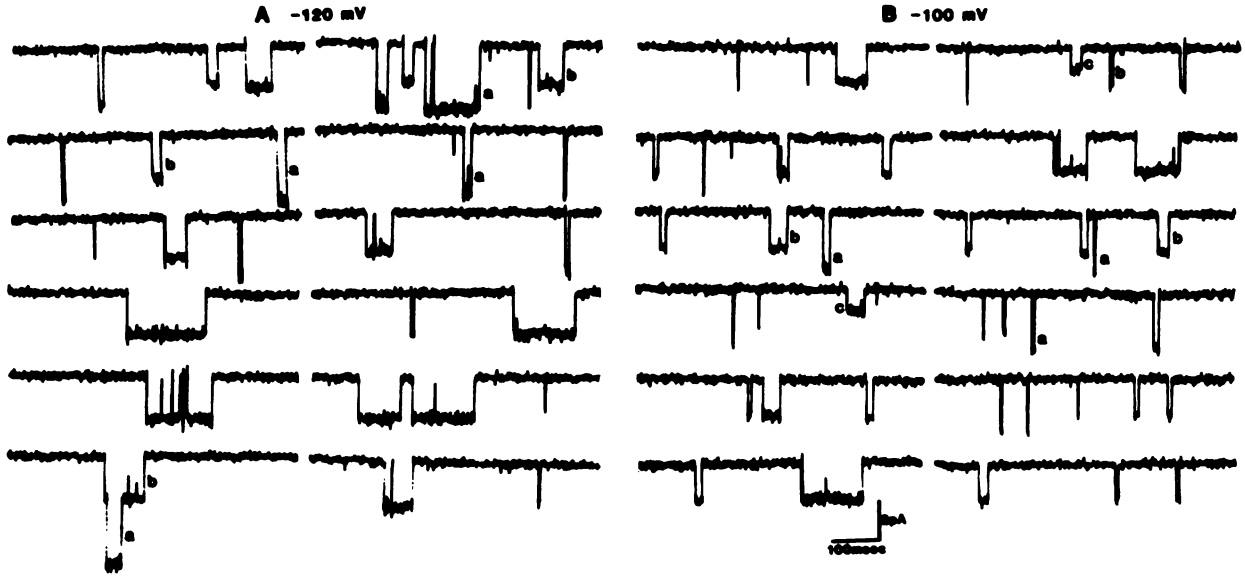

FIG. 1. *Samples of ACh-activated single-channel currents recorded at 10°*
The recordings were obtained under inside-out conditions; the micropipette contained 0.2 μM ACh. Holding potentials were −120 mV (A) and −100 mV (B). *a*, *b*, and *c* represent samples of channel-opening events with larger, intermediate, and smaller current amplitudes, respectively. Bandwidth = 1 KHz.

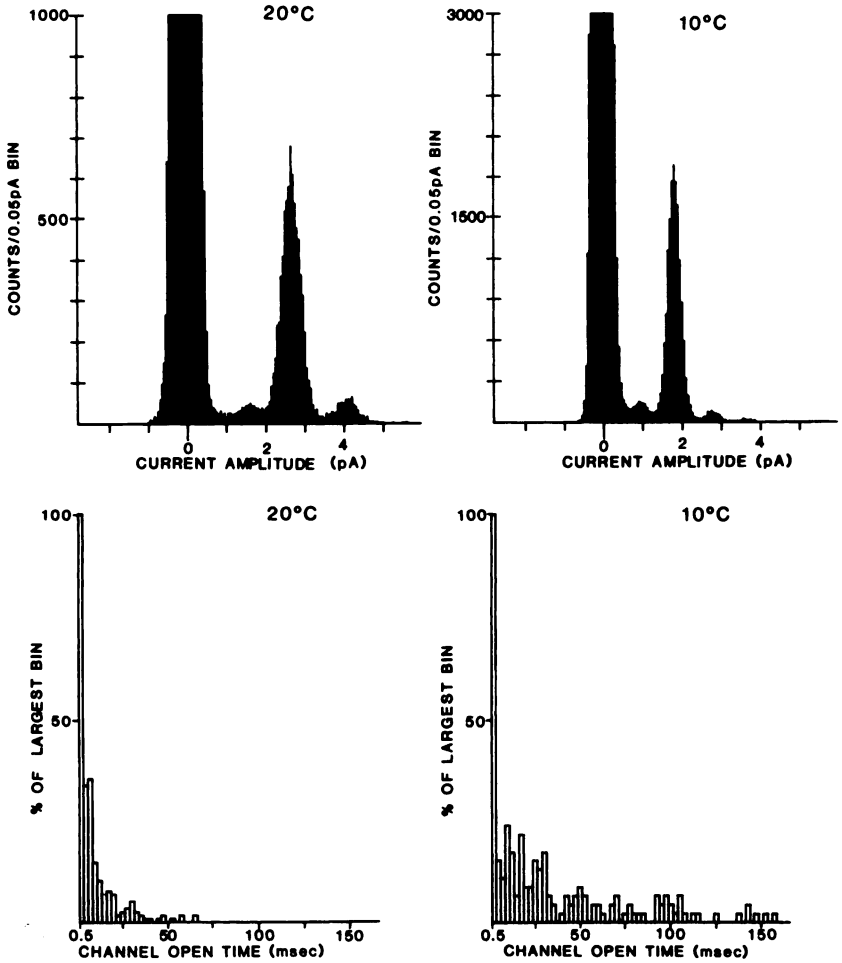

FIG. 2. *Total-amplitude (upper) and channel open-time (bottom) histograms of ACh-activated channel currents recorded at temperatures of 10° and 20°*

The micropipette contained 0.2 μM ACh, and the channel currents were recorded from inside-out patches at holding potentials of −100 mV for the left and −80 mV for the right histograms. In the amplitude histograms, the abcissa shows the current amplitude in pamp, converted from the difference between each point of the digitized signal and the baseline, and binned in fixed 0.05-pamp bins. The largest peak, centered in 0 pamp, represents the baseline or the noise of the closed-channel state. The location of the subsequent peaks on the abcissa reveals the current amplitude of the smaller, intermediate, and larger events, respectively. The channel open-time histograms refer only to the events with an intermediate current level. The average channel open times, estimated from the arithmetic mean, were 36.99 msec and 8.84 msec at 10° and 20°, respectively.

important to note that a "flicker" within an open channel, i.e., a short-duration transition from the open to closed state and back, terminated the open-channel event if the flicker reached the closing threshold. Thus, a long channel ("burst") could be broken up into several adjacent shorter channels by flickers. The maximal point within the interval between an opening and closing was then determined. If this value exceeded a given number of standard deviations above the current amplitude (as would be the case for a multiple-channel opening), the lifetime data for this event (the time between the opening and closing) were discarded. Otherwise, the lifetime data were stored in an array for later analysis. The average channel open time was determined either from the arithmetic mean of channel lifetimes or by taking the reciprocal of the slope of the regression line assuming a single-exponential distribution. In general, there was a very good agreement between these two determinations, except in case of channel open-time histograms obtained at 10° under control condition which showed a clear deviation from the single-exponential distribution.

Drug solutions. ACh chloride (crystalline salt, Sigma), bupivacaine hydrochloride (Sterling-Winthrop, Lot XBO-058), and bupivacaine methiodide (1) were used to prepare the stock solutions, which were diluted to desired concentrations with Hanks' solution containing TTX and passed through a Millipore filter (0.2 μm) prior to the experiments.

RESULTS

ACh-activated ionic channels in the absence of bupivacaine. Single channels activated by ACh contained in the patch micropipettes (0.02–0.3 μM) were recorded either from cell-attached or inside-out patches. Once the gigaohm seal was established, the currents flowing through individual channels activated by the agonist appeared as downward rectangular pulses (Fig. 1). When the frequency of channel-opening events was high, many individual currents superimposed in a stepwise fashion could be seen. In some cell cultures, large variations in the frequency of channel-opening events have been observed for a given concentration of ACh, probably resulting from the heterogeneity in the density or distribution of the AChRs in the rat myoballs. Moreover, consonant with a previous report (9), we found that, for

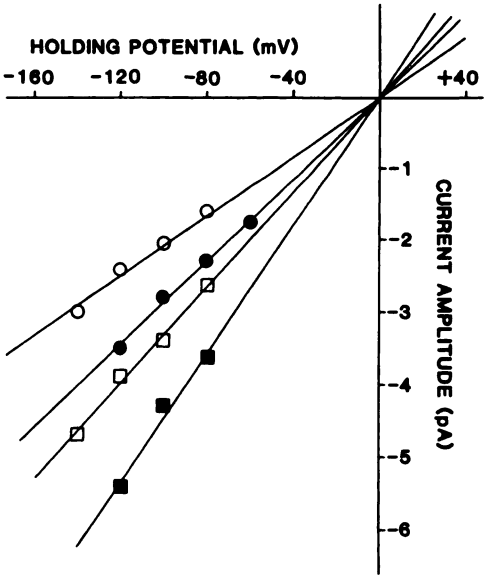

FIG. 3. *Current-voltage relationship of ACh-activated single-channel currents*

The single-channel currents were recorded from inside-out patches at 10° (○, □) and 20° (●, ■). The micropipette contained 0.2 μM ACh. Events with intermediate (○, ●) and larger (□, ■) current amplitudes were represented. Abcissa, holding potential; ordinate, amplitude of single-channel currents estimated from the total-amplitude histograms.

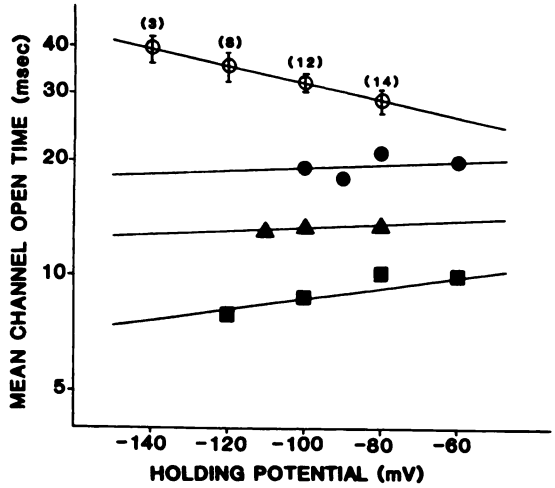

FIG. 4. *Concentration-dependent effect of bupivacaine on mean channel open time at various holding potentials*

Gigaohm seals were established with micropipettes filled with ACh alone (○) and ACh (0.2 μM) combined with bupivacaine, 5 μM (●), 10 μM (▲), or 20 μM (■) either in cell-attached or inside-out patches. Abcissa, holding potential; ordinate, mean channel open time. The numbers in parentheses, under control conditions, represent numbers of different patches at a given potential. Temperature 10°.

a given membrane patch and agonist concentration, the frequency of opening events increased with hyperpolarization.

In many records, either under cell-attached or inside-out patch conditions, channel openings with three different conductance states have been seen. The intermediate current level was the most prevalent and made up nearly 90% of the total recorded events. The larger and the smaller amplitude currents occurred either separately or superimposed upon the intermediate current level (Fig. 1). The total amplitude histograms obtained from inside-out records at −100 mV holding potential and temperature of 20° provided values of 4.2, 2.7, and 1.6 pamp for the amplitude of the larger, intermediate, and smaller current levels, respectively (Fig. 2). At 10° and −80 mV holding potential, the amplitudes of these currents were reduced to 2.7, 1.8, and 0.9 pamp. Figure 3 shows that the amplitude of both intermediate and larger channel currents is linearly dependent on voltage. The extrapolated reversal potential was close to 0 mV, and the slopes of these plots provided the ACh channel conductance. At 10° the conductances of the intermediate and largest events were 20.0 and 33.3 pS, respectively; at 20° the conductances of these events increased to 28.5 and 45.5 pS, therefore giving a Q_{10} of 1.4. The infrequent appearance of the smallest events prevented the precise determination of the conductance, but its value was about 10 pS at 10°. All of these conductance states of ACh-induced channel openings were abolished when the preparation was pretreated with a specific competitive blocker of the ACh receptor, α-bungarotoxin.

The same records seen in the Fig. 1 show a significant number of fast (<2 msec) events. In fact, at low temperature (10°), the distribution of the channel open times displayed a clear departure from the single-exponential function as seen in the histograms presented in the Fig. 2. At 20°, the channel open times were shortened and approached a single-exponential distribution (correlation coefficient ≃ 0.935). For example, at −120 mV holding potential, the arithmetic means of channel open times were 37.5 msec and 12.1 msec, respectively, at 10° and 20°, yielding a Q_{10} of 3.1, which is close to the value previously reported for EPC decay time constant (2, 11, 12). Figure 4 shows that the mean open time of ACh-activated channels depends exponentially on the membrane potential. Therefore, the rate constant for channel closing (k_{-2}; see the previous paper (1) for a schematic representation of AChR activation) is voltage-dependent, as shown in *inset A* of Fig. 7. At holding potentials of −80 and −120 mV, the values of k_{-2} were 34.7 sec^{-1} and 28.4 sec^{-1}, respectively, which gives an *e*-fold change per 200 mV. This voltage-dependency of k_{-2} is less marked than that observed in chronically denervated adult frog muscles (see ref. 5 in the preceding paper (ref. 1)), where k_{-2} shows an *e*-fold change per 85 mV.

Effect of bupivacaine applied with ACh through the micropipette. Single-channel currents were recorded at a temperature of 10° with a patch micropipette filled with ACh (0.2 μM) and bupivacaine (5–50 μM). Under this condition, bupivacaine caused a concentration-dependent shortening of the channel open time, without changing the single-channel current amplitude (Fig. 5). In contrast to observations with other local anesthetics, the abbreviation of the channel open times occurred without "bursting" (4). At −100 mV holding potential, bupivacaine (5, 10, 20, and 50 μM) decreased the mean channel open time to 50, 40, 25, and 10%, respectively, of the control values (see Figs. 4 and 6). As observed with the macroscopic events (1), in the presence of bupivacaine there was a loss of the voltage dependence of the mean

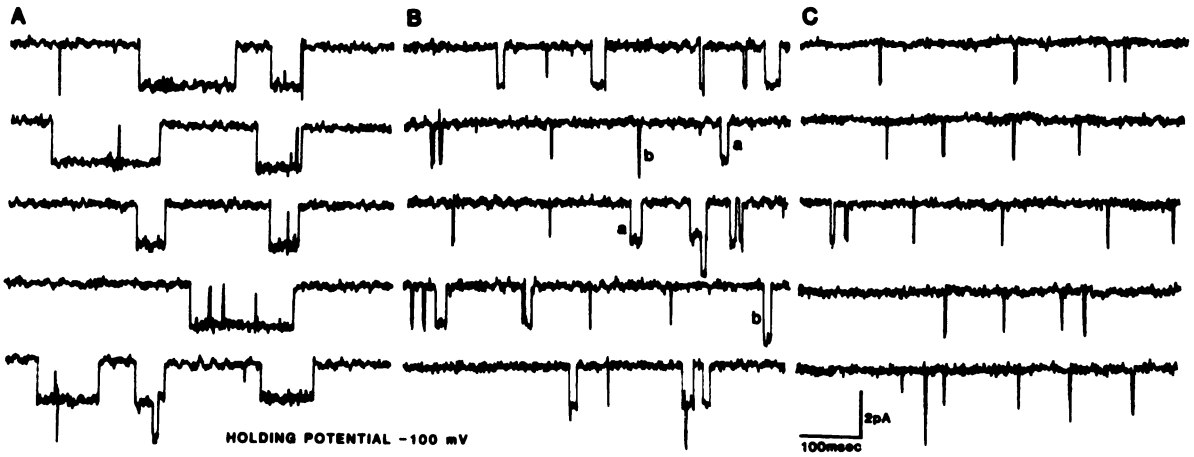

FIG. 5. *Samples of ACh-activated single-channel currents recorded in the presence of bupivacaine*
Single-channel currents were recorded from inside-out (A and B) and cell-attached (C) patches with micropipettes containing 0.2 μM ACh (A) or 0.2 μM ACh combined with bupivacaine, 10 μM (B) or 50 μM (C). *a* and *b* in B represent samples of intermediate and larger current levels, respectively.

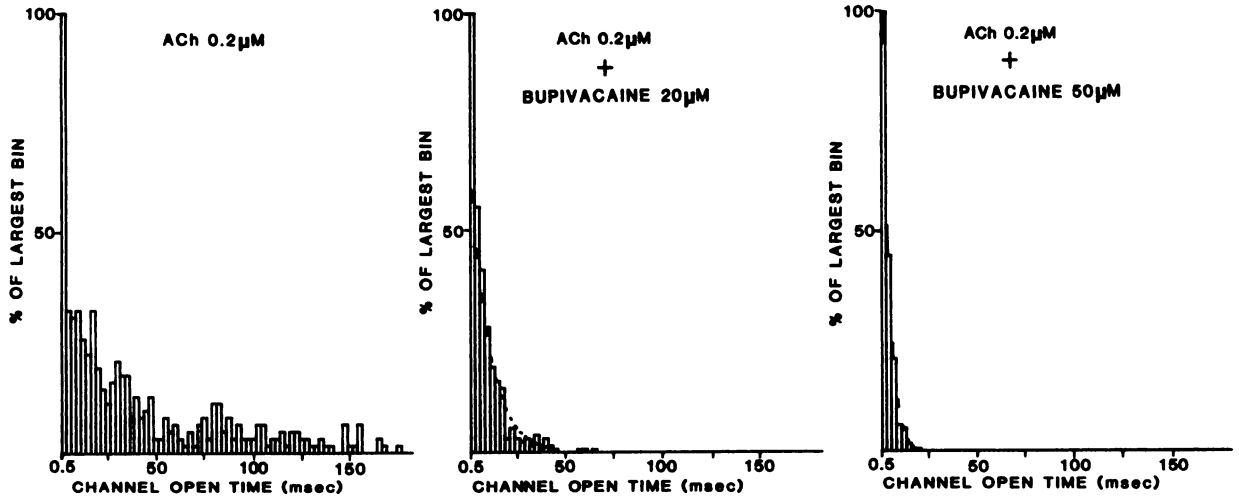

FIG. 6. *Open-time histograms of ACh-activated single channels in the absence and in the presence of bupivacaine*
The histograms represent channel currents recorded from inside-out patches with pipettes containing 0.2 μM ACh alone (*left*) and 0.2 μM ACh combined with bupivacaine, 20 μM (*middle*) or 50 μM (*right*). The holding potential was −100 mV. The averages of channel open time, estimated from the arithmetic mean of the total events analyzed, were 34.62, 8.87, and 4.0 msec for control and for 20 μM and 50 μM bupivacaine, respectively.

channel open time seen under control conditions. The shortening of the channel open time was more pronounced at more hyperpolarized potentials, such as at holding potentials of −80 mV and −120 mV, where bupivacaine (20 μM) decreased the mean channel open time to 32.4% and 23.3% of the control values, respectively (Fig. 4). The histograms of channel open times could be fitted to a single-exponential function (correlation coefficient of 0.92–0.995), as opposed to a more complex distribution seen under control conditions (Fig. 6). The plot of reciprocal of mean channel open time versus concentration of bupivacaine showed a linear relationship (Fig. 7). The slopes of these plots obtained at different potentials provided an estimate of the forward rate constant for the blocking reaction (k_3), which is exponentially dependent on the voltage (Fig. 7, *inset B*). The values of k_3 at −60 and −120 mV holding potentials were 3.6×10^6 and 4.75×10^6 sec^{-1} M^{-1}. From the voltage dependence of k_3 (1, 13), one can estimate that bupivacaine senses about 17.5% of the membrane potential at its rate-limiting energy barrier. The frequency of opening events gradually decreased in the presence of bupivacaine (Table 1). Although a marked shortening of the channel open times was seen in the presence of a high concentration (50 μM) of bupivacaine, single-channel conductance remained unaltered. As shown in Fig. 8, in the presence of bupivacaine the current-voltage relationship remained linear, and the reversal potential was at 0 mV.

Effect of bupivacaine application via the bathing solution. After the gigaohm seal was established with a micropipette containing only ACh, myoballs were exposed to bupivacaine in a concentration range of 12.5–100 μM. Under this condition, the effects of bupivacaine were qualitatively similar to those seen when the drug was applied together with the agonist through the patch

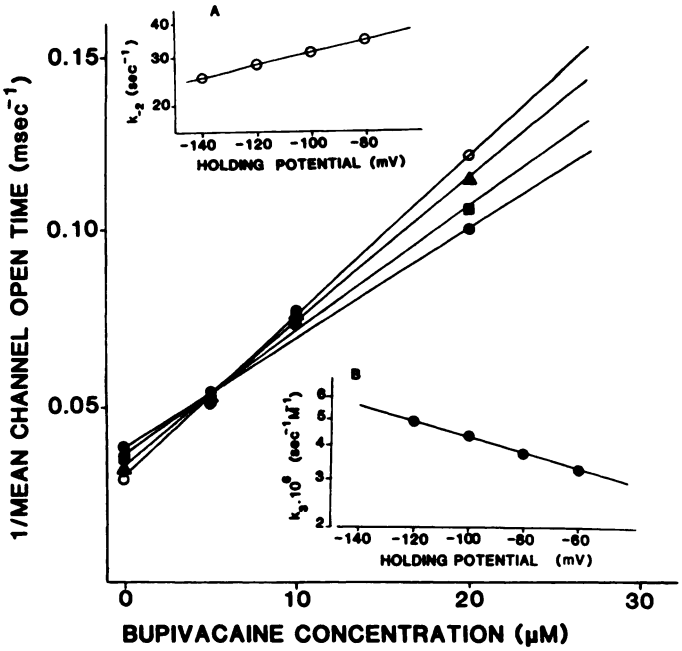

FIG. 7. *Reciprocal of mean channel open time versus bupivacaine concentration at various holding potentials.*

Holding potentials were −60 mV (●), − 80 mV (■), −100 mV (▲), and −120 mV (○). *Insets* represent the voltage dependence of the rate constant for channel closing (k_{-2}), under control conditions (A), and the voltage dependence of the rate constant for blocking reaction (k_3) in the presence of 5–20 μM bupivacaine (B).

micropipettes. However, in both cell-attached and inside-out patches, the onset of bupivacaine action was slower, and after 60 min of drug superfusion the decrease in channel open time was less pronounced. Figure 9 illustrates the concentration-dependent effect of bupivacaine on the mean channel open time. After 60 min of exposure to 12.5 μM bupivacaine, the mean channel open time decreased by 30%, whereas at 100 μM the mean channel open time was shortened by 80% of the control value.The "flickers" observed in some recordings under control conditions tended to disappear in the presence of bupivacaine, as seen in the records shown in the Fig. 10. The frequency of opening events was progressively decreased by the drug (Table 1), whereas the conductance of the single channels remained unchanged at all concentrations tested.

Effect of the quaternary derivative of bupivacaine on the ACh-activated channels. Whether the location of the bupivacaine binding site on the AChR is at the extracellular or intracellular surface of the cell membrane can be revealed more clearly with bupivacaine methiodide, a quaternary derivative with a permanent charge (see *inset*, Fig. 11). Moreover, this derivative would allow us to determine whether the charged or uncharged form of bupivacaine is responsible for the interactions with the sites at the AChR. Indeed, the superfusion of bupivacaine methiodide into the bathing solutions after the establishment of the gigaohm seals did not affect the AChR either when the agent was applied from the extracellular (cell-attached patch) or cytoplasmic (inside-out patch) face of the membrane. Even at a concentration of 100 μM, this compound neither changed the mean channel lifetime nor the conductance of the single channel. However, an immediate and marked shortening of the channel open times was seen when this drug was applied together with ACh inside the patch micropipette. As shown in Fig. 11, bupivacaine methiodide, at concentrations of 20 μM and 50 μM and at a holding potential −120 mV, decreased the mean channel open time to 35% and 10% of the control values, respectively, immediately after the gigaohm seal was achieved. The channel open times displayed the same voltage dependence as seen with the tertiary analogue; i.e., the shortening of the mean channel lifetime was more pronounced at more hyperpolarized potentials. The concentration-dependent decrease in mean channel open time is illustrated in the records displayed in Fig. 12. As observed with the tertiary compound, the shortening of the open state of ACh channels occurred without any flickering activity. The frequency of channel openings was decreased progressively with time of exposure to the drug (Table 1). As observed with the parent analogue, the conductance of the single channels was not altered by bupivacaine methiodide.

DISCUSSION

The myoballs from neonatal rats used for patch-clamp studies responded to ACh (0.02–0.3 μM) by activation of channels to yield different open states. An excessive

TABLE 1

Effect of bupivacaine and bupivacaine methiodide on the frequency of ACh-activated channel opening events

The frequency in A was expressed as the percentage of the initial frequency (1st min after establishment of the gigaohm seal) and in B as the percentage of the frequency under control conditions (i.e., with only ACh).

Conditions	% of initial frequency at drug concentrations of			
	10 μM	20 μM	50 μM	100 μM
A. Drug inside the micropipette[a]				
Bupivacaine	35% (40 min)[b]	22% (20 min)	10% (20 min)	
Bupivacaine methiodide	40% (40 min)	20% (20 min)	20% (10 min)	
B. Drug in bath superfusion				
Bupivacaine	—	—	—	35% (30 min)[c] 15% (60 min)

[a] ACh, 0.2 μM, combined with the drug inside the patch micropipettes.
[b] Time after establishment of the gigaohm seals.
[c] Time after starting superfusion of the drug.

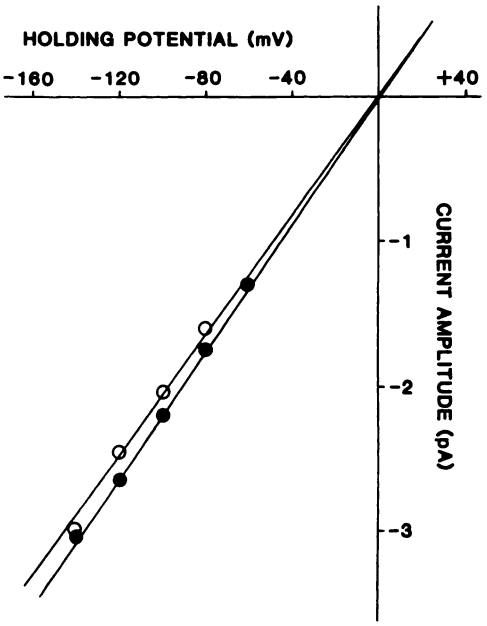

FIG. 8. *Current-voltage relationship of ACh-activated channel currents in the absence and in the presence of bupivacaine*

Single-channel currents were recorded from inside-out patches with pipettes filled with 0.2 μM ACh alone (O) and combined with 20 μM bupivacaine (●). Abcissa, holding potential; ordinate, channel current amplitude.

number of fast channel opening events (<2 msec) recorded at 10° and at a bandwidth of 1 KHz appears to account for the deviation of the channel open time distribution from the single-exponential function. Similar findings have been reported for several preparations, such as rat muscle cells (14), human muscle cells (15) in culture, and also for reconstituted AChR in lipid bilayers (16). It is difficult to discern whether these fast events seen under control conditions are related to a distinct population of channels with different kinetic properties or to a distinct open state of the same channel. At 20° the mean channel open time is abbreviated by almost 3 times in comparison to the values obtained at 10°. At the higher temperature (20°) the distribution of the channel open times approached a single exponential, probably because of a loss of detection of the very fast events at the same bandwidth filtering. In addition, single-channel current recordings showed that ACh-activated channels open with three different conductances. The events with intermediate conductance (20 pS, at 10°) were the most prevalent. In a previous report, Hamill and Sakmann (17) observed the low conductance state (~10pS) only after the opening of the channels with larger conductance. In our records, these three different current levels occurred either as independent events or superimposed in all possible combinations. Our findings are similar to those reported by Akaike *et al.* (9) for ACh-activated channels as well as by Trautmann (18), who used curare as an agonist. In frog adult muscle fibers, patch-clamp studies have revealed only two conductance states (20 and 32 pS) for ACh-activated channels.[5] The rare occurrence of the lowest and highest conducting states (10 and 33.3 pS, at 10°) precluded a systematic analysis of the significance of their appearance or of the functional properties of the channels. One could hypothesize that, at the molecular level, the subunits comprising the AChR could rearrange to yield different open states depending on factors, such as composition and organization of the lipid membrane. An alternative hypothesis based on the frequent appearance of either the larger or of the smaller current levels superimposed upon the prevalent intermediate current level could be that these events reflect different states of conductance of the same channel. An additional question is how these various conductance states are correlated to the different kinetics of channel activation which produce a complex distribution of the channel open times. We have observed that, in compar-

[5] C. N. Allen, A. Akaike, and E. X. Albuquerque, unpublished results.

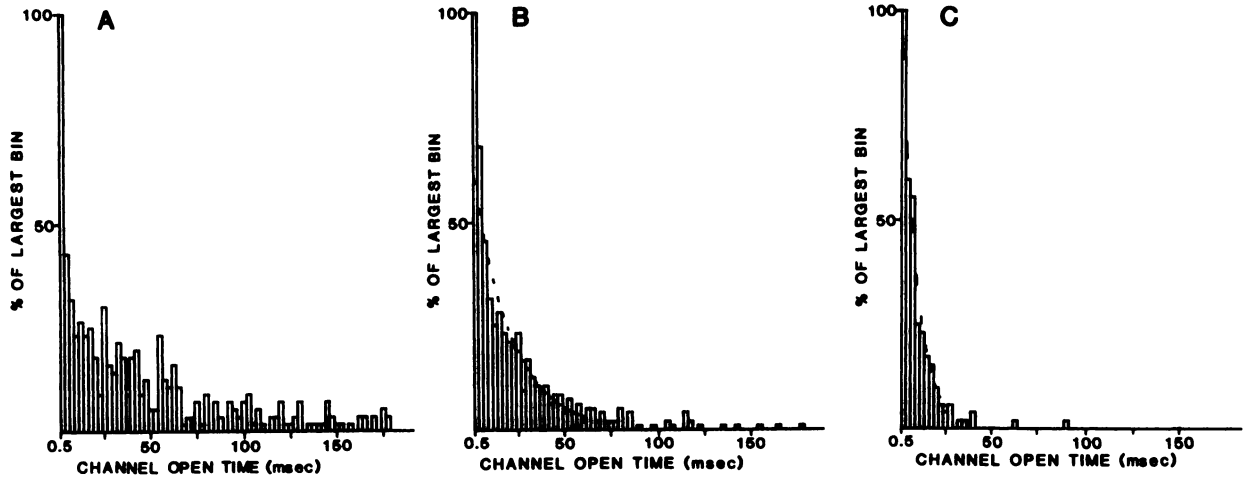

FIG. 9. *Channel open-time histograms of ACh-activated channels in the absence and in the presence of bupivacaine applied via bathing solution*

Histograms were obtained from single-channel currents recorded with micropipettes containing 0.1 μM ACh before (A) and after 60-min exposure to bupivacaine, 12.5 μM (B) and 100 μM (C). The mean channel open times estimated from the arithmetic mean were 48.63 msec (A), 23.88 msec (B), and 9.05 msec (C). A and C represent channel currents from inside-out patches and B from a cell-attached patch. The holding potential was −120 mV.

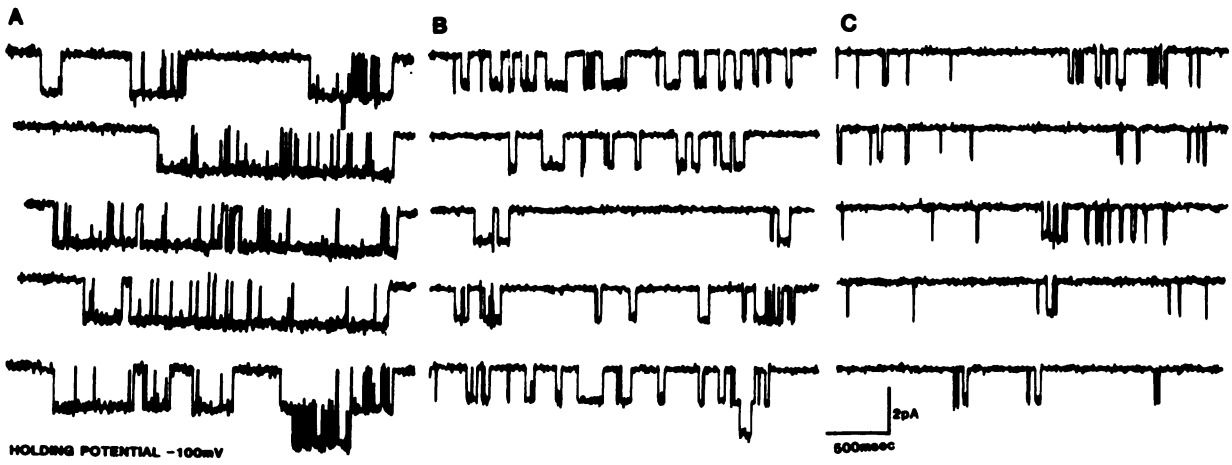

FIG. 10. *Samples of ACh-activated single-channel currents recorded in the absence and in the presence of bupivacaine applied through the bathing medium*

Single-channel currents were recorded from cell-attached patches with pipettes containing 0.05 μM ACh before (A) and after exposure of the myoballs to 100 μM bupivacaine for 20 min (B) and 40 min (C).

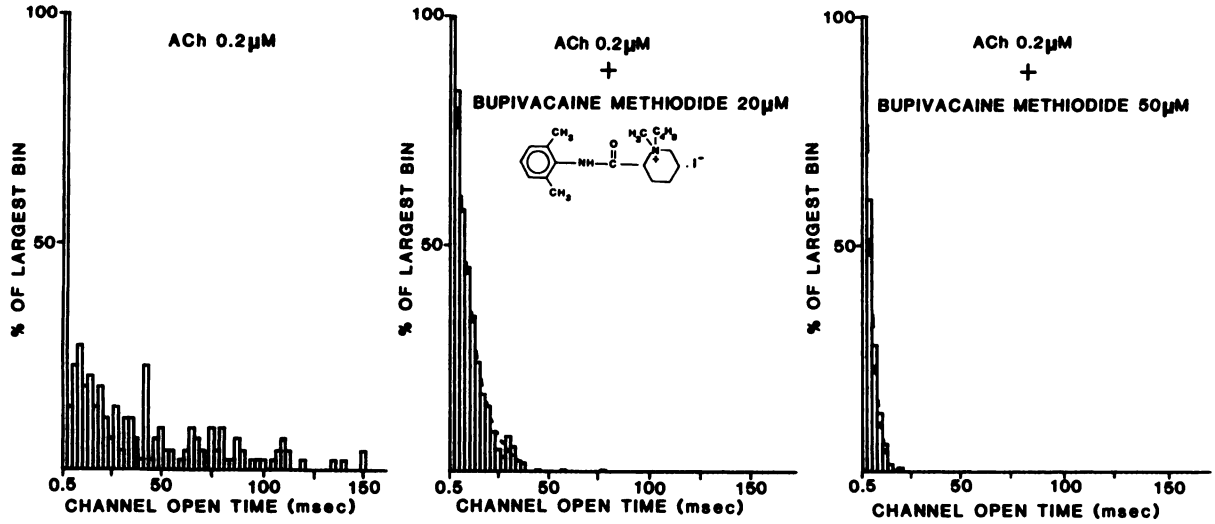

FIG. 11. *Channel open-time histograms in the absence and in the presence of a quaternary derivative, bupivacaine methiodide.*

Histograms represent single-channel currents recorded from cell-attached patches with micropipettes filled with 0.2 μM ACh alone (*left*) and 0.2 μM ACh combined with bupivacaine methiodide, 20 μM (*middle*) or 50 μM (*right*). The averages of channel open times estimated from the arithmetic mean were 40.38 msec (control) at a holding potential of −100 mV and 9.26 msec and 3.6 msec (20 μM and 50 μM bupivacaine methiodide, respectively) at a holding potential of −120 mV. *Inset*, Chemical structure of bupivacaine methiodide.

ison to the intermediate current level, the highest conductance state seems to have shorter lifetime. For example, in one experiment in which the number of larger events was frequent enough to be analyzed, at a −140 mV holding potential and a temperature of 10°, for the events with current amplitude of 2.7 and 4.2 pamp the mean channel open times were 32.8 and 18.3 msec, respectively. All of these questions could be addressed more precisely if a given patch of membrane contained only one active AChR. Such a goal perhaps can be achieved by recording a single channel in reconstituted membranes (16).

Patch-clamp studies demonstrated that bupivacaine abbreviates the open state of ACh-activated channels without changing the single-channel conductance. The distribution of the channel open times approximated a single exponential, in contrast to that seen under control conditions at 10° (Fig. 6). The flickers that appeared with agents such as pyridostigmine (9) and other local anesthetics (e.g., QX-222) were not observed. Instead, widely spaced short opening events appeared in the presence of bupivacaine. In the sequential model (3–6) shown in the accompanying paper (1), the rate constant (k_{-3}) for the unblocking reaction may be very small, and consequently the channels are kept in the blocked state for long periods. These blocked periods between short opening events cannot be distinguished from those in which the channels are in the closed state. If one assumes $k_3 \gg k_{-3}$ in the presence of bupivacaine, the channel open time is given by $1/(k_{-2} + [D]\, k_3)$, and the reciprocal of channel open times is expected to be linearly related to the drug concentration. Such a linear relationship is seen in Fig. 7 for bupivacaine concentrations up to 20 μM. These findings are in agreement with those of Ikeda *et al.* (1), where the action of bupivacaine on the EPC and MEPC decays were adequately described by a single-

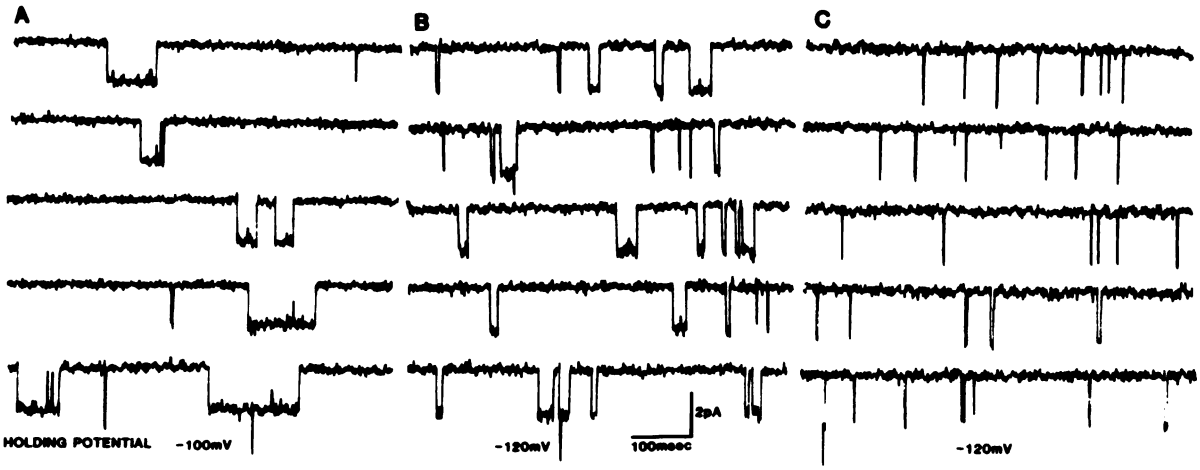

FIG. 12. *Traces of ACh-activated channel currents recorded in the absence and in the presence of bupivacaine methiodide*
Records of single-channel currents from cell-attached patches with pipettes containing 0.2 μM ACh alone (A) and combined with bupivacaine methiodide, 10 μM (B) and 50 μM (C).

exponential function. In contrast, QX-222, which induces bursting activity during the single-channel open state, yields multiple exponentials in the decay of macroscopic currents (2–4). In some records, when flickers were seen under control conditions, application of bupivacaine caused the disappearance of flickering, and only events with short open times were discerned. If this flickering-like activity is related to fast transitions between the closed and open states of ACh channels during a single occupancy of the agonist receptor (19), the disappearance of these flickers could result from the abbreviation of the open state of AChR in the presence of the anesthetic. In addition to the effects on the channel open time, bupivacaine decreased the frequency of channel-opening events in a concentration-dependent manner. This decrease could result either from the blockade of the open channel or from the interactions of bupivacaine with sites in the closed state (i.e., prior to the channel opening). The latter possibility is not very likely in light of the findings that, in contrast to bupivacaine, drugs such as meproadifen, which act mainly on the channel before opening, caused a marked decrease in the frequency of channel opening without changing either the mean channel open time or the single-channel conductance (20). Moreover, these drugs cause an increase in the affinity of the agonist for its binding site and at the macroscopic level produce a significant time- and voltage-dependent depression of the peak EPC amplitude (21), effects which were not observed in the presence of bupivacaine (1). It also could be argued that some of the decrease in channel frequency observed in the presence of bupivacaine could be accounted for by an abbreviation of the channel lifetimes to a point where they cannot be detected because of the limits imposed on the recording system by the filtering bandwidth of 1–3 KHz. However, bupivacaine caused an immediate shortening of the channel open times which remained constant during prolonged exposure to the drug while the frequency of channel opening continued to decline over the same period. It seems more likely that the decrease in frequency of channel opening events is due to a prolonged blockade of the open channel.

The selective application of agents to either side of the cell membrane, as allowed by the patch-clamp technique (10, 22), provided a definitive answer to the question concerning the location of the binding sites and the drug access to these sites. In comparison to bath application, an immediate and more marked effect on the properties of ACh-activated channels was seen when bupivacaine, combined with ACh inside the micropipette, was in direct contact with the extracellular surface of the myoballs, suggesting an externally located binding site for this anesthetic at the AChR. Indeed, the analysis of the influence of the voltage on the forward rate constant (k_3) of the blocking reaction allows an estimation of the apparent location of the bupivacaine binding site. In rat myoballs, bupivacaine senses about 17.5% of the membrane potential at its rate-limiting energy barrier. In frog neuromuscular junction, a maximal value of 11% was obtained from the macroscopic event measurements (1), which may not be discrepant considering the number of transformations required to obtain this estimate and the fact that two different biological preparations were used. A similar superficial binding site has been proposed for drugs such as phencyclidine methiodide and piperocaine methiodide (23), and gephyrotoxin (24). In contrast, the binding site for the local anesthetics procaine and QX-222 seems to be more deeply located at the AChR (4, 5). The delay in the appearance of the effects and the reduced potency of bupivacaine seen when this agent was superfused via a bathing medium suggest the existence of some barrier for the drug access. Most likely, diffusion through the lipid phase of the cell membrane accounts for this behavior, since passage through the pipette-membrane gigaohm seal is improbable (9, 10), and diffusion through the ACh-activated channels may be limited by the size of the drug molecules and other factors (25). Bupivacaine has a pK_a of 8.1. Thus, protonated bupivacaine, the predominant form at pH 7.2 in which the experiments were done, would appear to be responsible for the drug action at the nicotinic AChR. This was

borne out by the present patch-clamp studies using the quaternary derivative (bupivacaine methiodide). Modification of the kinetics of ACh-activated ionic channels occurred only when the drug was applied to the extracellular segment of the membrane, inside the patch micropipette (Figs. 11 and 12). The superfusion of this quaternary compound in both cell-attached and inside-out patches after the establishment of the gigaohm seals had no effect on the properties of ACh-activated channels. Thus, in contrast to another quaternary compound such as pyridostigmine, which has significant effects on the AChR under any condition of drug application (9), the quaternary derivative of bupivacaine and most likely the protonated form of the tertiary parent anesthetic have limited or no access to its binding site at the nicotinic AChR through the cell membrane. A reduced effect of the tertiary anesthetic bupivacaine, seen when the drug was superfused in the bathing solution in both cell-attached and inside-out patches, undoubtedly results from the ability of its uncharged form to diffuse through the cell membrane. The results suggest that, at the cytoplasmic (internal) portion of the AChR, there are no sites for bupivacaine interactions, since the kinetics of ACh-activated channels were not affected when bupivacaine methiodide was applied from the intracellular face of the cell membrane, under the inside-out patch condition. Similar findings using voltage clamping and internal perfusion of QX-314 have been reported (26).

In summary, patch-clamp studies of the AChR on rat myoballs have shown that the nicotinic AChR has various conductance states and those events with about 20 pS conductance were the most predominant; moreover, the histograms of open times displayed an excessive number of very fast events, which contribute to the deviation from a single-exponential distribution. The results of studies using bupivacaine and bupivacaine methiodide indicate that the charged form is responsible for the interactions with a site of the AChR located close to the external surface of the membrane. Finally, the results indicate that bupivacaine blocks the ACh-activated ionic channels primarily in its open conformation, producing a species with little or no conductance.

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