

Multiple Mechanisms of Ketamine Blockade of N-methyl-D-aspartate Receptors

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Background: The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor is blocked by ketamine, and this action likely contributes to ketamine's anesthetic and analgesic properties. Previous studies suggest that ketamine occludes the open channel by binding to a site located within the channel pore. This hypothesis was examined by investigating the effects of ketamine on single-channel currents from NMDA receptors.

Methods: The cell-attached and outside-out configurations of the patch clamp technique were used to study NMDA-activated currents recorded from cultured mouse hippocampal neurons.

Results: In cell-attached patches, NMDA evoked currents that had an apparent mean open time (τ_o) of 3.26 ms. The probability of at least one channel being open (P_o') was 0.058. The addition of ketamine (0.1 μM or 1 μM) to the pipette solution decreased P_o' to 53% and 24% of control values, respectively. At 1 μM ketamine, this reduction was due to a decrease in both the frequency of channel opening and the mean open time (44% and 68% of control values, respectively). Ketamine did not influence channel conductance and no new components were required to fit the open- or closed-duration distributions. Ketamine (50 μM), applied outside the recording pipette, reduced the opening frequency of channels recorded in the cell-attached configuration. This observation suggests that ketamine gained access to a binding site by diffusing across the hydrophobic cell membrane. In outside-out patches, ketamine

potency was lower than that observed in cell-attached patches: 1 μM and 10 μM ketamine reduced P_o' to 63% and 34% of control values, respectively, and this reduction was due primarily to a decrease in the frequency of channel opening with little change in mean open time.

Conclusions: These observations are consistent with a model whereby ketamine inhibits the NMDA receptor by two distinct mechanisms: (1) Ketamine blocks the open channel and thereby reduces channel mean open time, and (2) ketamine decreases the frequency of channel opening by an allosteric mechanism. (Key words: Anesthetic. Blockade. Ion channel. Ketamine. N-methyl-D-aspartate.)

GLUTAMATE is the major excitatory neurotransmitter in the mammalian central nervous system. Based on pharmacologic sensitivity, ion permeability, and channel kinetics, at least two major classes of ionotropic glutamate receptors have been identified: the N-methyl-D-aspartate (NMDA) receptors, and the non-NMDA or α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) kainate receptors.¹ The NMDA receptor is a unique neurotransmitter receptor in that channel activation requires the binding of both glutamate and the co-agonist glycine.² The channel is blocked in a voltage-dependent manner by physiologic concentrations of Mg^{2+} and undergoes calcium-dependent and calcium-independent forms of inactivation.³⁻⁵ Receptors for NMDA are thought to play a pivotal role in various physiologic functions, including synaptic plasticity and neuronal development. In addition, NMDA receptors may contribute to pathologic processes such as seizure, ischemia-related neuronal death, and several neurodegenerative diseases.⁶

Ketamine (2-O-chlorophenyl-2-methylamino cyclohexanone) blocks NMDA receptors, and this action is thought to contribute to ketamine's potent anesthetic and analgesic properties.⁷⁻¹⁰ Electrophysiologic and binding studies have revealed that various other antagonists, including phencyclidine, memantine, amantadine, and MK-801, induce a "use-dependent" inhibition of the NMDA receptor.¹¹⁻¹⁵ The blockade is use dependent in that the rates of onset and the recovery from blockade are increased by applying agonist. These observations have been interpreted as indicating that the chan-

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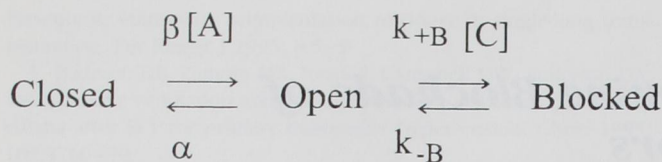


Fig. 1. Scheme 1.

nel must be in the open state before ketamine can bind to or dissociates from a blocking site possibly located within the channel pore. This type of blockade is called "uncompetitive inhibition" because it is contingent on the binding of agonist to the receptor.¹⁶ In contrast, a "noncompetitive" blocker binds independently of the presence of agonist.

Previous single-channel studies of the NMDA channel have provided insights into the mechanisms by which dissociative anesthetics block the NMDA receptor. Heuttner and Bean¹³ examined the effects of MK-801 on NMDA-activated single-channel currents and found that MK-801 produced a dose-dependent decrease in the mean duration of channel opening.¹³ Similarly, phencyclidine decreased the frequency and duration of NMDA channel openings.¹⁷ These actions are consistent with a sequential open channel blocking mechanism that can be represented by scheme 1.

The rates of channel opening and closing are β and α , the rates of association and dissociation of the blocker are k_{+B} and k_{-B} , and $[A]$ and $[C]$ are the concentrations of agonist and blocker, respectively.¹⁸ Because this model assumes that the receptor exists in a limited number of discrete states, the rates governing the transitions between these states can be estimated from the time constants obtained from the dwell time distributions. According to scheme 1, the mean duration of channel opening is decreased by the blocking drug because the open (ion-conducting) state can be terminated by either channel closure (α) or transitions into the open, nonconducting state referred to as "blocked" ($k_{+B}[C]$). Thus the mean single-channel open time (τ_o) will be inversely related to the sum of the two transition rates leading from the open state; that is, $\tau_o = 1/(k_{+B}[C] + \alpha)$. Accordingly, open-channel blockade is characterized by a decrease in the mean duration of the open state of the channel.

Scheme 1, however, cannot account for agonist-dependent recovery from channel blockade because the model assumes that the agonist remains bound until the blocker dissociates from the receptor. Thus the rate of recovery from blockade should be not be influenced by the extracellular concentration of agonist. To ac-

count for this inconsistency, a model of "drug trapping" was postulated, as summarized in scheme 2. Here the agonist can dissociate and the receptor close with ketamine bound to the receptor. Closing of the channel "traps" ketamine on the receptor, and the probability of dissociation of ketamine remains low until the channel is stimulated to reopen after agonist binding.¹⁹ In its simplest form, this model proposes that ketamine does not directly influence channel "gating" or the transition rates between the open and closed states. The true affinity of the binding site remains unchanged even though the site is "guarded" (that is, access is restricted by the conformation of the receptor). Scheme 2, which is summarized here, is discussed in detail by MacDonald *et al.*⁷

Use-dependent blockade does not necessarily imply occlusion of the open pore or preferential binding to the open state. Receptors for NMDA can exist in several agonist-associated closed states, such as the desensitized form of the receptor. Preferential binding of ketamine to a agonist-associated closed state would also be use dependent (scheme 3). This scheme resembles the modified-receptor model proposed by Hille²⁰ that describes the effects of certain local anesthetics on voltage-activated sodium channels. In scheme 3, the normal channel is represented as making transitions between three states: closed, open, and desensitized. The NMDA channel resides in more than three states, but for our purpose the scheme has been simplified. The rates of entry into, and recovery from, a nonconducting desensi-

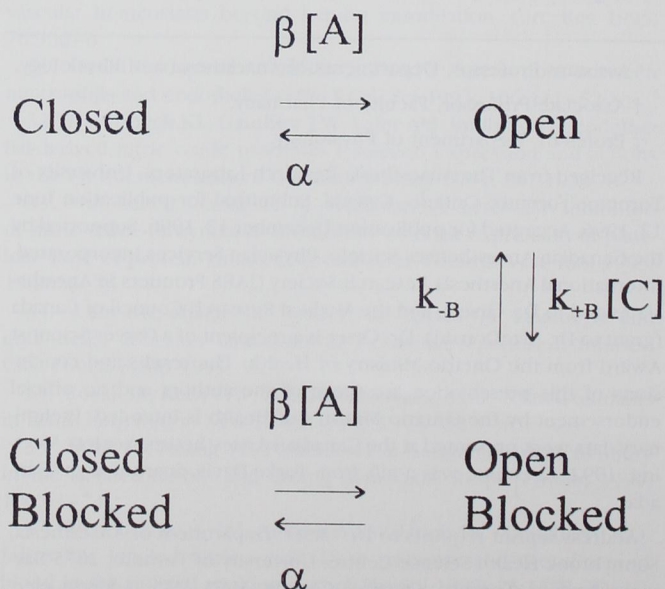


Fig. 2. Scheme 2.

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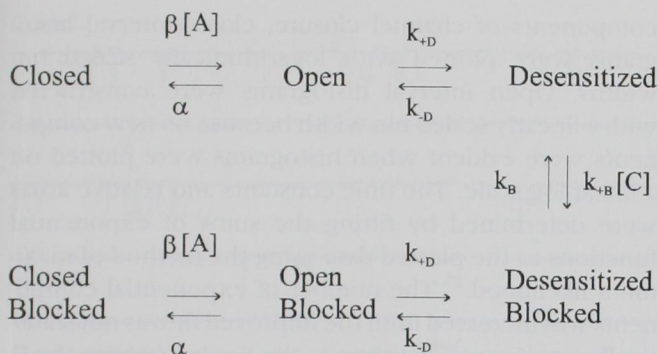


Fig. 3. Scheme 3.

tized state are indicated by k_{+D} and k_{-D} , respectively. The rates of association and disassociation of ketamine are assumed to be negligible when the channel is in a non-desensitized state.

Similar to scheme 2, ketamine can be trapped when the agonist is removed and the channel reverts to the non-ligand-bound state so that the onset and recovery from blockade will be agonist dependent. However, in contrast to scheme 2, scheme 3 predicts that ketamine will reduce the number of channels available for opening without influencing mean channel-open time.

The kinetic properties of the ketamine blockade of the NMDA receptor have not been examined previously at the single-channel level. It is plausible that the mechanism of ketamine block differs substantially from that of phencyclidine or MK-801: Ketamine's actions on the nicotinic acetylcholine receptor and non-NMDA (quisqualate)-type glutamate receptor in locust suggest that it interacts with both the open and closed conformational state of these channels.²¹⁻²³ Furthermore, the physical site(s) of binding may not be identical for all uncompetitive antagonists of the NMDA receptor. Molecular studies of the binding sites for phencyclidine and MK-801 indicate that the phencyclidine binding site appears to be confined to the putative M2 segment, whereas blockade by MK-801 requires an interplay between residues associated with the M2 and M3 regions of the protein.²⁴ Different binding sites and mechanisms of action might account for the diverse clinical properties of uncompetitive NMDA receptor antagonists and represent potential targets for the development of new pharmaceutical agents.

Here we report the effects of ketamine on single-channel currents activated by NMDA and examine the

validity of the various kinetic schemes intended to describe the blockade. Our results suggest that there are two mechanisms of inhibition: an open-channel blockade and a closed-channel blockade that involves a site accessible from the hydrophobic membrane phase. Part of this work was published previously in abstract form.[§]

Materials and Methods

Dissociation and Culture of Hippocampal Neurons

Animal protocols were approved by the University of Toronto Animal Care Committee. Cultures of fetal hippocampal neurons were prepared from Swiss white mice, as previously described.²¹ Briefly, hippocampal neurons were dissected from fetal mice pups. Neurons were dissociated by using enzymatic digestion and trituration. Cells were plated on collagen-coated dishes, incubated at 37°C in 7% carbon dioxide, and maintained in culture for 10–21 days.

Solutions and Single-channel Current Recordings

Before recording, cells were rinsed thoroughly with a standard extracellular recording solution containing 140 mM NaCl, 1.3 mM CaCl_2 , 5.4 mM KCl, 25 mM *N*'-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulphonic acid, 33 mM glucose, and 300 nM tetrodotoxin. The solution was buffered to a pH of 7.4 with NaOH and the osmolality was adjusted to 297–300 mOsm. Glycine (1 μM) was added to the solution because it is an essential co-agonist for gating of the NMDA receptor.⁵ Patch electrodes were constructed from thin-walled borosilicate glass (1.5 mm outer diameter; World Precision Instruments) using a two-stage vertical puller (Narishige PP-83). Pipettes were coated near the tips with Sylgard 184 (Dow Corning, Midland, MI) and fire polished to a final resistance of 2–5 M Ω . The electrodes used for cell-attached recordings were filled with a solution containing 70 mM NaCl, 70 mM Na_2SO_4 , 1 mM CaCl_2 , 10 μM NMDA, and 1 μM glycine. Ketamine (0 μM , 0.1 μM , or 1.0 μM) was added to the pipette solution. In a second series of cell-attached experiments, ketamine (50 μM) was added to the bath solution. For outside-out patch recordings, the pipettes were filled with solutions containing 140 mM CsF, 10 mM *N*'-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulphonic acid, 11 mM ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid, and 1 mM CaCl_2 . The pH was adjusted to 7.4 with CsOH. Patches were voltage clamped using an Axopatch 1B or Axopatch 200 amplifier (Axon Instruments). For the outside-out

§ Orser BA, MacDonald JF: Ketamine blocks NMDA channels via a hydrophobic pathway. (Abstract) Can J Anaesth 1992; 39:A12.

patch recordings, drugs and agonist were applied through a perfusion barrel that was positioned close to the membrane patch.² Patches were exposed to NMDA for approximately 5–15 min before records were sampled for kinetic analysis. We assumed that channels were functioning under near steady-state conditions and the extent of glycine-insensitive desensitization had stabilized.^{25,26} All experiments were conducted at room temperature (20–25°C).

Single-channel currents were filtered at 2 kHz and stored on VHS tape for off-line analysis. Data were digitized (T1-1 interface, Axon Instruments, Inc., Foster City, CA) and sampled every 100 μ s using pCLAMP software (Axon Instruments, Inc., Foster City, CA). Current records were selected for detailed kinetic analysis based on the stability of the recording and the number of channels present in the patch. Only patches that remained stable over a 5- to 45-min period were analyzed. It was assumed that patches contained at least as many channels as conductance levels observed and that each channel behaved independently. Only records with infrequent (<10%) openings to a second open level were used for the analysis. However, most patches had at least two open levels.

The threshold method was used to detect transitions between the open and closed states.²⁷ The threshold was set at one half the maximal current amplitude, and all records were monitored visually to ensure stability of the baseline. To minimize the effect of false events (such as transitions due to random noise), the minimum event duration was set at 300 μ s. This strict criteria, as well as the limitations of the recording system, would inevitably result in “missed” or undetected events. For example, if the duration of channel closure was shorter than the rise time of the recording system or the specified minimum event duration, closed events would be missed.^{27,28} This would result in an overestimation of channel open time. Conversely, missed brief openings would result in an overestimation of closed times. Methods to correct for missed events were not used. However, the effect of missed events on our interpretation of ketamine’s effects was minimized by using identical analysis criteria for records obtained in the absence and presence of ketamine. Furthermore, all records were analyzed at least twice to ensure that a substantial number of brief events were not excluded from the analysis. Data were binned into open- and closed-interval histograms and plotted using the pSTAT program (pCLAMP 6.0, Axon Instruments, Inc., Foster City, CA). We observed that the duration of closed events spanned several orders of magnitude. To detect multiple kinetic

components of channel closure, closed-interval histograms were plotted with logarithmically scaled bin widths. Open interval histograms were constructed with a linearly scaled bin width because no new components were evident when histograms were plotted on a linear-log scale. The time constants and relative areas were determined by fitting the sums of exponential functions to the plotted data using the method of maximum likelihood.²⁷ The number of exponential components was increased until the improved fit was not statistically significant according to the F-value (where the F-value compares the sum of the squared errors extracted from the fit for the various models). Critical values of F corresponding to $P < 0.05$ were obtained from standard tables. Results are presented as means \pm SEM unless otherwise stated.

The mean duration of channel opening and closure were extracted from the event lists. Unfortunately, the number of channels in the patch cannot be determined accurately if the channels take long sojourns in desensitized or inactivated states.²⁹ Our records reflect the steady-state activity of a population of receptors, and prolonged closings of individual channels cannot be distinguished. Because we could not determine accurately the number of channels present in each patch, we defined a modified open probability (P_o') in which P_o' is the probability that at least one channel is open. P_o' was calculated as t_o/t_i where t_o was the total dwell time at the open level and t_i was the time interval over which P_o' is measured. The frequency of channel opening was determined by the number of open events divided by the duration of a specified time interval. A discrepancy between P_o' and the open probability of a single channel (calculated by multiplying the frequency of channel opening and mean open time) is expected because P_o' does not distinguish between single or double openings, whereas the mean open time was calculated from single open events.

Drugs and Other Chemicals

Ketamine is an arylcyclohexylamine that contains an asymmetric carbon atom. It exists as two isomers: the (R) and (S) form, where the (S) isomer is the more potent general anesthetic and NMDA receptor antagonist.^{30,31} The commercially available preparations of ketamine used in these experiments are racemic mixtures (Parke-Davis, Scarborough, Canada). All other agonists and compounds were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

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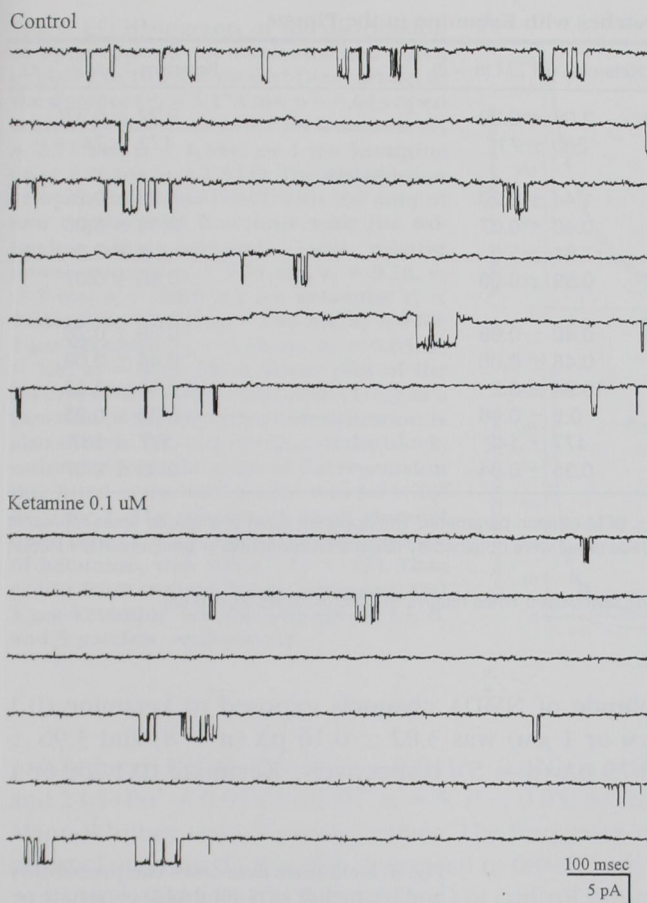


Fig. 4. Single-channel currents, evoked by applications of 10 μM *N*-methyl-D-aspartate to cell-attached patches from hippocampal neurons, are shown. Each patch was voltage clamped at the resting membrane potential of the cell. Currents were recorded from two different cells, in the absence and presence of 0.1 μM ketamine. Inward currents are represented by downward deflections.

Results

NMDA-activated Currents Recorded in the Cell-attached Configuration

The addition of NMDA (10 μM) to the pipette solution resulted in single-channel openings, as illustrated in figure 4. With the membrane voltage clamped at the resting potential of the cell (pipette potential = 0 mV), currents were characterized by bursts of openings interrupted by brief and long channel closures. The mean current amplitude for seven patches was 4.33 ± 0.32 pA. The current-voltage relation, measured at pipette holding potentials ranging from -30 to $+30$ mV, was linear with an estimated slope conductance of 43.2 ± 9.4 pS.

We observed a large variation in the frequency of channel opening among different patches (1.66 s^{-1} to 55.5 s^{-1}). However, in individual patches, the frequency of channel opening remained stable during the duration of each recording. Therefore we assumed that this variability resulted from differences in the number of channels present in each membrane patch. The probability of at least one channel being open (P_o') was 0.058 ± 0.015 . The mean frequency of channel opening was $13.4 \pm 4.4 \text{ s}^{-1}$, and the mean open time (τ_o) was $3.26 \pm 0.25 \text{ ms}$ ($n = 13$).

The duration of channel openings and closings were measured and event lists were used to construct open- and closed-duration histograms. Table 1 summarizes the mean time intervals, time constants, and relative areas estimated from the dwell-time distributions.

Nowak and Wright³² reported that membrane hyperpolarization caused a decrease in the probability of NMDA channel opening. To hyperpolarize the membrane patch, a positive holding potential of $+30$ mV was applied to the recording electrode. Conversely, the membrane was depolarized by applying a pipette potential of -30 mV. The value of P_o' was 0.03 ± 0.02 ($n = 8$) at a pipette potential of $+30$ mV and 0.07 ± 0.04 ($n = 6$) at a pipette potential of -30 mV. The mean channel-open time also decreased with membrane hyperpolarization (V pipette = $+30$ mV, $\tau_o = 2.85 \pm 0.48 \text{ ms}$; V pipette = -30 mV $\tau_o = 9.97 \pm 2.00 \text{ ms}$).

Ketamine Modulation of Single-channel Currents from Cell-attached Patches

Ketamine was shown previously to reversibly inhibit NMDA-evoked whole-cell currents recorded from hippocampal and striatal neurons. The median inhibitory concentration for blockade is reported to range from 0.43 to 10 μM .^{8,12,33} To determine if this inhibition by ketamine resulted from a decrease in channel conductance, frequency of channel opening or channel-open time, we studied, in the cell-attached configuration, the effects of ketamine on single NMDA receptors. We selected ketamine concentrations in the range of previously reported IC_{50} (concentration that inhibits 50% of the maximal response) of whole-cell currents.

Inclusion of ketamine (0.1 μM or 1.0 μM) in the recording electrode produced a pronounced reduction in the frequency of channel opening but no change in single-channel conductance (fig. 5). We also tried to record single-channel events with 10 μM ketamine in the pipette solution, but channel openings were so infrequent that kinetic analysis of these records was not possible.

Table 1 summarizes the distributions of open and

Table 1. Distribution of Open and Closed Intervals, Cell Attached Patches with Ketamine in the Pipette

	Control (n = 13)	Ketamine 0.1 μ M (n = 8)	Ketamine 1 μ M (n = 5)
Mean open time	3.26 \pm 0.25	3.06 \pm 0.20	2.22 \pm 0.37*
Mean closed time	130 \pm 39	260 \pm 71*	173 \pm 74
Open intervals			
τ_1 (ms)	1.23 \pm 0.18	1.44 \pm 0.20	0.83 \pm 0.26
Area 1	0.34 \pm 0.04	0.40 \pm 0.07	0.49 \pm 0.06
τ_2 (ms)	11.51 \pm 2.45	17.61 \pm 5.0	6.8 \pm 2.5
Area 2	0.64 \pm 0.04	0.59 \pm 0.08	0.55 \pm 0.07
Closed intervals			
τ_1 (ms)	0.29 \pm 0.04	0.40 \pm 0.08	0.38 \pm 0.26
Area 1	0.48 \pm 0.03	0.48 \pm 0.05	0.44 \pm 0.09
τ_2 (ms)	2.6 \pm 1.5	10.94 \pm 3.2	6.91 \pm 4.17
Area 2	0.23 \pm 0.02	0.5 \pm 0.06	0.23 \pm 0.05
τ_3 (ms)	296 \pm 78	477 \pm 142	377 \pm 137
Area 3	0.28 \pm 0.02	0.35 \pm 0.04	0.32 \pm 0.07

The membrane was voltage clamped to the resting potential. Shown is the mean \pm SEM of each parameter. The apparent open and closed times represent the mean values obtained from the events lists. The time constants (τ) and relative areas (area) were obtained by fitting a biexponential or triexponential function to the dwell time histograms.

* The Mann-Whitney test was used to determine significant differences in mean open and closed times relative to control values ($P < 0.05$).

closed intervals measured with ketamine (0 μ M, 0.1 μ M, or 1 μ M) in the pipette. Ketamine did not significantly influence channel conductance as the mean current am-

plitude of NMDA channels exposed to ketamine (0.1 μ M or 1 μ M) was 3.82 \pm 0.16 pA (n = 8) and 3.96 \pm 0.73 pA (n = 5), respectively. Ketamine (0.1 μ M or 1

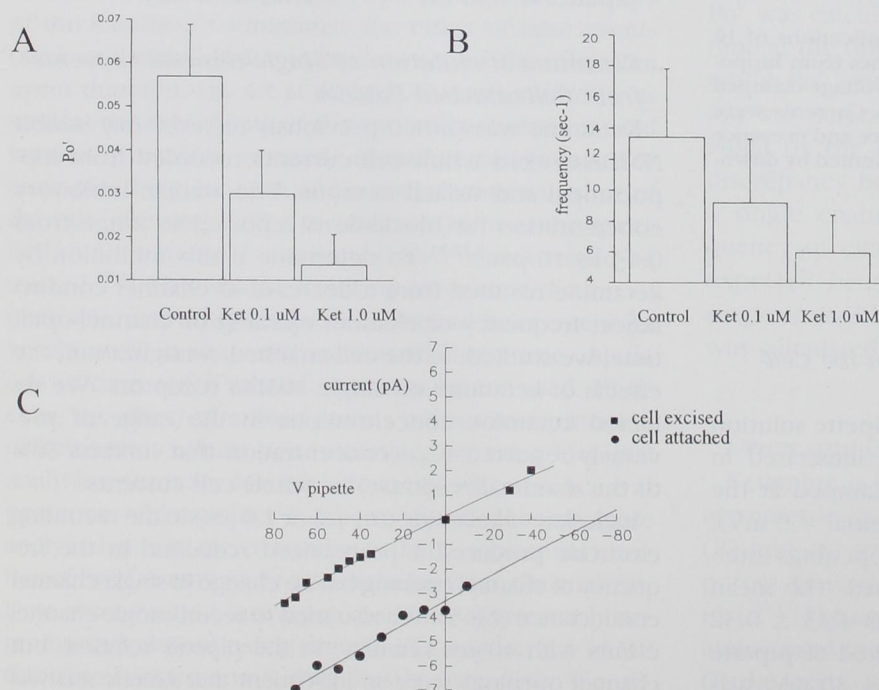
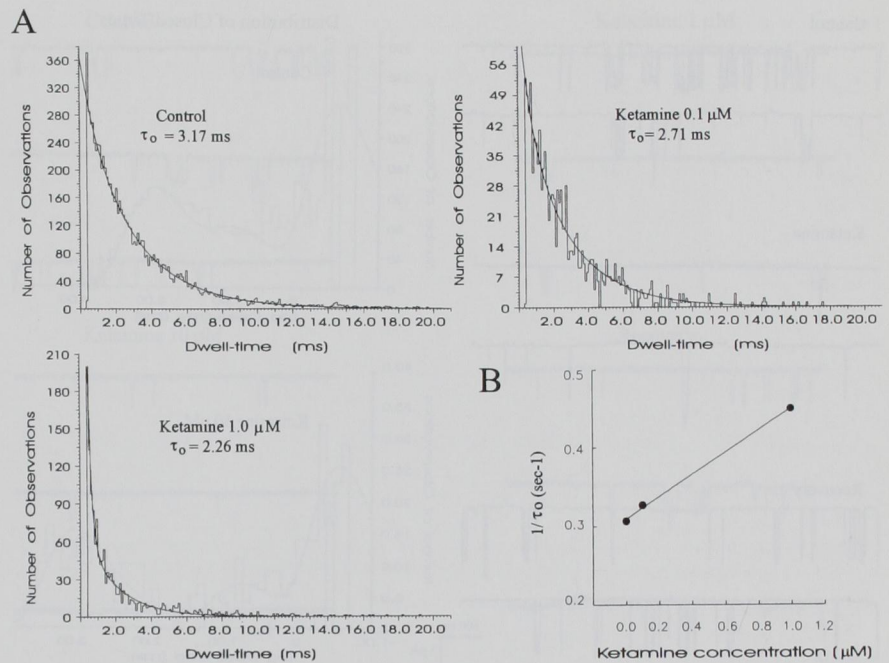


Fig. 5. Ketamine decreases the probability and frequency of *N*-methyl-D-aspartate receptor channel opening in cell-attached patches. (A) The steady-state probability of at least one channel being open (P_o') was 0.058 \pm 0.015 (n = 13), 0.031 \pm 0.01 (n = 8), and 0.014 \pm 0.007 (n = 5), in the presence of 0 μ M, 0.1 μ M, and 1 μ M ketamine, respectively. **(B)** The frequency of channel opening (f) decreased from a control value of 13.4 \pm 4.4 s⁻¹ to 9.1 \pm 4.11 s⁻¹ and 5.95 \pm 2.45 s⁻¹ in patches exposed to 0.1 μ M and 1.0 μ M ketamine, respectively ($P < 0.05$, by the Mann-Whitney test). **(C)** The current-voltage relations for single-channel currents recorded in the cell-attached and inside-out configurations are shown. Currents were recorded from the same cell before and after patch excision. $V_{pipette}$ refers to the pipette holding potential (the resting potential of the cell is not known, so current amplitude is shown relative to the various pipette potentials). The slope conductance, estimated from the regression lines fitted to the data points for the cell-attached and the inside-out patches were 51 pS and 45 pS, respectively. The resting membrane potential of the cell estimated from the reversal potential of currents recorded in the cell-attached configuration was -70 mV. In the inside-out configuration, currents reversed polarity at 0 mV.

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Fig. 6. (A) Histograms of the distribution of open times from a cell-attached patch exposed to $10\ \mu\text{M}$ *N*-methyl-D-aspartate, in the absence ($\tau_0 = 3.174\ \text{ms}$, $n = 8,643$ open events) or presence of $0.1\ \mu\text{M}$ ketamine ($\tau_0 = 2.71\ \text{ms}$, $n = 1,144$) or $1\ \mu\text{M}$ ketamine ($\tau_0 = 2.26\ \text{ms}$, $n = 1,542$). The distribution of open times was fitted with the sum of two exponential functions with the following time constants (and relative areas): control $\tau_1 = 1.05\ \text{ms}$, $a_1 = 0.16$, $\tau_2 = 3\ \text{ms}$, $a_2 = 0.84$; $0.1\ \mu\text{M}$ ketamine $\tau_1 = 0.65\ \text{ms}$, $a_1 = 0.11$, $\tau_2 = 2.69\ \text{ms}$, $a_2 = 0.89$; $1\ \mu\text{M}$ ketamine $\tau_1 = 0.20\ \text{ms}$, $a_1 = 0.65$, $\tau_2 = 1.8\ \text{ms}$, $a_2 = 0.35$. (B) A linear plot of the inverse of the mean open time ($1/\tau_0$) as a function of the ketamine concentration is also shown. The rate of onset of the block, estimated from the slope of the regression line fitted to the data points, was $1.4 \times 10^8\ \text{m}^{-1} \cdot \text{sec}^{-1}$. The inverse of mean channel open time ($1/\tau_0$), measured in the absence of ketamine, was $306\ \text{s}^{-1}$ ($n = 13$). Data points from control, $0.1\ \mu\text{M}$ ketamine, and $1\ \mu\text{M}$ ketamine are the average of 13, 8, and 5 patches, respectively.



μM) reduced Po' to 53% ($Po' = 0.031 \pm 0.010$, $n = 8$) and 24% ($Po' = 0.014 \pm 0.007$, $n = 5$, $P < 0.05$, by the Mann-Whitney test) of control values. The frequency of channel opening (f) was also decreased to 68% ($f = 9.1 \pm 4.11\ \text{s}^{-1}$) and 44% ($f = 5.9 \pm 2.4\ \text{s}^{-1}$) of control values ($P < 0.05$, by the Mann-Whitney test). A significant decrease in channel-open time was observed at the higher concentration of ketamine. With $1\ \mu\text{M}$ ketamine, channel-open time was reduced to 68% of control. The difference in the fractional reduction of Po' compared with f with $1\ \mu\text{M}$ ketamine can be attributed to the decrease in channel-open time.

The concentration-dependent decrease in mean open time is consistent with a bimolecular interaction between ketamine and a site on the open channel, as illustrated in scheme 1. The relation between drug concentration (ketamine $0\ \mu\text{M}$, $0.1\ \mu\text{M}$, and $1\ \mu\text{M}$) and channel-open time was used to approximate the forward rate constant (k_{+B}) of ketamine blockade (fig. 6). The rate of association, calculated from the slope of the plot of $1/\tau_0$, was $1.4 \times 10^8\ \text{m}^{-1} \cdot \text{s}^{-1}$.

In the Cell-attached Configuration, Addition of Ketamine to the Bath Solution Inhibits NMDA Channels Recorded in the Cell-attached Configuration

The uncharged form of ketamine is highly lipid soluble.³⁴ Thus it is plausible that ketamine can diffuse into

the cell membrane and gain access to a hydrophobic binding site on the NMDA receptor. Therefore we tried to determine if ketamine, added to the bath solution, could inhibit NMDA receptors isolated in the tip of the recording electrode.

Stable single-channel currents were recorded with $10\ \mu\text{M}$ NMDA in the pipette solution. Adding $50\ \mu\text{M}$ ketamine to the bath solution caused a gradual, reversible inhibition of channel opening (fig. 7). The probability of channel opening was significantly reduced compared with control values (Po' , control = 0.046 ± 0.010 , Po' , ketamine = 0.051 ± 0.003 , $n = 5$, $P < 0.05$, by the Wilcoxon signed rank test).

This inhibition was primarily characterized by a decrease in the frequency of channel opening, rather than by a reduction in the mean channel open time (table 2). Three of the five patches remained stable for a prolonged period, and perfusion of the cell with the ketamine-free solution resulted in a gradual increase in the probability of channel opening (Po' , recovery = 0.011 ± 0.002).

Ketamine Inhibition of NMDA Channels Recorded in the Outside-out Configuration

A major advantage of the cell-attached recording method is that the plasma membrane is not disrupted after seal formation. This results in minimal disruption of cytosolic factors that might influence channel

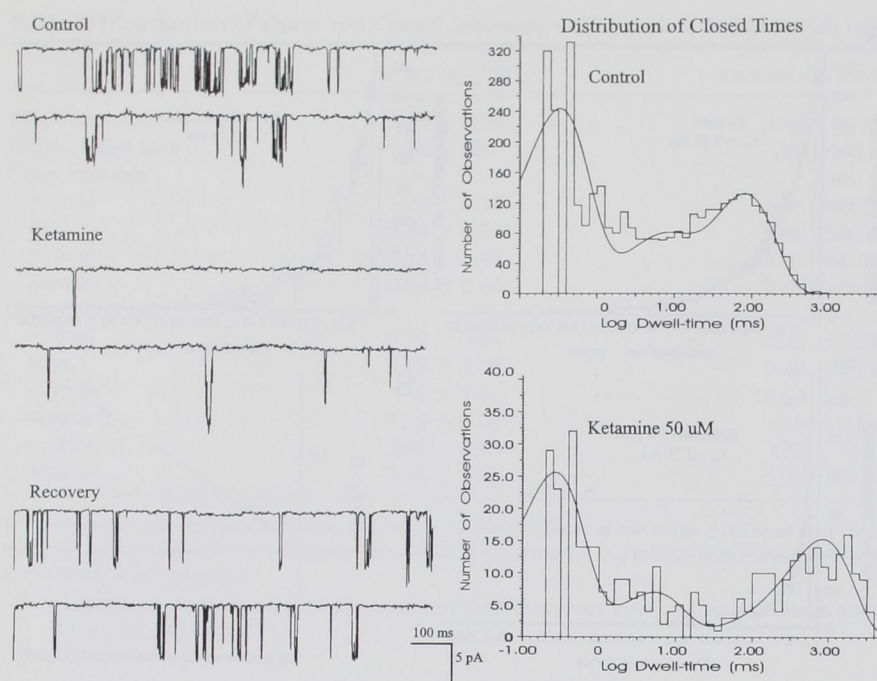


Fig. 7. Ketamine (50 μM) added to the bath solution reversibly inhibited channels present in cell-attached patches. Single-channel currents were recorded from the same cell, in the absence and presence of ketamine. The durations of closed times are plotted as the distribution of log time versus the bin frequency. In the absence of ketamine, the distribution was fitted with the sum of three exponential functions, with time constants and relative areas of $\tau_1 = 0.32$ ms, $a_1 = 0.56$, $\tau_2 = 5.3$, $a_2 = 0.13$, $\tau_3 = 80.7$ ms, and $a_3 = 0.32$, whereas $\tau_1 = 0.23$ ms, $a_1 = 0.53$, $\tau_2 = 5.1$ ms, $a_2 = 0.14$, $\tau_3 = 838.93$ ms, $a_3 = 0.33$ for currents recorded in the presence of ketamine. The mean closed times for the illustrated tracings were $\tau_{c \text{ control}} = 38.9 \pm 1.21$ ms ($n = 3662$ events) and $\tau_{c \text{ ketamine}} = 412 \pm 36.86$ ms ($n = 390$), whereas the mean channel-open times were $\tau_{o \text{ control}} = 2.82 \pm 0.48$ ms ($n = 3,539$) and $\tau_{o \text{ ketamine}} = 1.61 \pm 0.07$ ms ($n = 482$).

gating or drug sensitivity.³⁵ However, the pipette solution cannot be easily exchanged, so the same population of receptors cannot be examined before and

Table 2. Ketamine Added to the Bath Solution

	Control ($n = 5$)	Ketamine 50 μM ($n = 5$)
Po'	0.046 ± 0.01	$0.0051 \pm 0.003^*$
Frequency	19.6 ± 9.3	$2.2 \pm 0.9^*$
Mean open time	2.71 ± 0.10	2.26 ± 0.27
Mean closed time	151 ± 95.3	$730.9 \pm 472^*$
Open intervals†		
τ_1 (ms)	0.86 ± 0.17	0.75 ± 0.14
Area 1	0.34 ± 0.05	0.46 ± 0.06
τ_2 (ms)	6.4 ± 3.4	5.2 ± 1.1
Area 2	0.63 ± 0.06	0.53 ± 0.06
Closed intervals†		
τ_1 (ms)	0.20 ± 0.04	0.30 ± 0.09
Area 1	0.48 ± 0.06	0.56 ± 0.03
τ_2 (ms)	6.5 ± 3.6	6.7 ± 2.8
Area 2	0.25 ± 0.04	0.16 ± 0.03
τ_3 (ms)	376 ± 170	571 ± 174
Area 3	0.26 ± 0.04	0.25 ± 0.02

The effects of 50 μM ketamine applied to the bath solution on single channels recorded in the cell attached configuration.

* The Wilcoxon Rank Sum Test for paired data was used to determine significant differences between values measured before and after drug application ($P < 0.05$). The frequency of channel opening and the probability of channel opening were significantly reduced whereas the mean closed time was increased by ketamine.

† The frequency of channel opening was too low in one of the four recordings to acquire a sufficient number of events to create dwell time histograms.

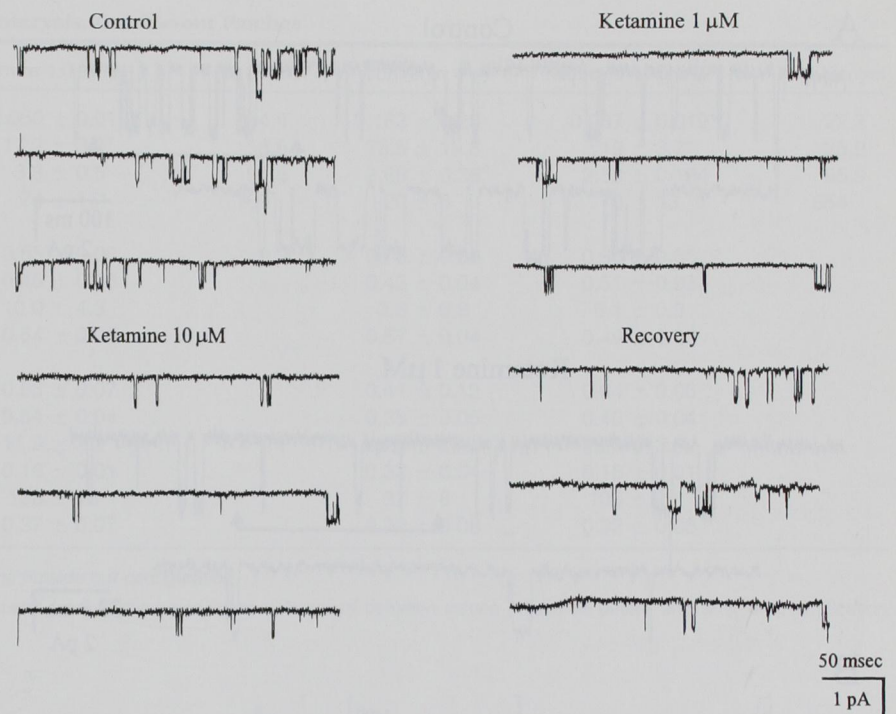
after exposure to a known concentration of drug. To study the same receptor population in the absence and presence of ketamine, we examined the effects of ketamine on NMDA receptors in outside-out membrane patches (fig. 8).

In patches held at -60 mV, applications of NMDA (10 μM) evoked channel openings with a mean current amplitude of 2.69 ± 0.11 pA ($n = 9$); the estimated single channel conductance was 44.8 pS. Application of ketamine (1 μM or 10 μM) did not change the single-channel current amplitude (2.47 ± 0.13 pA, $n = 5$, and 2.50 ± 0.16 pA, $n = 4$, respectively), (fig. 9).

As summarized in table 3, ketamine (1 μM or 10 μM) reduced Po' to 63% and 34% of control values, respectively. For five patches, the steady-state probabilities of at least one channel being open, measured before and after exposure to ketamine (1 μM), were 0.087 ± 0.028 and 0.056 ± 0.019 ($P < 0.05$, by the Wilcoxon signed rank test), respectively. Thus 1 μM ketamine reduced Po' to 64% of control, and 10 μM ketamine decreased Po' to 23% of control. These reductions in Po' were due primarily to a decrease in the frequency of channel opening, rather than to a reduction in the mean channel-open time (table 3). Applications of the competitive NMDA receptor antagonist, DL-2-amino-5-phosphoaleric acid (10–100 μM), reversibly inhibited residual channel openings

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Fig. 8. *N*-methyl-D-aspartate-evoked inward currents from an outside-out patch voltage clamped at -60 mV. Sample traces of single-channel currents show ketamine inhibition and recovery from blockade. Traces were successively recorded from the same cell.



($n = 3$), indicating that the currents were indeed mediated by the NMDA receptor.

Similar to the dwell-time histograms constructed for currents recorded in the cell-attached configuration, open-interval histograms were best fit with the sum of two exponential functions, whereas the closed-duration histograms were best fit with the sum of three exponential functions (fig. 10). A possible decrease in channel open time was apparent with the higher concentration of ketamine ($10 \mu\text{M}$), but this effect was not significant ($P = 0.437$, by the Wilcoxon signed rank test).

Discussion

Single-channel studies were undertaken to identify the mechanisms underlying ketamine blockade of the NMDA receptor. In the absence of ketamine, channel conductance was similar to that previously reported for NMDA receptors in hippocampal neurons.^{36,37} Furthermore, the duration of NMDA channel openings was within the range reported for native hippocampal receptors and cloned NR1a-NMε1 and NR1-NR2A subunits expressed in HEK 293 cells and *Xenopus* oocytes.³⁶⁻⁴¹ The effects of ketamine on single-channel NMDA currents are summarized here.

In cell-attached patches, the inclusion of ketamine ($0.1 \mu\text{M}$ or $1 \mu\text{M}$) in the pipette solution caused a concentration-dependent decrease in both the duration and frequency of NMDA channel openings. These actions are similar to the effects of phencyclidine and its analogs and are consistent with an open-channel mechanism of blockade.¹⁷ However, the decreases in channel-open times induced by $0.1 \mu\text{M}$ and $1 \mu\text{M}$ ketamine (6% and 32%, respectively) were small relative to the reduction in open-channel frequency (32% and 56%, respectively). Furthermore, in cell-attached patches, externally applied ketamine ($50 \mu\text{M}$) caused an 89% decrease in the frequency of channel opening without a significant change in channel-open time. Ketamine blockade of receptors recorded in the outside-out patch configuration was similarly characterized by a decrease in the frequency of open events with little change in open time.

Mechanisms of Ketamine Blockade

As discussed previously, several mechanisms could account for ketamine blockade of the NMDA receptor. Schemes 1, 2, and 3 will now be considered in light of our observations. Scheme 1 is not consistent with the use-dependent recovery from block previously described for ketamine.⁷ In addition, scheme 1 predicts that a new closed state with a mean duration of $1/k_B$

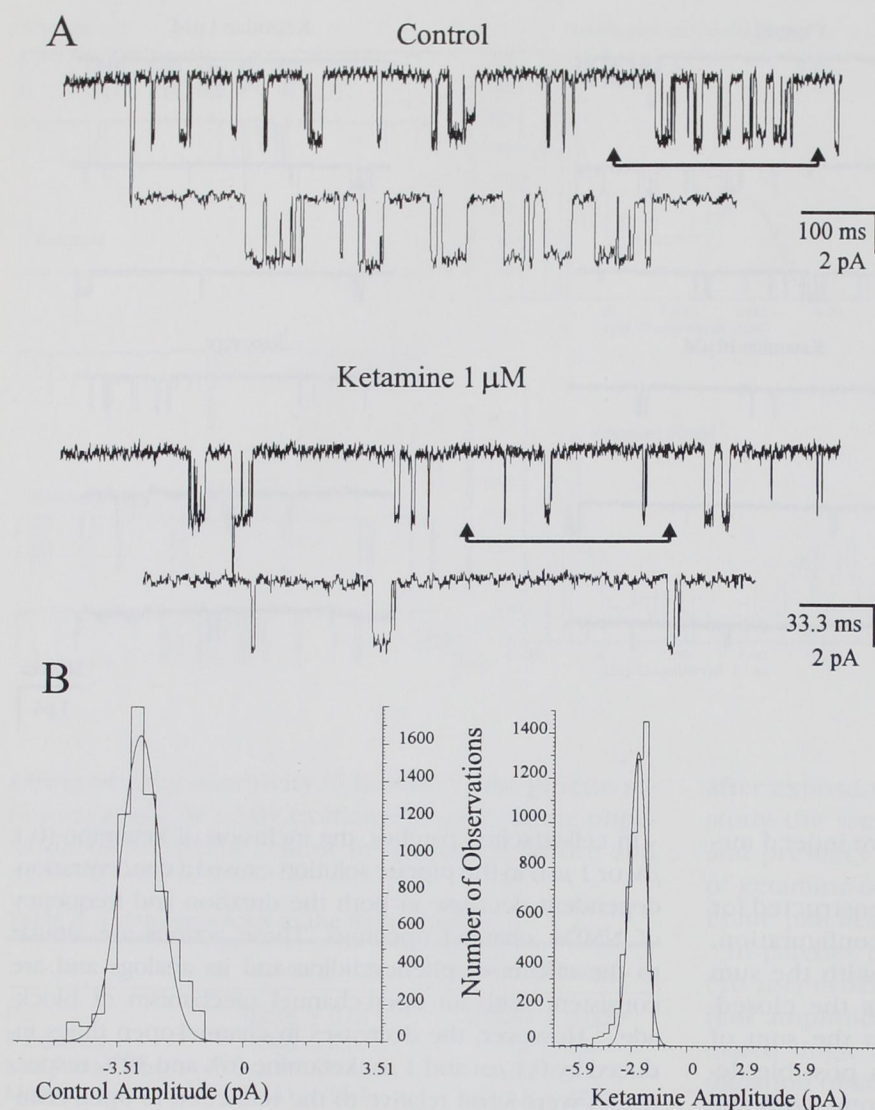


Fig. 9. Currents were recorded from a single outside-out patch, in the absence and presence of ketamine. (A) Current traces are displayed at two different time scales (100 ms and 33.3 ms). The solid bars with arrows indicate the data selected for higher resolution. (B) Histograms of the current amplitude for all openings longer than 300 μ s, recorded in the absence and presence of ketamine (1 μ M), are shown. The distributions were fitted by a single Gaussian curve with mean values of 3.03 ± 0.54 pA ($n = 2,450$ events) and 2.95 ± 0.47 pA ($n = 6,345$ events), respectively.

should be evident in the distribution of the closed times. We observed no change in the fast or intermediate components of the closed-time distributions and no new closed-time components. Thus we rejected scheme 1 as a possible model of ketamine blockade. Both scheme 2 and 3 are consistent with use-dependent recovery from blockade; however, scheme 2 predicts a reduction in mean open time, whereas scheme 3 does not.

In the cell-attached configuration with ketamine added to the recording electrode, there was a concentration-dependent decrease in channel-open time, consistent with scheme 2. The rate of association of ketamine with the open channel was estimated to be $1.4 \times 10^8 \text{ m}^{-1} \cdot \text{sec}^{-1}$. This value is considerably greater than that previously reported for ketamine inhibition

of whole-cell currents ($1.7 \times 10^4 \text{ m}^{-1} \cdot \text{sec}^{-1}$ and $9 \times 10^4 \text{ m}^{-1} \cdot \text{sec}^{-1}$).^{7,33} However, the time course of inhibition of whole-cell currents depends not only on the association rate constant but also on the probability of channel opening (P_o) where $k_{on} = 1/P_o (\alpha + k_{+B} [B])$. The maximum probability of NMDA channel opening is not known, and an overestimation of P_o results in an underestimation of k_{+B} . Thus molecular rate constants are more directly determined from single-channel experiments. Although our value of $1.4 \times 10^8 \text{ m}^{-1} \cdot \text{sec}^{-1}$ is presented only as an approximation, it suggests that the rate of ketamine binding is of the order of magnitude expected for a fast channel blocker. However, the observed decrease in channel-open time accounted, only in part, for the decrease

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Table 3. Distribution of Open and Closed Intervals, Outside-out Patches

	Control (n = 5)	Ketamine 1.0 μ M (n = 5)	% Control	Control (n = 4)	Ketamine 10 μ M (n = 4)	% Control
Po'	0.087 \pm 0.028	0.056 \pm 0.019*	64.4	0.163 \pm 0.25	0.037 \pm 0.019*	22.7
Frequency	29.4 \pm 4.4	17.2 \pm 3.8*	58.5	73.5 \pm 19.8	19 \pm 3.7*	25.9
Mean open time	3.6 \pm 0.3	3.3 \pm 0.5	91.6	2.69 \pm 0.36	2.58 \pm 0.164	95.9
Mean closed time	44 \pm 12	74 \pm 15*	169	20 \pm 6	70 \pm 33	354
Open Intervals						
τ_1 (ms)	0.60 \pm 0.05	0.55 \pm 0.06		0.73 \pm 0.08	0.98 \pm 0.65	
Area 1	0.33 \pm 0.03	0.46 \pm 0.06		0.43 \pm 0.04	0.51 \pm 0.03	
τ_2 (ms)	11.5 \pm 2.5	10.0 \pm 4.3		3.6 \pm 0.8	6.1 \pm 0.9	
Area 2	0.65 \pm 0.05	0.54 \pm 0.06		0.57 \pm 0.04	0.49 \pm 0.03	
Closed intervals						
τ_1 (ms)	0.29 \pm 0.04	0.55 \pm 0.07		0.41 \pm 0.12	0.64 \pm 0.06	
Area 1	0.48 \pm 0.03	0.54 \pm 0.04		0.38 \pm 0.05	0.40 \pm 0.04	
τ_2 (ms)	2.6 \pm 1.50	11.9 \pm 3.2		3.6 \pm 2.1	13.0 \pm 3.8	
Area 2	0.23 \pm 0.02	0.18 \pm 0.01		0.33 \pm 0.04	0.18 \pm 0.01	
τ_3 (ms)	83 \pm 19	138 \pm 16		37 \pm 8	106 \pm 24	
Area 3	0.10 \pm 0.06	0.37 \pm 0.07		0.32 \pm 0.08	0.32 \pm 0.06	

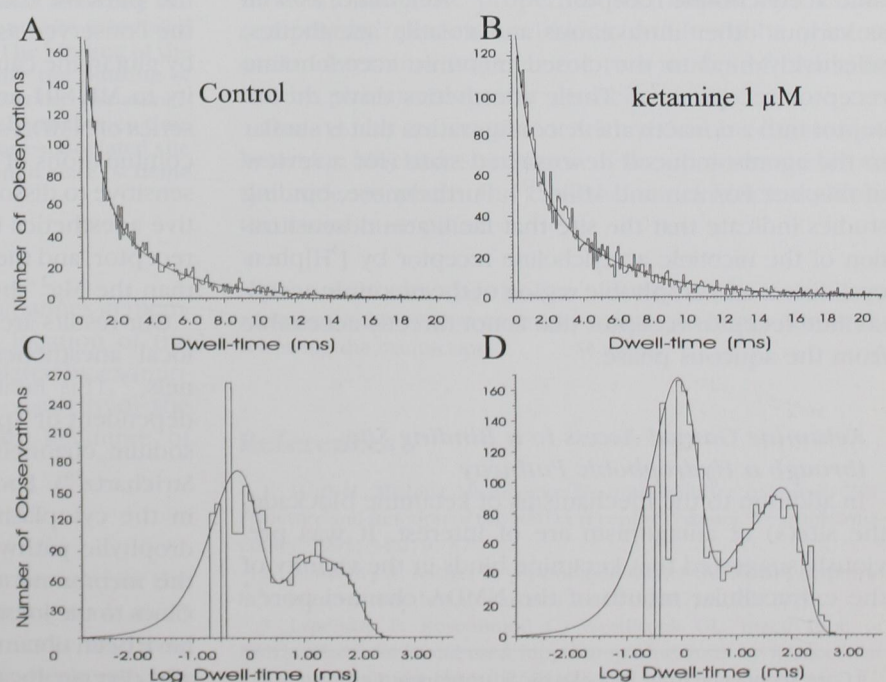
Ketamine inhibition of NMDA receptors recorded in the outside out configuration.

* The Wilcoxin Rank Sum Test for paired data was used to determine significant differences between values measured before and after drug application ($P < 0.05$).

in Po', suggesting that the channel could close with ketamine bound to the receptor. This closed-blocked state would reduce the number of channels available for activation and thereby reduce the frequency of channel opening. These observations are consistent with scheme 2.

In outside-out patches or cell-attached patches with ketamine added outside the recording electrode, a large reduction in opening frequency was observed with no significant change in mean open time (despite the presence of high concentrations of ketamine). Detailed single-channel studies on NMDA

Fig. 10. Open- and closed-time distributions of N-methyl-D-aspartate receptors recorded in the outside-out configuration. (A) The open-duration histogram was fitted with the sum of two exponential functions that had fast and slow time constants (ms) and relative areas of $\tau_1 = 0.74$ ms, $a_1 = 0.26$, $\tau_2 = 3.6$ ms, $a_2 = 0.75$. (B) In the presence of 1 μ M ketamine, $\tau_1 = 0.81$ ms, $a_1 = 0.2$, $\tau_2 = 4.1$ ms and $a_2 = 0.81$. (C) The closed-duration histograms were best fit with three exponential functions. Under control conditions, $\tau_1 = 0.60$ ms, $a_1 = 0.6$, $\tau_2 = 8.11$ ms, $a_2 = 0.16$, $\tau_3 = 39.47$ ms, $a_3 = 0.25$, whereas $\tau_1 = 0.8$ ms, $a_1 = 0.6$, $\tau_2 = 7.6$ ms, $a_2 = 0.09$, $\tau_3 = 65$ ms and $a_3 = 0.34$ in the presence of ketamine (D).



receptor function suggest that the receptor kinetics are characterized by several agonist-bound inactive states.⁴²⁻⁴⁷ Any one of these states might preferentially enhance ketamine binding and lead to blocked states. This would reduce the number of channels available for opening but produce no change in mean open time. We observed that only the longest closed time was prolonged by ketamine, as expected from a decrease in the number of channels available for normal activation. The lack of change in the open-time constants and the fast and intermediate closed-time constants suggests that ketamine, acting through a membrane delimited pathway does not modulate channel gating.

Our data suggest that ketamine acts by at least two mechanisms: an open-channel block, as evidenced by the decrease in τ_o , and a closed block characterized by a decrease in open frequency with no change in open time. It is not known if distinct sites mediate open and closed blockade. Furthermore, the kinetics of the two mechanisms appear to depend on the configuration of the membrane patch. There was an apparent decrease in drug sensitivity after patch excision. Factors that could contribute to changes in drug sensitivity include disruption of the cytoskeleton and alterations of cytosolic factors that influence the sensitivity of the receptor to antagonists.³⁵

Open and closed mechanisms of blockade were previously proposed for ketamine inhibition of the nicotinic acetylcholine receptor.^{21,22,47-50} Ketamine, as well as various other intravenous and volatile anesthetics, selectively bind to the closed nicotinic acetylcholine receptor.^{17,22} These anesthetics drive the receptor into a nonactivatable configuration that is similar to the agonist-induced desensitized state (for a review of this, see Forman and Miller⁵¹). Furthermore, binding studies indicate that the site that facilitates desensitization of the nicotinic acetylcholine receptor by [³H]phenylcyclidine is a hydrophobic region of the nicotinic acetylcholine receptor that is not directly accessible from the aqueous phase.⁵²

Ketamine Gained Access to a Binding Site through a Hydrophobic Pathway

In addition to the mechanisms of ketamine blockade, the site(s) of antagonism are of interest. It was previously suggested that ketamine binds in the vicinity of the extracellular mouth of the NMDA channel pore.⁷

However, we observed that ketamine added to the bath solution inhibited NMDA receptors recorded in the cell-attached configuration. It is unlikely that ketamine gained access to the extracellular mouth of the channel by diffusing across the high-resistance seal (>10 G Ω) and accumulating in the tip of the recording electrode. Ketamine is formulated as a hydrochloride salt and is highly water soluble, with a pK_a (negative logarithm of the acid ionization constant) of 7.5.³⁴ Under physiologic conditions, a large fraction of the drug exists in the lipid-soluble form (44%). The octanol/buffer partition coefficient was reported to be 398 (pH 7.4).^{||} Therefore the concentration of ketamine in the lipid phase is several orders of magnitude greater than in the aqueous phase. Our results suggest that ketamine gained access to a blocker site associated with the lipid membrane or the lipid-protein interface, as indicated in figure 11. Indeed, the presence of a hydrophobic pathway for dissociated anesthetics was predicted from binding studies of the antagonist [³H]MK-801.⁴⁶ Javitt and Zukin⁴⁶ postulated that [³H]MK-801 gained access to the slow hydrophobic pathway, which was independent of channel gating and a fast hydrophilic pathway associated with channel opening.

The site(s) of blockade by uncompetitive antagonists, including ketamine, have been investigated using cloned NMDA receptor subunits and site-directed mutagenesis.^{53,54} Dissociative anesthetics are thought to bind to a site associated with the Mg^{2+} binding location on the putative transmembrane domain. Replacement of the conserved asparagine 598 residue in the M2 region by glutamine caused a decrease in the receptor sensitivity to MK-801 and Mg^{2+} .⁵⁴ However, examination of a series of NMDA subunit heteromers revealed that not all combinations of NMDA receptor subunits were equally sensitive to dissociative anesthetics: Not all the dissociative anesthetics bind to an identical site on the NMDA receptor, and their effects were mediated by a site other than the Mg^{2+} binding domain.⁵³

Our results are somewhat analogous to the actions of local anesthetics on voltage-activated sodium channels.⁵⁵ This family of compounds induce both a use-dependent or "phasic" block and "tonic" inhibition of sodium channels (for a review, see Butterworth and Strichartz⁵⁶). Local anesthetics are thought to dissolve in the cytoplasm and reach the channel *via* the hydrophilic pathway. In addition, they may diffuse into the membrane and bind *via* a hydrophobic pathway. Clues to the location of binding sites of local anesthetics have been obtained by examining the effects of structurally diverse local anesthetics and their effects under

|| Correspondence from Parke-Davis, Scarborough, Canada.

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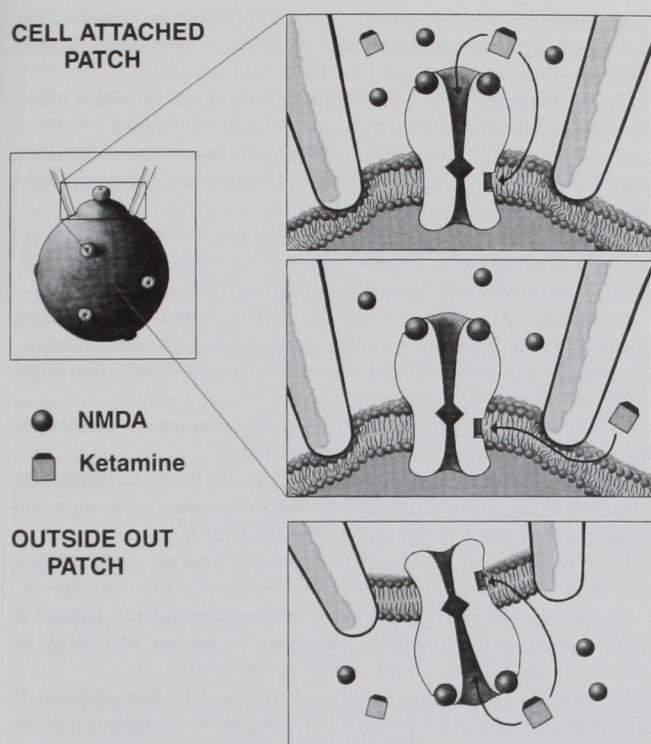


Fig. 11. Patch configurations used to investigate ketamine blockade of the *N*-methyl-D-aspartate (NMDA) receptor. For the cell-attached patches, NMDA was included in the pipette solution. Ketamine was included either in the pipette or added externally to the bath solution. In the outside-out patch configuration, both ketamine and NMDA were added to the external solution. Changes in NMDA receptor activity are schematically interpreted. Our results suggest that ketamine may interact with the receptor at two potentially distinct sites: one site located within the channel pore and a second site associated with a hydrophobic domain of the protein. The binding of the agonist to the receptor is assumed to modify the binding of ketamine to both sites. Binding of ketamine at the site associated with the channel pore would decrease channel-open time (scheme 2), whereas binding to the membrane-associated site does not require the channel to be in the open state (scheme 3).

different recording conditions (such as changes in membrane potential or pH). Similarly, the location of the ketamine-binding site(s) might be elucidated by examining the stereoselectivity and voltage sensitivity of the closed- and open-channel blockade by ketamine of NMDA receptors.

Clinical Implications

Ketamine has unique clinical properties and is the anesthetic of choice in specific situations, such as in patients with a compromised hemodynamic status or asthma.¹⁰ More recently, ketamine has been used in

subanesthetic doses as a potent analgesic. In humans, inhibition of the NMDA receptor is thought to mediate ketamine's clinical properties.^{57,58} During anesthesia, the peak plasma concentration in humans is approximately 8.5–9.5 μM , whereas analgesia is associated with plasma concentrations of approximately 0.55 μM .^{10,59} In rats, the peak plasma concentration associated with anesthesia is less than 36 μM .⁶⁰ Ketamine in the plasma exists in two states: a free unbound form and a form bound to plasma proteins.^{61,62} The free fraction of ketamine determines the rate of diffusion to a site of action. In humans, as much as 47% of ketamine is bound to plasma proteins.⁶¹ However, ketamine is highly lipid soluble and the brain-to-plasma ratio for ketamine is estimated to be 6.5:1, suggesting that ketamine preferentially accumulates in the brain.⁶³ Thus it is possible that, during anesthesia, the concentration of ketamine present at the NMDA receptor is considerably higher than the plasma concentration.

We observed that clinically relevant concentrations of ketamine induced both an open and closed blockade of the NMDA receptor. It is of interest to determine which of these mechanisms contribute to ketamine's clinical actions. The predominance of closed-channel blockade observed at low concentrations of ketamine suggests that ketamine's analgesic properties might result from closed- rather than open-channel blockade. In this regard it is notable that the anti-Parkinsonian drugs, memantine and amantadine, have no appreciable anesthetic or analgesic properties and inhibit the NMDA receptor by open-channel blockade.⁶ It will be of interest to determine whether the two modes of blockade can be pharmacologically dissociated.^{39,64–66}

Our results are consistent with a dual mechanism of ketamine blockade: occlusion of the open channel from the aqueous phase and closed-channel blockade from the membrane phase.

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