Anesthesiology
68:563–570, 1988

Ketamine Decreases the Open Time of Single-channel Currents Activated by Acetylcholine

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Patch clamp techniques have been used to study the effects of ketamine on single-channel currents activated by acetylcholine from cell-attached patches of BC_3H1 mouse tumor cells grown in culture. Ketamine decreased the average lifetime of the channels, although its effects were not consistent with a sequential blocking model in which molecules of drug bind to the open channel to occlude it. The reduction in channel lifetime produced by ketamine was dose dependent and occurred at clinically relevant concentrations. At 3 μM, which is the plasma level attained after an intravenous dose of 2 mg/kg, average channel lifetime should be reduced by about 17%. This finding may help to explain clinical reports that ketamine can potentiate neuromuscular block produced by vecuronium or *d*-tubocurarine. In addition, similar effects on transmitter-activated channels in the central nervous system may underlie some of the clinical properties of ketamine. (Key words: Acetylcholine, channels: ketamine. Anesthetics, intravenous: ketamine. Neurophysiology: patch clamping; single-channel recording.)

KETAMINE, an intravenous general anesthetic structurally similar to phencyclidine, is of limited usefulness as an anesthetic because of unpleasant emergence reactions.

The mechanisms by which ketamine acts on the nervous system to produce anesthesia and psychological reactions are not yet understood. However, ketamine has been shown to interact with several different types of ion channels, and alterations in channel properties may underlie some of the clinical effects of ketamine.

In nerve membranes ketamine depresses both peak inward sodium currents and steady-state potassium currents underlying the action potential.[1,2] Because it blocks excitable sodium channels and prevents action potential conduction, ketamine can be considered to have local anesthetic properties. The local anesthetic properties of ketamine also have been demonstrated *in vivo.* Ketamine injected into the subarachnoid space of dogs produces anesthesia[3] similar to that produced by local anesthetics. In humans, ketamine has been used for intravenous regional anesthesia,[4] and can produce loss of pain and thermal sensations when injected subcutaneously.[5]

Ketamine, like other local anesthetics, also depresses postsynaptic responses to acetylcholine (ACh). In frog sympathetic ganglia, ketamine depresses responses to iontophoretically applied ACh and blocks ganglionic transmission mediated by ACh.[6] Ketamine also reduces both excitatory and inhibitory responses to applied ACh in *Aplysia* neurons.[7] In frog sciatic-nerve/sartorius-muscle preparations, ketamine blocks twitches elicited by indirect (nerve) but not direct (muscle) stimulation, suggesting a postsynaptic action of the drug.[8] Ketamine decreases the amplitude of miniature end-plate potentials (mepps) and decreases the time constant of decay of miniature endplate currents (mepcs).[8–10] A decrease in decay rate of mepcs is indicative of a reduction in mean channel lifetime. In addition, several clinical reports have suggested that ketamine may potentiate neuromuscular block produced by vecuronium[11] and *d*-tubocurarine (*d*Tc).[12,13]

Ligand binding studies have shown that ketamine appears to interact with the open state of ACh-activated channels. Ketamine has only minimal effects on binding of labeled ACh, *d*Tc, and α-bungarotoxin, which bind to the ACh recognition site.[10,14] Ketamine inhibits binding of [^{3}H]perhydrohistrionicotoxin, which is thought to label the conducting portion of the ion channel.[14]

The experiments described in this paper were designed to study further the effects of ketamine on ion channels activated by ACh in BC_3H1 mouse tumor cells in culture. The purpose of this work is to provide a better understanding of the interactions between ketamine and ion channels at the motor endplate and in the central nervous system. Because ketamine has local anesthetic properties, results were compared with the predictions of a sequential channel-blocking model, which has been proposed to explain local anesthetic effects on ACh-activated channels.

Results show that ketamine decreases the average lifetime of ion channels activated by ACh, and does so at clinically relevant concentrations. Effects of ketamine on ACh-activated channels, however, do not resemble those of other local anesthetics.

Methods

Patch clamp techniques[15] have been used to record single-channel currents activated by ACh from BC_3H1

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Received from the Veterans Administration Medical Center, Iowa City, Iowa; and the Department of Anesthesia, College of Medicine, University of Iowa, Iowa City, Iowa. Accepted for publication November 17, 1987. Supported in part by a grant from the Veterans Administration and by an allocation of computing funds from the College of Medicine, University of Iowa.

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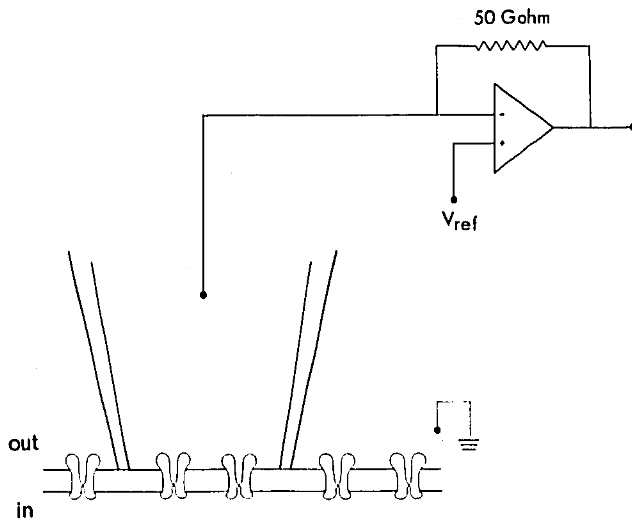

FIG. 1. Schematic drawing for single-channel recording from cell-attached patch. Components are not to scale. An electrode is pressed against a cell membrane containing acetylcholine receptors. The patch clamp circuit is a current-to-voltage converter having a feedback resistance of 50 Gohm (1 Gohm = 10^9 ohm).

mouse tumor cells grown in culture. In patch clamping, a blunt microelectrode with a tip opening of about 2 μ is pressed against the membrane of a cell. (See fig. 1.) Upon application of gentle suction, an extremely high resistance seal is formed between electrode and membrane, effectively isolating the patch of membrane underneath the electrode. By varying the electrical potential of the electrode, the potential across this patch can be "clamped" to any desired voltage. If the membrane patch remains attached to the rest of the cell (cell-attached patch) then the imposed electrode potential will add to the resting potential of the cell.

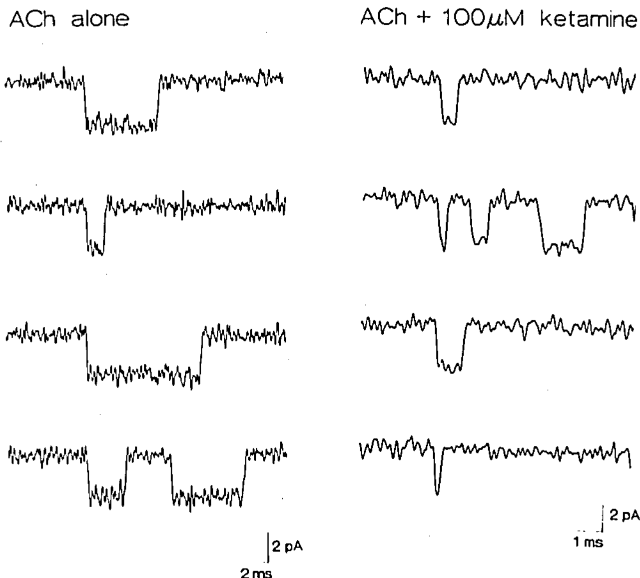

FIG. 2. Sample recordings of single-channel currents from cell-attached patches exposed to 200 nM ACh or 200 nM ACh plus 100 μM ketamine. Opening events are shown as downward deflections of the trace. Patch potential was +75 mV. Note different time scales.

ACh that has been placed in the electrode will bind to receptors and open ion channels in the membrane patch underneath the electrode, thus allowing current to flow across the cell membrane. This current, corresponding to the opening of single-ion channels, will be recorded by the patch clamp circuit.

CELL CULTURE

BC_3H1 cells were derived from a mouse cerebrovascular tumor, and express nicotinic receptors for ACh when incubated in serum-free media.[16,17] Cells were grown in tissue culture flasks[18] at 37° C in a 5% CO_2-enriched atmosphere using Dulbecco's modified Eagles's medium containing 1 mg/l glucose, and supplemented with 10% heat-inactivated fetal calf serum, 0.32 mg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. When the cells had grown to confluence, they were induced to promote synthesis of ACh receptors by incubation in a serum-free N2SF modified hormone-supplemented culture medium containing RPMI 1640, 5 μM bovine serum albumin, 5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM Na_2SeO_3, and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid), *p*H 7.2. After 3 to 5 days in serum-free medium, the cells were treated with 0.5% trypsin in 0.1% EDTA phosphate-buffered saline to lift them off culture flasks, then subcultured onto microscope cover slips. Cells were used 1 to 3 days later.

PATCH CLAMPING

Cells were visualized with a Nikon Diaphot® inverted microscope equipped with Hoffman Modulation Contrast® optics. Cells were bathed in a solution containing 100 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$, and 10 mM HEPES, at *p*H 7.4. Experiments were performed at controlled room temperature (18–23° C).

Patch clamp electrodes were pulled in two stages on a David Kopf® Model 720C vertical puller, coated near the tip with Sylgard® (Dow Corning, Midland, MI), then polished on a microforge. Electrodes had tip diameters of 0.5 to 2 μm, and resistances of 2 to 5 Mohm (1 Mohm

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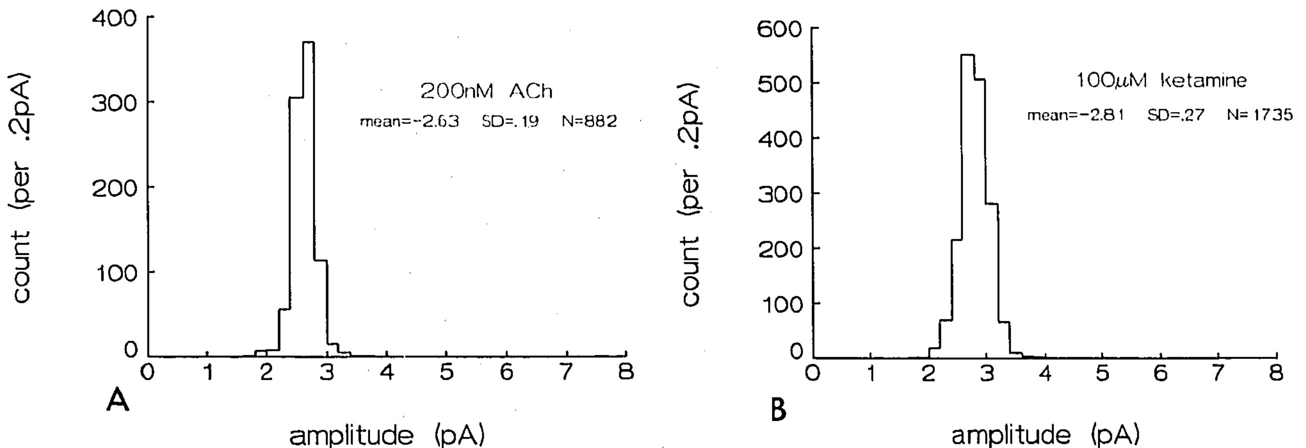

FIG. 3. Histograms showing the distribution of single-channel amplitudes. *A*. ACh alone. *B*. ACh plus 100 μM ketamine. Each histogram represents data from a single patch. Patch potential was +75 mV.

= 10^6 ohm). They were filled with the bathing solution plus 100 to 400 nM ACh (Sigma) and 1 to 200 μM ketamine (Vetalar, Parke-Davis). Since ketamine has a pK_a of 7.5, both cationic and uncharged forms were present in solution. The concentrations of ACh used in this study are well below 1 mM, the estimated equilibrium dissociation constant for block of the open channel by ACh under conditions of these experiments.[19]

Signals were measured using a List® Model EPC-7 patch clamp circuit, then low pass filtered at 3 kHz with an 8-pole Bessel filter (−3dB, Frequency Devices, Haverhill, MA). Channels were recorded with the patch pipet hyperpolarized +75 mV relative to cell resting potential, which was estimated to be −59 mV. Thus, the potential across the membrane patch was −134 mV. Whenever possible, at least 1,000 events were collected for analysis from each patch.

DATA ANALYSIS

Single-channel events, together with a segment of baseline prior to each event, were digitized at 25 to 50 μs per point, either on-line or from digital tape (Unitrade, Philadelphia, PA), using an interrupt-driven sampling routine (pCLAMP®, Axon Instruments, Burlingame, CA). Records of 512 points per event were stored by an IBM PC-AT® equipped with a 20-megabyte fixed disk and a Tecmar LabMaster® analog-to-digital conversion board.

Once events were stored on disk, the computer searched each record to locate transition times when each channel opened and closed, and to determine the current level associated with the open state. Records then were displayed for user approval or rejection. This information was used to generate an idealized record file containing channel open times, closed times (intervals between opening events), and current amplitudes.

The idealized record file was analyzed to determine average single-channel current amplitude and open-time and closed-time distributions. Average channel amplitude was calculated from the mean amplitude of events >500 μs in duration. Channel open-time and closed-time distributions were described as the sum of one or two exponential components estimated by the method of maximum likelihood.[20,21] This involved a nonlinear least-squares approximation according to the P3R function of BMDP (University of California) statis-

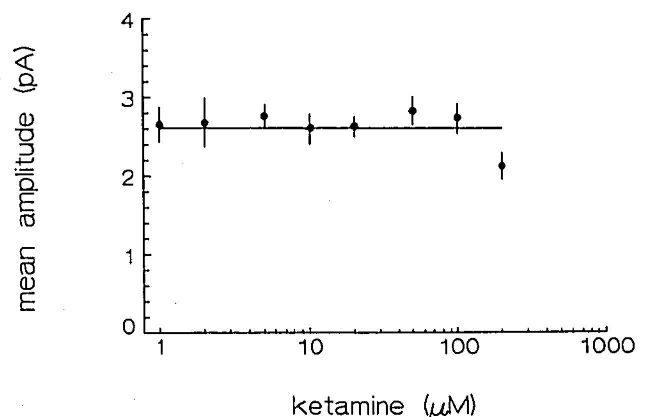

FIG. 4. Relationship between average single-channel amplitude at +75 mV and ketamine concentration. Each point is the mean ± SD of 4 to 13 patches. Line is a least-squares fit.

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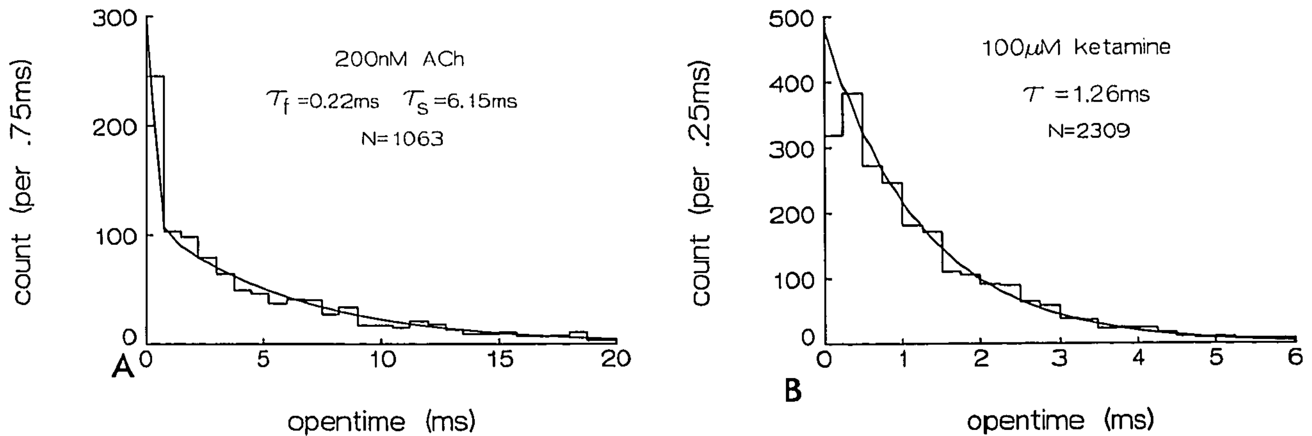

FIG. 5. Histograms showing the distribution of channel open times. *A*. ACh alone. *B*. ACh plus 100 μM ketamine. Note different time scales. Smooth curves superimposed on the histograms are maximum likelihood estimates for double-exponential (ACh alone) or mono-exponential (ketamine) distributions. For ketamine, the first bin, which contains a count of the number of events with lifetimes < 250 μs, is smaller than predicted because of the limited bandwidth of the recording system.

tical package. Distributions were corrected for missed events less than 100 μs in duration. A single component to the distributions was considered adequate if the correlation between the two time constants generated by a two-component fit was greater than 0.5.

For each patch in which the closed-time distribution consisted of two components that differed by more than 50-fold, total burst length, number of openings per burst, and open time per burst also were determined. A burst was defined as a group of openings that are separated by closed times shorter than a critical time t_c. The value of t_c for each patch was determined by solving the

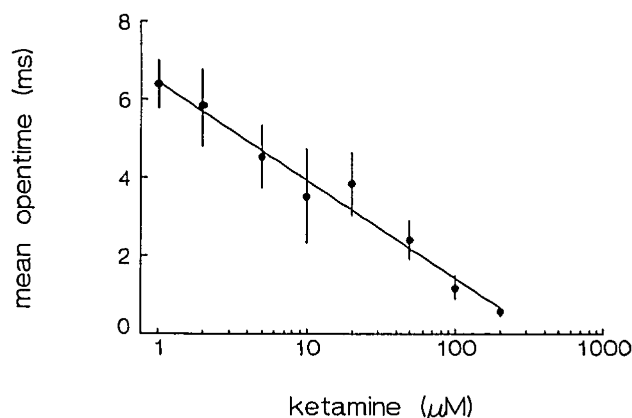

FIG. 6. Relationship between average channel lifetime at +75 mV and ketamine concentration. At concentrations less than 5 μM, the time constant of the slower component of the open-time distribution was used. Each point is the mean ± SD of 4 to 13 patches. Line is a least-squares fit.

equation:

$$e^{-t_c/\tau_s} + e^{-t_c/\tau_f} = 1$$

where τ_f and τ_s are the time constants of the fast and slow components of the closed-time distribution. With this formula, the proportion of long closed times that are misclassified as short will equal the proportion of short closed times that are misclassified as long.[22] Although most patches contained more than one active channel, openings within a burst were assumed to arise from activation of a single channel.

Results

Figure 2 illustrates typical recordings of single-channel currents activated by 200 nM ACh in the absence and presence of ketamine. Each opening event was analyzed to determine amplitude, open time (duration), and closed time (time since previous event). Some of these results with ACh have been described previously.[23]

AMPLITUDES

Figure 3 illustrates analysis of amplitudes from patches that were exposed to 200 nM ACh with or without ketamine. For each patch, the amplitude from every event was sorted into a bin, or group, 0.2 pA in width, in order to generate a histogram. The vertical axis represents a count of the number of events in each bin, while the horizontal axis shows the amplitude associated with each bin. Each histogram exhibits only a single narrow peak, indicating that only one conductance level is present. Channel amplitude, therefore, is expressed simply as a mean of 2.63 pA ± 0.19 (SD) in the absence of ketamine, or 2.81 pA ± 0.27 at 100 μM ketamine. At

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all concentrations of ketamine tested, only a single conductance level was present in each patch (N = 60 patches).

Ketamine, at concentrations up to 100 μM, did not change the amplitude of single-channel currents (fig. 4). Average channel amplitude at 100 μM was 2.72 pA ± 0.19 (mean, 9 patches ± SD) compared with 2.70 pA ± 0.16 (N = 24) in the absence of ketamine. Although single-channel amplitude appears slightly reduced at 200 μM, channel amplitude was difficult to measure at this concentration. The duration of the currents was extremely short, and, thus, limitations in bandwidth and effects of low-pass filtering may be responsible for the apparent decrease in amplitude.

Open Times

Figure 5 illustrates open-time data from the same patches whose amplitudes were analyzed earlier. Open times were sorted into bins 0.75 ms (ACh alone) or 0.25 ms (ACh plus 100 μM ketamine) in width to produce a histogram, analogous to that created for amplitudes.

With ACh alone, or at low concentrations of ketamine (<5 μM), the distribution of channel open times often required two time constants τ_f and τ_s ($\tau_f < \tau_s$) to describe the data. In the example of figure 5*A*, the time constants are 0.22 ms for τ_f and 6.15 ms for τ_s. The presence of two components in the open-time distribution suggests that there are at least two open states of the channel. The significance of the faster component is unclear, while the slower component would correspond to average channel lifetime as measured by fluctuation analysis[22] or miniature endplate current decay. The time constant of this closed slower component, therefore, was used as an indication of mean open time for comparison with the ketamine data.

At higher concentrations of ketamine, channel open-time distributions were well described by a single exponential, requiring only one time constant. The time constant of the distribution is then equal to average channel lifetime. In the patch shown in figure 5*B*, average channel lifetime at 100 μM ketamine is 1.26 ms. A faster component to the open-time distribution may still exist, but it would not be detectable because of the limited bandwidth of the recording system.

Figure 6 illustrates the relationship between channel lifetime, or the slower component of the open-time distribution, and ketamine concentration. Channel open time was decreased by ketamine in a dose-dependent manner. At 100 μM, ketamine decreased mean channel open time to 1.19 ms ± 0.30 (N = 9), compared with 6.32 ms ± 0.83 (N = 20) in the absence of ketamine.

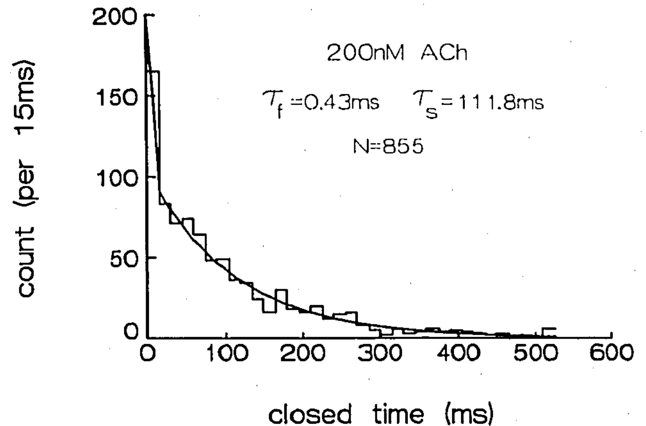

FIG. 7. Representative closed-time histogram. The data are well described by two exponential components that differ greatly in magnitude, 0.43 ms and 111.8 ms.

Closed Times

Closed times (or intervals between events) were analyzed in the same fashion as open times, and usually were described as a sum of two exponential components (fig. 7). When τ_f and τ_s differed greatly in magnitude (more than 50-fold), opening events were grouped into bursts (fig. 8). A burst was defined as a group of openings separated by very short closed times less than a critical time t_c. (See the Methods section.) Rapid times represent transient closures (or blockages) of the channel, and are termed gaps within bursts. Longer closures occur when agonist dissociates from its receptors, and are termed gaps between bursts. At 100 μM ketamine, t_c = 8.6 ± 3.9 ms (N = 8).

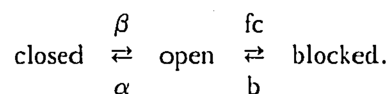

Figure 9 shows how the number of openings per burst increases with ketamine concentration. At 100 μM, the number of openings per burst was 1.61 ± 0.16 (N = 8), compared with 1.10 ± 0.05 (N = 16) with ACh alone.

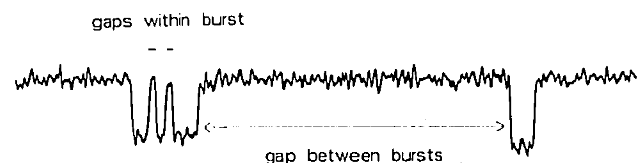

FIG. 8. Sample recording shown to illustrate the grouping of openings into bursts. Rapid closed times are termed gaps within a burst, while a longer closure is a gap between bursts.

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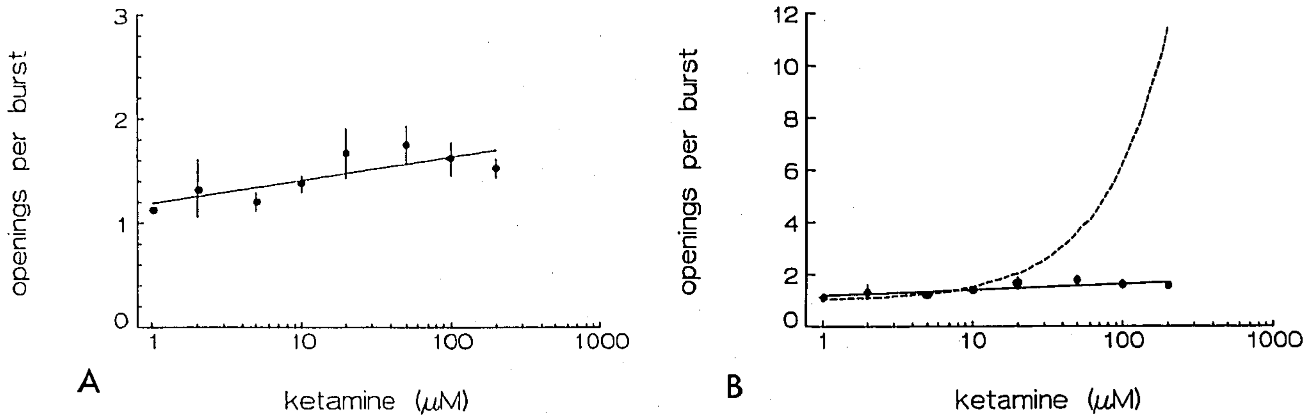
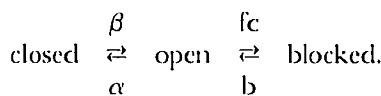


FIG. 9. *A*. Relationship between the number of openings per burst and ketamine concentration. *B*. A plot of the same data on a different vertical axis. Each point is the mean ± SD of four to 13 patches. Solid lines are least squares fits. The dotted line represents the predictions of the sequential blocking model.

SEQUENTIAL BLOCKING MODEL

A sequential channel-blocking model has been proposed to explain the effects of several drugs, especially local anesthetics, on ACh-activated channels.[24–26] (See also references 27–29.) Channel blockers appear to bind to the channel in its open state to occlude the channel and prevent the flow of ions. Further analysis therefore was undertaken to determine whether ketamine effects were consistent with a sequential blocking model:



$$\text{closed} \underset{\alpha}{\overset{\beta}{\rightleftarrows}} \text{open} \underset{b}{\overset{fc}{\rightleftarrows}} \text{blocked.}$$

This sequential model predicts that the number of openings per burst should increase with drug concentration as the channel chatters back and forth between open (conducting) and blocked (nonconducting) states. The mean number of openings per burst (m) should be[21]:

$$m = 1 + fc/\alpha$$

where c is the concentration of the blocking drug and α is the rate constant for channel closing in the absence of blocker, equal to the reciprocal of mean open time (1/6.32 ms). A linear plot of inverse open time as a function of ketamine concentration (fig. 10) yields a straight line with slope $f = 7.9 \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}$, the forward rate constant for the proposed blocking reaction. The sequential model then predicts an average of 6.0 openings per burst at 100 μM ketamine. This number does not compare well with the observed value of 1.61 (fig. 9*B*).

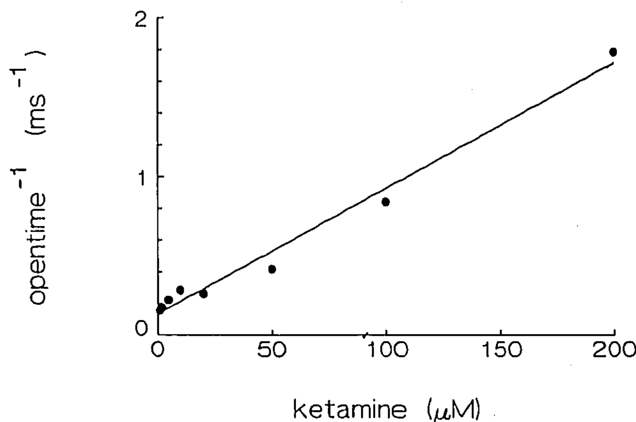


FIG. 10. Plot of open time^{-1} as a function of ketamine concentration. The slope of the line is the forward rate constant f for the proposed blocking reaction. $f = 7.9 \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}$.

Discussion

BC_3H1 mouse tumor cells in culture have been used as a model system to study the effects of ketamine on ion channels activated by ACh. The kinetics of channel activation in these cells has been studied previously in detail by Sine and Steinbach,[30,31] although their results are slightly different from those presented here. They found two distinct types of channel activity, brief duration openings that appeared as isolated events and bursts of long duration openings separated by short-duration closed periods. Both open- and closed-time histograms contained three resolvable exponential components.

Differences in experimental conditions are sufficient to explain the discrepancies between their findings and those reported here. The experiments of Sine and Steinbach were designed to maximize the temporal res-

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olution of their recording system in order to study the kinetics of channel activation. Cells were exposed to a high K^+ solution, in which K^+ rather than Na^+ became the predominant permeant cation. This high K^+ solution also reduced cell resting potential close to zero. Recording electrodes often contained zero Ca^{++} with 1 mM EGTA, and currents were measured at 9 to 11° C.

For the experiments described in this paper, conditions were maintained closer to a normal physiologic state. Cell resting potential was estimated at −59 mV, and Na^+ was the predominant charge carrier. Experiments were performed at room temperature rather than 37° C, however, because of bandwidth limitations. Under these conditions, short-lived open or closed states were detectable only if they were longer than about 100 μs in duration. The extremely fast components to open- and closed-time distributions reported by Sine and Steinbach, with time constants in the range 50 to 100 μs, were therefore not resolvable.

Such rapid components, however, contribute very little to the total charge transferred during transmitter action. The faster component of open-time distributions reported here, τ_f, was present in only 13 of 20 cells, and accounted for only 2 to 3% of the net current. An even faster component, not detectable here, would have contributed even less. Effects of ketamine therefore have been considered mainly in terms of changes in the slower component of open-time distributions. Currents that flow during these longer open states associated with the slower component will be the main determinant of synaptic efficacy.

Ketamine decreases the average time channels stay open once they have been activated by ACh, thus decreasing the time constant of the slower component of open-time distributions. This effect of ketamine occurs at clinically relevant concentrations. Peak plasma levels after an intravenous dose of 2 mg/kg ketamine average about 0.75 μg/ml, or 3 μM (Parke-Davis product information on Ketalar®), while values two to three times higher also have been reported after a single intravenous dose of 75 mg/m² body surface.[13] Results of these experiments suggest that 3 μM ketamine should reduce channel lifetime by about 17%.

This finding has immediate implications for explaining enhancement by ketamine of neuromuscular block produced by vecuronium or *d*Tc. A similar reduction in the lifetime of endplate channels would reduce the efficacy of released ACh, and decrease the amplitude of both spontaneous miniature end-plate potentials and evoked end-plate potentials. However, the depressant effects of ketamine on neuromuscular transmission may not always be clinically apparent. Ketamine also may act at other sites, possibly within the central nervous system, to increase skeletal muscle tone. The safety factor for neuromuscular transmission is high, and a substantial decrease in endplate potential size must occur before neuromuscular transmission will fail. In addition, a failure of neuromuscular transmission at a limited number of motor units within a muscle may not have a measurable effect on twitch tension. Thus, the postsynaptic blocking action of ketamine may only be unmasked in the presence of other neuromuscular blockers such as vecuronium or *d*Tc.

Although ketamine antagonizes the postsynaptic actions of ACh by decreasing channel lifetime, it cannot necessarily be considered a "channel blocker." Data presented here demonstrate that the actions of ketamine are not consistent with a sequential channel blocking model in which molecules of drug bind to the open channel to occlude it. Additional experiments are in progress to explore other models that may explain alterations in channel lifetime produced by ketamine.

Ketamine effects on ACh-activated channels have been discussed in terms of alterations in cholinergic transmission at the endplate. In addition, ACh-activated channels in BC_3H1 cells also may be considered as a model system for studying anesthetic effects on other ion channels, such as those in the central nervous system. Anesthetics interact with several different types of channels, including those activated by ACh, glutamate, and GABA (γ-aminobutyric acid).[32,33] Knowledge of the mechanism by which ketamine alters channel properties in BC_3H1 cells may provide insight into its effects on other channels more closely linked to its site of action in the central nervous system.

The author thanks V. Matulionis for technical assistance and L. Schenkel for typing the manuscript.

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