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Arcaine Blocks *N*-Methyl-D-aspartate Receptor Responses by an Open Channel Mechanism: Whole-Cell and Single-Channel Recording Studies in Cultured Hippocampal Neurons

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SUMMARY

Arcaine, a putative competitive antagonist at the polyamine site on the *N*-methyl-D-aspartate (NMDA) receptor complex, not only inhibits polyamine enhancement of NMDA-induced [^{3}H]dizocilpine (MK-801) binding but also depresses binding in the absence of polyamines. In the present experiments, we investigated the mechanism of this latter effect in whole-cell and single-channel recordings from cultured rat hippocampal neurons. Arcaine produced a concentration-dependent block of NMDA-evoked inward currents (K_D, 61 μM at −60 mV) but not those induced by kainate, α-amino-3-hydoxy-5-methyl-4-isoxazolepropionic acid, or γ-aminobutyric acid. The arcaine block was strongly voltage dependent and was almost completely relieved at positive holding potentials. Analysis of the voltage dependence indicated that the arcaine acceptor site appeared to sense 67% of the transmembrane electric field. In support of an open channel blocking mechanism, arcaine, like Mg^{2+}, prevented dizocilpine from blocking the NMDA receptor channel. Moreover, increasing the dizocilpine concentration partially overcame the arcaine effect, indicating a competitive interaction between arcaine and dizocilpine. Spermine, which in our preparation usually produced only an arcaine-like voltage-dependent block of NMDA currents at high concentrations (>100 μM), had no effect on the block by arcaine at lower concentrations. In single-channel recordings, arcaine caused a concentration- and voltage-dependent decrease in apparent channel amplitude. Assuming a simple model of open channel block, we estimate the arcaine binding and unbinding rates as 4.4×10^8 M^{-1} sec^{-1} and 1.8×10^4 sec^{-1}, respectively, which are comparable to the rates for open channel block by Zn^{2+} and substantially faster than those of Mg^{2+}. These results indicate that arcaine inhibits NMDA-induced [^{3}H]dizocilpine binding by blocking the open NMDA receptor channel, an action that is independent of the polyamine site.

The NMDA receptor is the most well characterized of the various subtypes of glutamate receptor. A great deal of interest has been focused on this receptor because of its importance in normal brain function and in pathophysiological conditions such as epilepsy and cerebral ischemia (1). Electrophysiological and biochemical studies have identified a number of regulatory sites on the NMDA receptor complex at which drugs can act to modulate NMDA receptor-mediated responses (2, 3). Certain structural analogs of L-glutamate act as competitive antagonists by binding to the NMDA recognition site (4). Other amino acid analogs compete with glycine at the strychnine-insensitive glycine coagonist site and, thus, act as noncompetitive antagonists of NMDA receptor-mediated responses (4). Divalent cations such as Mg^{2+}, Co^{2+}, and Mn^{2+} bind to a site in the ionophore of the NMDA receptor complex to produce a voltage-dependent block (5–7), whereas other, group IIb, cations such as Zn^{2+} and Cd^{2+} bind to a site outside the transmembrane electric field to produce a voltage-independent antagonism of NMDA responses (8–10) (but see Ref. 11). A number of other drugs, such as the dissociative anesthetics dizocilpine (MK-801), PCP, and ketamine (12–14), tricyclic antidepressants (15), and THA (16), also block NMDA-evoked currents by binding to a site in the open channel.

Recently, another site on the NMDA receptor complex at which drugs can act to regulate its functional activity has been identified. This is the polyamine recognition site, which has been characterized on the basis of radioligand binding studies showing that the polyamines spermine and spermidine enhance [^{3}H]dizocilpine binding to the NMDA receptor channel at low concentrations (with a reversal of the effect at higher concentrations) (17). Moreover, electrophysiological studies have demonstrated that low concentrations (~1 μM) of spermine and spermidine enhance NMDA-evoked currents in cultured stria-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; KA, kainic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, γ-aminobutyric acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCP, phencyclidine; DA10, diaminodecane; THA, tetrahydroaminoacridine.

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tal (18) and hippocampal (19) neurons and also in oocytes transfected with rat brain RNA (20, 21). Binding and electrophysiological studies have identified a number of antagonists at the polyamine site, including putrescine, dielthylenetriamine, and DA10 (19, 22, 23). Recent binding studies have indicated that arcaine (diguanidinobutane) is also a competitive antagonist at the polyamine site (24). Thus, arcaine produces a parallel shift to the right in the concentration-response curve for spermine (24). However, in addition to inhibiting spermine responses, arcaine also reduced NMDA-induced [^{3}H]dizocilpine binding in the absence of exogenous spermine (24). Possible explanations for this effect are that low levels of spermine may still be present in the washed membranes or, alternatively, that arcaine acts as an inverse agonist at the polyamine site, as may be the case for DA10 (19, 22). On the other hand, arcaine could reduce NMDA-induced [^{3}H]dizocilpine binding by a mechanism unrelated to its actions at the polyamine site.

In the present study, we used electrophysiological techniques to examine the interaction of arcaine with the NMDA receptor. Our results demonstrate that arcaine produces a selective, concentration-dependent blockade of NMDA-evoked currents. This is due to a voltage-dependent interaction of arcaine with open NMDA channels that is similar to but more rapid than that produced by Mg^{2+}. The arcaine block was unaltered by spermine, indicating that it is not mediated through an action at the polyamine site.

Materials and Methods

Cell culture. Hippocampal neurons from 19-day-old Sprague-Dawley rat embryos were grown in primary culture, as described by Segal (25). Briefly, hippocampal tissue was suspended by passage through a Pasteur pipette, and the cell suspension was plated onto polyornithine- or Matrigel (Collaborative Research Inc., Bedford, MA)-coated culture dishes containing modified Eagle's medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 5% horse serum, and 1% glutamine. In addition, transferrin, insulin, selenium, corticosterone, triiodothyronine, progesterone, and putrescine were added to the medium, as described by Guthrie *et al.* (26). The cultures were incubated at 37° in a humidified atmosphere containing 10% CO_2. The culture medium was replaced every 7 days with a similar medium as described above, but lacking fetal calf serum. Cells were used for electrophysiology 7–12 days after plating.

Whole-cell electrophysiology. Electrophysiological recordings were carried out at room temperature on the stage of an inverted phase-contrast microscope. Before each experiment, the culture medium was removed, the cells were rinsed completely, and the culture dish was partially filled with bathing solution containing (in mM) 140 NaCl, 5 KCl, 0.1 $CaCl_2$, and 10 HEPES. The bathing medium also contained 1 μM tetrodotoxin to block voltage-activated Na^+ currents and 1 μM strychnine to block glycine-activated Cl^- channels. The osmolality was adjusted to 315–325 mOsm with sucrose, and the pH was adjusted to 7.4 with NaOH.

Patch pipettes (4–10 MΩ) were prepared from filament-containing thin-wall glass capillary tubes (1.5-mm outer diameter) (World Precision Instruments, New Haven, CT), using a two-stage vertical pipette puller (model L/M-3P-A; Adams & List Associates, Westbury, NY). The electrodes were filled with recording solution containing (in mM) 145 potassium gluconate, 2 $MgCl_2$, 5 HEPES, 0.1 $CaCl_2$, and 1 EGTA. In experiments in which the holding potential was varied, the potassium gluconate was replaced with 145 mM CsCl. The osmolality was adjusted to 310 mOsm with sucrose, and the pH was adjusted to 7.4 with KOH (or CsOH in the CsCl recording solution).

Whole-cell currents were recorded with an Axopatch 1B or 1C patch-clamp amplifier (Axon Instruments, Burlingame, CA) and displayed on a high-speed ink pen recorder (Gould Electronics, Cleveland, OH). The holding potential was maintained at −60 mV unless otherwise noted. Drugs were dissolved in buffer on the day of use and applied via a rapid perfusion system similar to the one described by Tang *et al.* (27), which allowed rapid switching between solutions (<100 msec exchange time). The perfusion device consisted of a seven-barrel array of glass capillary tubes (0.32-mm outer diameter) (J & W Scientific, Folsom, CA), which all emptied via a common tip approximately 200 μm in diameter. The tip was positioned approximately 200 μm from the cell. Flow through each barrel was gravity fed and regulated by microvalves controlled by a microprocessor-based programmable clock. One barrel contained buffer and the others contained various agonists and antagonists alone and in combination. Only one valve was opened at a time, and buffer was applied continuously between drug applications. All NMDA-containing solutions also contained 10 μM glycine (except as otherwise noted).

Single-channel recording. Recordings were obtained from single NMDA channels in excised outside-out membrane patches. The patch pipette contained either the potassium gluconate or CsCl solutions described above. Once an outside-out patch was obtained, it was placed near the mouth of the perfusion pipette. Membrane currents were filtered at 1 kHz (−3 dB; four-pole, low-pass Bessel filter), digitally sampled at 10 KHz, and acquired on a microcomputer using the pClamp (CLAMPEX) or Axotape software packages (Axon Instruments, Burlingame, CA). Data were collected in 30–60-sec epochs.

Data analysis. The fractional block (B) of NMDA-, KA-, AMPA-, or GABA-evoked currents by arcaine in the whole-cell experiments was calculated according to the formula $B = 1 - I_{ARC}/I$, where I is the steady state current evoked by the agonist and I_{ARC} is current evoked by the agonist in the presence of arcaine. Differences between two means were compared with the Student's t test; the criteria for statistical significance was $p < 0.05$. All quantitative data are expressed as mean ± standard error; n is the number of neurons or membrane patches tested.

Concentration-effect data were fit to the logistical equation:

$$B = \frac{1}{1 + (K_D/[\mathrm{ARC}])^{n_H}} \quad (1)$$

where [ARC] is the arcaine concentration, K_D is the concentration of arcaine resulting in 50% block (IC_{50}), and n_H is an empirical parameter describing the steepness of fit and having the same meaning as the Hill coefficient.

The single-channel data were analyzed using the FETCHAN module of the pClamp software package. Analyses were performed on continuous 20–30-sec runs of channel activity. Openings and closings of channels were detected using a 50% threshold criterion with respect to the main open state (except for the patch shown in Fig. 6, in which the threshold was set at a sufficiently low level to detect the small-amplitude substate events). Amplitude histograms were fitted to the sum of one or more Gaussian functions using the least-squares curve-fitting routine in PSTAT. Openings or closings briefer than 200 μsec were ignored except in the determination of burst durations, where a dwell time threshold of 3 msec was used (see Results).

The rates of channel block and unblock were estimated from single-channel current records using the method described by Yellen (28). Our analysis assumes that the reduction in channel amplitude produced by arcaine can be described by a two-state process, in which the open channel alternates rapidly (flickers) between the blocked and unblocked states (30), with a blocking rate β and an unblocking rate α. If the current signal is passed through a first-order filter of time constant τ, the amplitude distribution of the filtered output is a β distribution described by the probability density function:

$$f(y) = y^{(a-1)}(1-y)^{(b-1)}/B(a,b)$$

where $a = \alpha\tau$ and $b = \beta\tau$ and

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$$B(a,b) = \int_0^1 y^{(a-1)}(1-y)^{(b-1)}dy$$

Because our events were filtered using the multistage filter of the patch-clamp amplifier, we estimated the parameter τ by passing simulated single-channel records for a two-state blocking process (generated by a QS-1 Quantipore Stochastic Simulator, Instrutech Corp., Elmont, NY) through the patch-clamp amplifier and filter. The point by point amplitude histograms developed from the simulated signals could be well described by a β distribution. The parameters a and b of the β distribution generated by the simulation provided an estimate of τ as 502 μsec.

The actual single-channel records were analyzed in a similar fashion. Point by point amplitude histograms were generated from portions of experiments (10–30-sec epochs) that contained only one active channel with little substate channel activity and that maintained a stable baseline current level. The histograms were normalized to the full channel amplitude in the absence of arcaine. The β distribution was then fit by eye to the histograms, by adjusting the two parameters a and b.

Drugs. AMPA was obtained from Tocris Neuramin (Essex, England) and dizocilpine hydrogen maleate [(+)-10,11-dihydro-5-methyl-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate] (MK-801) from Research Biochemicals Inc. (Natick, MA). All other drugs were from Sigma Chemical Co. (St. Louis, MO).

Results

NMDA responses were evoked in the whole-cell experiments with a perfusion solution containing 10 μM NMDA and 10 μM glycine; the Ca^{2+} concentration was 0.1 mM, as normally present in the bath solution. Under these conditions, NMDA elicited a rapidly rising inward current that showed little desensitization during prolonged (20–30-sec) applications (Fig. 1A). At a holding potential of −60 mV, inward currents evoked by 10 μM NMDA ranged from 180 to 820 pA in amplitude (mean ± standard error of 452 ± 42 pA, in 24 representative cells).

Arcaine block. As illustrated in Fig. 1A, arcaine caused a concentration-dependent block of the NMDA-evoked inward current. Arcaine diminished the amplitude of the NMDA responses without altering their shape, indicating that the inhibitory effect of arcaine was rapid in onset (<100 msec) and recovery (<100 msec). This suggests that, if the block occurs by an open channel mechanism (see below), the rate of binding and unbinding must be rapid, compared with the gating of the channel. Arcaine concentrations as low as 10 μM reliably reduced NMDA-evoked currents, and complete blockade of the response occurred at 600 μM. The concentration-effect data were fit to a sigmoidal curve (Fig. 1B) according to eq. 1, with K_D = 61 μM and n_H = 1.2.

Specificity of block. In order to assess the specificity of the arcaine block, we tested the drug on currents evoked by the excitatory amino acids KA and AMPA and the inhibitory amino acid GABA. As shown in Fig. 2, arcaine (300 μM) only minimally affected currents evoked by 30 μM KA or 5 μM AMPA, whereas in the same cell it almost completely blocked the NMDA-evoked current. In a series of similar experiments, the fractional block by 300 μM arcaine of KA- and AMPA-evoked currents was 0.06 ± 0.02 (n = 5) and 0.08 ± 0.03 (n = 7), respectively. Similarly, 300 μM arcaine had minimal effects on GABA-evoked currents (fractional block, 0.03 ± 0.03; n = 4).

Mechanism of block. To determine whether arcaine blocked NMDA currents by competitively displacing NMDA or glycine from their binding sites on the NMDA receptor complex, we investigated the block by 50 μM arcaine at different concentrations of NMDA or glycine. As shown in Fig. 3A, raising the concentration of NMDA from 3 to 50 μM (which resulted in a 10-fold increase in the amplitude of the NMDA-evoked current) did not affect the block of the NMDA current by 50 μM arcaine (n = 6). As the glycine concentration was lowered from 1 to 0.1 μM, there was a slight but nonsignificant increase in the fractional block of the NMDA current by arcaine (Fig. 3B; n = 4). These observations suggest that arcaine does not block NMDA-evoked currents by displacing NMDA or glycine from their binding sites on the NMDA receptor complex.

It has been proposed that arcaine acts as a competitive antagonist at the polyamine site on the NMDA receptor (24). The fact that arcaine is able to block NMDA-evoked current in the absence of added polyamines makes it difficult to interpret actions of arcaine at this site. Nevertheless, if arcaine does act as a competitive antagonist at the polyamine site, then increasing the polyamine concentration should reverse the ar-

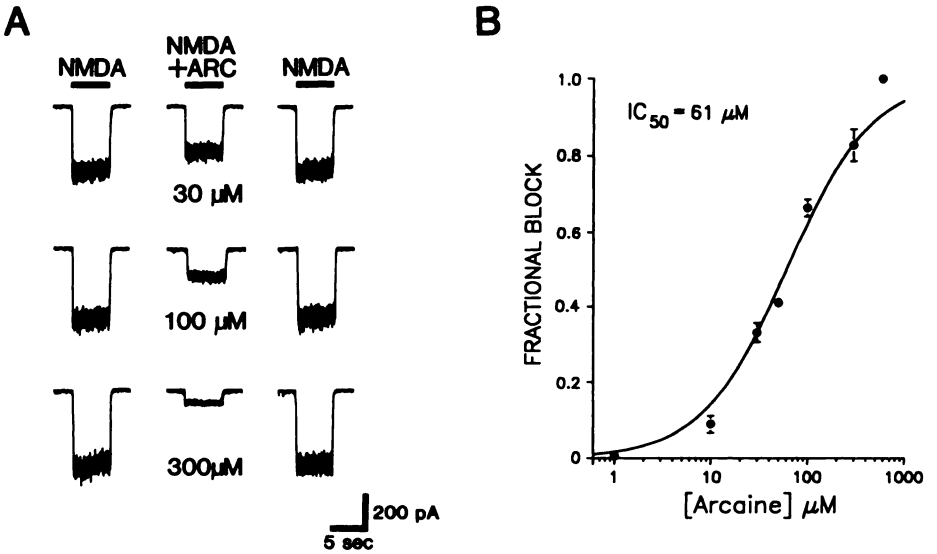

Fig. 1. Concentration-dependent antagonism of NMDA-evoked inward currents by arcaine. A, Responses to 5-sec pulses of 10 μM NMDA in the presence and absence of 30, 100, and 300 μM arcaine (*ARC*). Each *row* shows three consecutive pulses; all nine records are from the same cell. Holding potential, −60 mV. B, Concentration-response curve for the reduction of NMDA-evoked inward currents by arcaine. Each *point* is the mean ± standard error of 4–13 neurons. The data were fit the logistical equation given in the text (Eq. 1), with K_D = 61 ± 5 μM and n_H = 1.2 ± 0.1.

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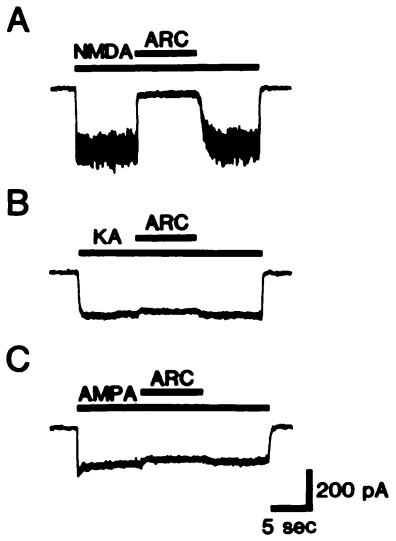

Fig. 2. Selectivity of the arcaine block. Comparison of the effect of 300 μM arcaine (*ARC*) on inward currents induced by 10 μM NMDA (A), 30 μM KA (B), and 5 μM AMPA (C). All three traces are from the same neuron. Holding potential, −60 mV.

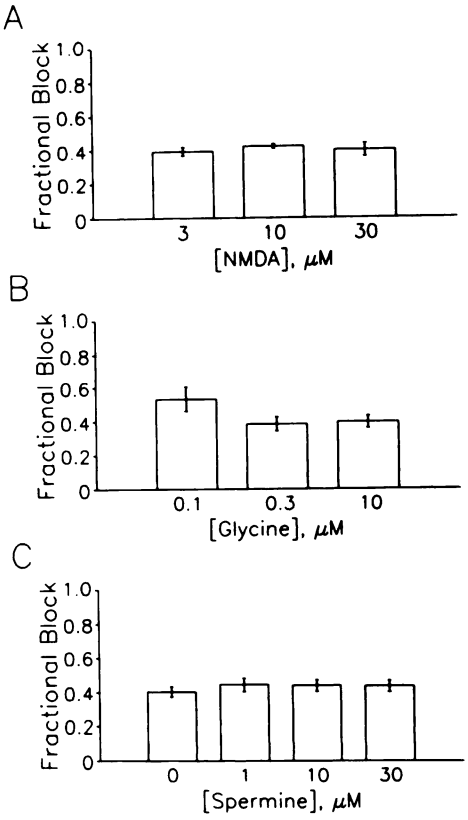

Fig. 3. Arcaine does not act at the NMDA, glycine, or polyamine recognition sites. The fractional block of NMDA-induced currents (−60 mV) by 50 μM arcaine is shown for increasing concentrations of NMDA (A) (n = 6), glycine (B) (n = 4), and spermine (C) (n = 7). Except as indicated, the concentration of NMDA was 10 μM, glycine was 10 μM, and the perfusion solution was nominally spermine-free. *Bars*, standard error.

caine block. Therefore, we examined the effect of spermine on the arcaine block of NMDA-evoked currents. Spermine, at concentrations that in our preparation usually had no direct effect on NMDA currents (1–30 μM), was not able to reduce the block of NMDA currents by 50 μM arcaine (Fig. 3C). (In a small proportion of experiments, spermine caused a potentiation of NMDA responses, but this occurred too infrequently to permit a detailed analysis.) We did not test the effects of higher concentrations of spermine on the block produced by arcaine, because at high concentrations (300 μM) spermine itself caused a voltage-dependent block of NMDA currents (n = 5), in a manner similar to that of arcaine (see below). Because polyamines may act at an intracellular site (29), we tested the effect of intracellular spermine on the arcaine block of NMDA currents. The fractional block produced by 50 μM arcaine in recordings with 10 μM spermine in the patch pipette (0.44 ± 0.06; n = 3) was no different than that in the absence of spermine.

Voltage dependence of block. The voltage dependence of the block by 100 μM arcaine was examined in eight neurons. The current traces in Fig. 4A illustrate the results in one such cell. At −60 mV, arcaine caused a substantial block of the NMDA current. As the holding potential was raised to more depolarized levels the fractional block of the NMDA current was reduced, and at positive holding potentials it was almost completely relieved. The mean NMDA current (normalized to control at −60 mV) for the eight cells, in the presence and absence of arcaine, is plotted against holding potential in Fig. 4B. The current-voltage curve was linear in the absence of arcaine but showed marked rectification in the presence of arcaine at negative potentials. The mean normalized NMDA current in the presence of arcaine was significantly different from control only at holding potentials more negative than −20 mV.

Arcaine, a diguanidine, is likely to be charged at physiological pH. Therefore, the voltage dependence of block may occur because the apparent affinity with which arcaine binds to its blocking site is modified by the membrane electric field. The voltage dependence of the block was analyzed according to the model of Woodhull (31), which is based upon the binding of a single blocking ion to a site within the transmembrane field, under the condition that there is no interaction between the blocking particle and other ions in solution or bound to the channel. The model allows the fraction of the transmembrane field sensed by the ion at its binding site to be calculated, based upon an exponential dependence of apparent binding affinity on membrane potential. Specifically, in the present experiments, the ratio of the NMDA current in the absence and presence of arcaine (I/I_{ARC}) can be related to the transmembrane potential difference (V) by the relationship

$$I/I_{ARC} = 1 + \{[ARC]/K_D(0)\} \exp(-zFV\delta/RT) \quad (2)$$

where $K_D(0)$ is the dissociation constant of the arcaine-acceptor site complex at 0 transmembrane potential, δ is the fraction of the electric field experienced at the acceptor site, z is the charge of arcaine, and F, R, and T have their usual meanings. Eq. 2 can be linearized by rearranging and taking the natural logarithms of both sides, resulting in the following equation for a straight line

$$\ln(I/I_{ARC} - 1) = \ln\{[ARC]/K_D(0)\} - (z\delta F/RT) \times V \quad (3)$$

in which $K_D(0)$ and δ can be determined from a plot of $\ln(I/I_{ARC} - 1)$ against V. The data from Fig. 4B are expressed in this fashion in Fig. 4C. A linear least-squares fit gives a $K_D(0)$ of 278 μM and δ of 0.67 (assuming the charge of arcaine at

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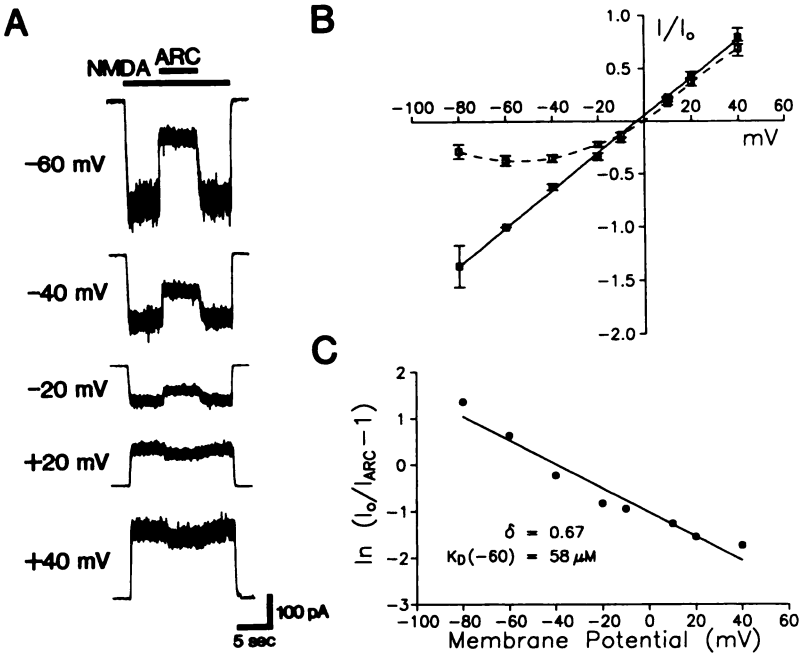

Fig. 4. Voltage-dependent block of NMDA-evoked currents by arcaine. A, Effect of 100 μM arcaine (*ARC*) at holding potentials of −60, −40, −20, +20, and +40 mV. All traces are from the same cell. B, Current-voltage curves for NMDA-evoked currents in the absence (■) and presence (□) of arcaine (n = 8). The currents were normalized to the control current at −60 mV. C, Woodhull analysis of the voltage dependence of the arcaine block (same data as in B).

physiological pH is +1). The K_D at an arbitrary voltage is related to the $K_D(0)$ by

$$K_D(V) = K_D(0)\exp(z\delta FV/RT) \quad (4)$$

The calculated K_D(−60 mV) was 59 μM, which is very close to the K_D of 61 μM determined from the concentration-response data obtained at −60 mV (Fig. 1B). Thus, the voltage dependence of the arcaine block can be fully accounted for by the action of the electrostatic field on the charged arcaine molecule at its acceptor site in the channel.

Comparison with Mg^{2+}. To further characterize the blocking effect of arcaine on NMDA channels, we investigated the interaction of arcaine with the open channel blocker dizocilpine. It has previously been shown that the long-lasting blocking effect of dizocilpine on NMDA currents can be prevented by coapplication of Mg^{2+}, presumably because Mg^{2+} interferes with access of dizocilpine to its binding site within the ionophore of the NMDA receptor (12). We observed a similar interaction between arcaine and dizocilpine. As shown in Fig. 5A, 3 μM dizocilpine, when applied together with NMDA, caused a persistent blockade of the NMDA-evoked inward current response (presumably due to trapping of the drug in the channel) that was evident when NMDA was applied alone after the termination of the dizocilpine coapplication. However, coapplication of 1 mM arcaine (Fig. 5B) or 10 mM Mg^{2+} (Fig. 5C) prevented dizocilpine from producing a long-lasting blockade of the NMDA channel, so that the subsequent NMDA response recovered to near-control levels (84.0 ± 2.7%; n = 4). When the concentration of dizocilpine was raised to 10 μM, the recovery of the NMDA response was reduced (47.6 ± 4.8%; n = 4; $p < 0.01$ with respect to 3 μM dizocilpine), indicating a competitive interaction between arcaine and dizocilpine.

Single-channel recordings. We further examined the block of NMDA responses by arcaine in recordings of unitary NMDA receptor channel currents in outside-out membrane patches. At a holding potential of −60 mV, perfusion of patches with 2 μM NMDA evoked inward single-channel currents that were sensitive to Mg^{2+}. The channels had a large main conductance state and a number of smaller subconductance states (Fig. 6, A and B, *top*). The conductance of the main open state was approximately 58 pS. To remain consistent with the whole-cell studies, an external solution containing 0.1 mM Ca^{2+} was used. Ca^{2+} has previously been demonstrated to block the NMDA channel as well as permeate it (6, 7). Therefore, the somewhat larger conductance of the main open state we observed, in comparison with previous studies (32), is likely to be due to the diminished Ca^{2+} block in our low-Ca^{2+} solution. In fact, changing to an NMDA solution containing 2 mM $CaCl_2$ resulted in a reduction in the conductance of the main open state to approximately 50 pS (three patches tested at both Ca^{2+} concentrations).

The most obvious and consistent effect of arcaine on NMDA channels at hyperpolarized holding potentials was a concentration-dependent decrease in the apparent single-channel amplitude and an increase in open channel noise (Fig. 6A). This was not due to an alteration in channel gating resulting in the appearance of more frequent subconductance openings. Rather, there was a gradual overall decrease in channel amplitude, reflected as a progressive rightward shift in the amplitude histograms with increasing concentrations of arcaine (Fig. 6B). Fig. 6C summarizes the effect of increasing the concentration of arcaine on the amplitude of the main conductance state in 10 outside-out patches at −60 mV. In the presence of 100 μM arcaine, channel amplitude was reduced by 69%. This is almost identical to the 67% block of whole-cell currents produced by 100 μM arcaine, indicating that the reduction in unitary current amplitude completely accounts for the block of the macroscopic current.

Fig. 7 illustrates the effect of voltage on the arcaine-induced decrement in apparent unitary current amplitude. At −60 mV, there was a clear reduction in single-channel amplitude in the presence of 50 μM arcaine. In contrast, 30 μM Mg^{2+} caused a flickery block of the single-channel currents, as has been observed previously (5, 7). The striking difference in the appear-

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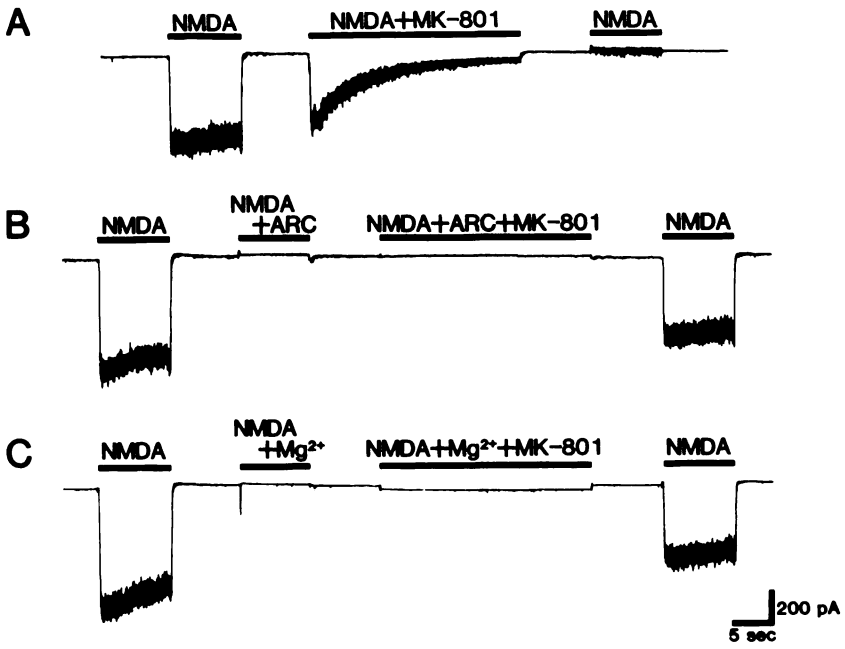

Fig. 5. Arcaine prevents blockade of NMDA channels by dizocilpine (MK-801). A, 3 μM MK-801 produces a slow, use-dependent block of NMDA-activated current. The drug is trapped within the channels and has a slow unbinding rate, as indicated by the persistent block of the NMDA current (recovery occurred in a use-dependent fashion with ~6-min cumulative NMDA application). B, In the same cell as in A, coapplication of 1 mM arcaine (*ARC*) with 3 μM MK-801 prevented channel blockade by MK-801, as indicated by the prompt recovery of the NMDA response. C, Similarly, in a different neuron, 10 mM Mg^{2+} was also able to prevent channel blockade by 3 μM MK-801.

ance of the drug-blocked single-channel currents in the presence of arcaine and Mg^{2+} is likely due to differences in the rate of binding and unbinding (see below). At +20 mV, the reduction in amplitude produced by arcaine was completely relieved. Similarly, at this positive potential, the Mg^{2+}-induced flickery block of the NMDA channel was no longer apparent. Thus, as in the whole-cell experiments, the arcaine block was strongly voltage dependent.

In an attempt to estimate the blocking and unblocking rates of the NMDA channel by arcaine, we analyzed the current records of NMDA-evoked single-channel events in the presence of different concentrations of arcaine, using the simple model of open channel block formulated by Neher and Steinbach (30). The all-points amplitude histograms obtained in the presence of arcaine were fit to a β distribution (see Materials and Methods), from which the rates of block and unblock of the NMDA channel could be derived. The apparent blocking rate increased in a linear fashion, from 5.4 to 23.1 $\times$ 10^3 sec^{-1}, as the concentration of arcaine was increased, whereas the unblocking rate remained relatively constant, at approximately 1.9 $\times$ 10^4 sec^{-1} (Fig. 8). The rate constant for block derived from the slope of the best fit line to the apparent blocking rate data was 4.5 $\times$ 10^6 M^{-1} sec^{-1}.

In contrast to the clear effect of arcaine on the apparent amplitude of single-channel currents activated by NMDA, arcaine had no significant effect on the burst duration. In patches perfused with NMDA, there was an obvious and consistent difference between short closed times, corresponding to gaps within bursts, and longer closed times, corresponding to gaps between bursts. This was reflected in the distribution of closed times, which exhibited a natural separation between short intervals (<3 msec) and longer events. The short closings were fit to two exponentials, with time constants of 0.18 $\pm$ 0.02 msec and 1.08 $\pm$ 0.1 msec. Therefore, to estimate burst durations, we analyzed single-channel records by ignoring closed intervals slower than 3 times the slower time constant. The burst duration histograms of four patches exposed to 2 μM NMDA were well fit by a two-exponential function, with time constants τ_1 = 0.51 $\pm$ 0.06 msec and τ_2 = 5.1 $\pm$ 0.2 msec. These values were not significantly different from those obtained in the same patches in the presence of NMDA plus 50 μM arcaine, in which τ_1 = 0.65 $\pm$ 0.12 msec and τ_2 = 6.2 $\pm$ 1.1 msec.

Discussion

Our initial interest in arcaine stemmed from the observation of Reynolds (24) [see also the work by Sacaan and Johnson (33)] that arcaine acts as a competitive antagonist of spermine-induced increases in [^{3}H]dizocilpine binding. However, in these studies arcaine also reduced [^{3}H]dizocilpine binding in the absence of added spermine, suggesting that it could interact directly with the NMDA receptor complex. The results of the present study provide support for this concept. Thus, arcaine produced a polyamine-independent block of NMDA responses in hippocampal neurons but had little effect on currents evoked by the non-NMDA glutamate receptor agonists KA or AMPA or by GABA, indicating a selective effect on the NMDA receptor complex. Although there are a number of potential sites at which arcaine could exert its blocking action (see the introduction), our results indicate that arcaine acts by binding in the NMDA channel, in a manner similar to that of Mg^{2+} (5–7), dissociative anesthetics such as PCP, ketamine, and dizocilpine (12–14), tricyclic antidepressants (15), and THA (16).

This mechanism is supported by the data obtained in both the whole-cell and single-channel recordings. The block of whole-cell NMDA currents by arcaine occurred in a voltage-dependent manner, i.e., the strong block of the NMDA current at hyperpolarized membrane potentials was almost completely relieved at positive potentials. An analysis of the voltage dependence of the arcaine block according to the Woodhull model (31) indicated that the arcaine blocking site appeared to sense approximately 67% of the transmembrane field. Thus, the voltage dependence is similar to that of other low affinity cationic open channel blockers such as Mg^{2+} (10), tricyclic antidepressants (15), and THA (16). Whereas the strong voltage dependence of the arcaine block provides suggestive evidence that the arcaine binding site may be within the ionophore

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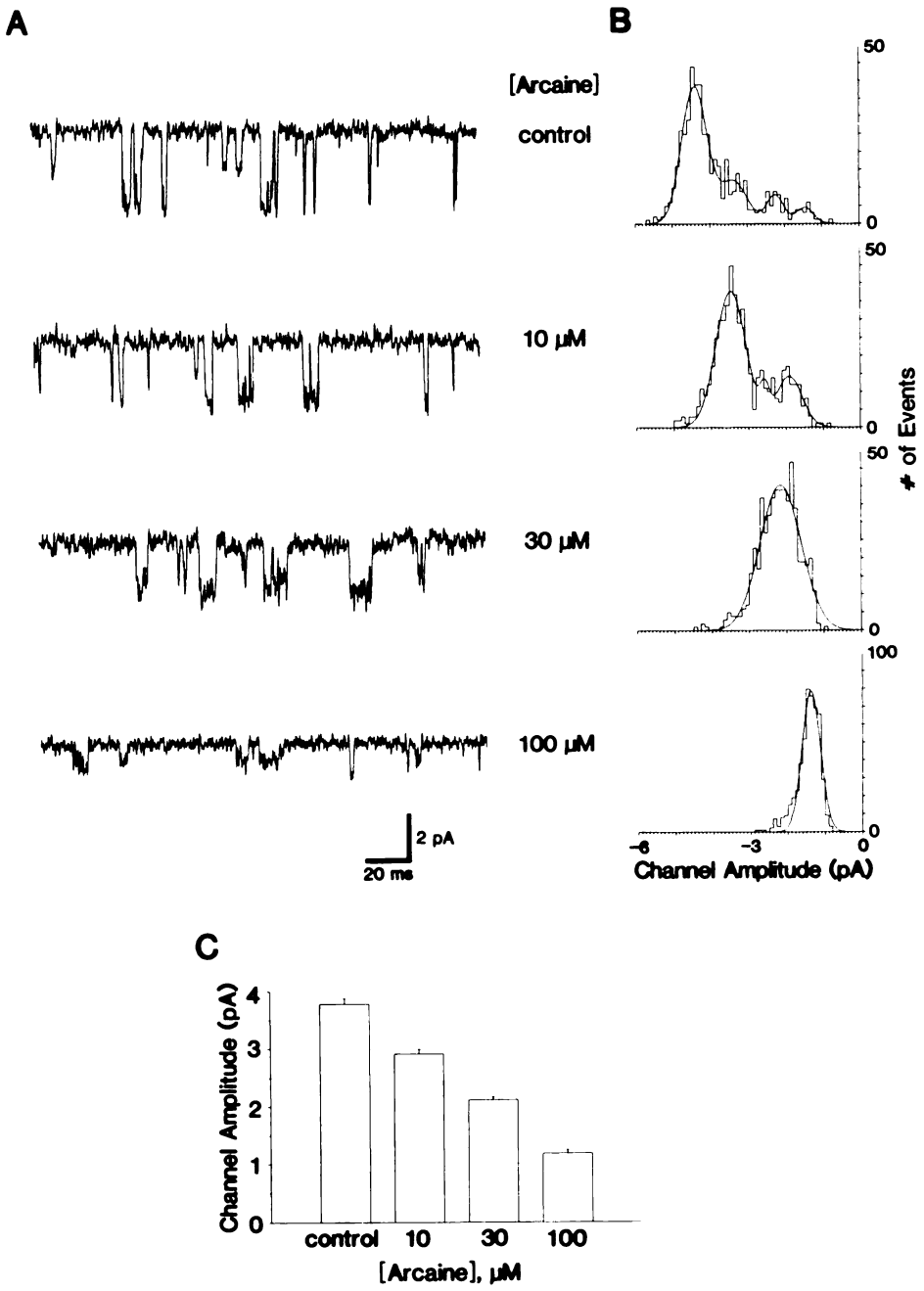

Fig. 6. Arcaine reduces the apparent amplitude of NMDA-activated single-channel currents in a concentration-dependent manner. Traces in A show single-channel currents evoked by 2 μM NMDA in the absence (control) and presence of increasing concentrations of arcaine (10–100 μM), at a holding potential of −60 mV. The corresponding amplitude histograms for this patch are shown in B (bin width = 0.1 pA). C, Apparent unitary current amplitude (main conductance state) versus arcaine concentration in 10 patches.

of the NMDA receptor complex, this was confirmed by competition studies with dizocilpine. Thus, arcaine, like Mg^{2+}, was able to prevent the binding of dizocilpine, which is well known to occur in the channel pore (12). This result, by itself, is insufficient to allow the conclusion that arcaine acts within the channel, because competitive antagonism at the NMDA or glycine recognition sites, or allosteric block of the channel such as that produced by low concentrations of Zn^{2+} (8, 9), would also prevent dizocilpine binding and block. However, because the block by arcaine was not affected by changes in the concentration of either NMDA or glycine, we can conclude that arcaine is not an antagonist at the NMDA or glycine sites. In addition, the voltage dependence of the arcaine block is inconsistent with an action at the NMDA or glycine recognition sites or the Zn^{2+} allosteric blocking site (4, 8, 9). Finally, the concentration-dependent competition between arcaine and dizocilpine provides particularly convincing evidence supporting an open channel mechanism. We note that the Hill coefficient near 1 in the isotherm of Fig. 1B is consistent with a 1:1 binding reaction between arcaine and its channel acceptor site.

In our preparation, spermine (added either extracellularly or intracellularly) failed to cause a consistent potentiation of NMDA responses. Therefore, we were unable to study the interaction of arcaine with the polyamine site. The only consistent effect of spermine that we observed was an arcaine-like voltage-dependent block of NMDA currents [which may explain the observed decrease in spermine-enhanced [^{3}H]dizocilpine binding that occurs with high (>100 μM) spermine concentrations (17, 19)]. Although the reason for the lack of stimulatory polyamine responses in the bulk of our recordings is unclear, this situation allowed us to examine the direct action of arcaine at the NMDA receptor. Moreover, because the po-

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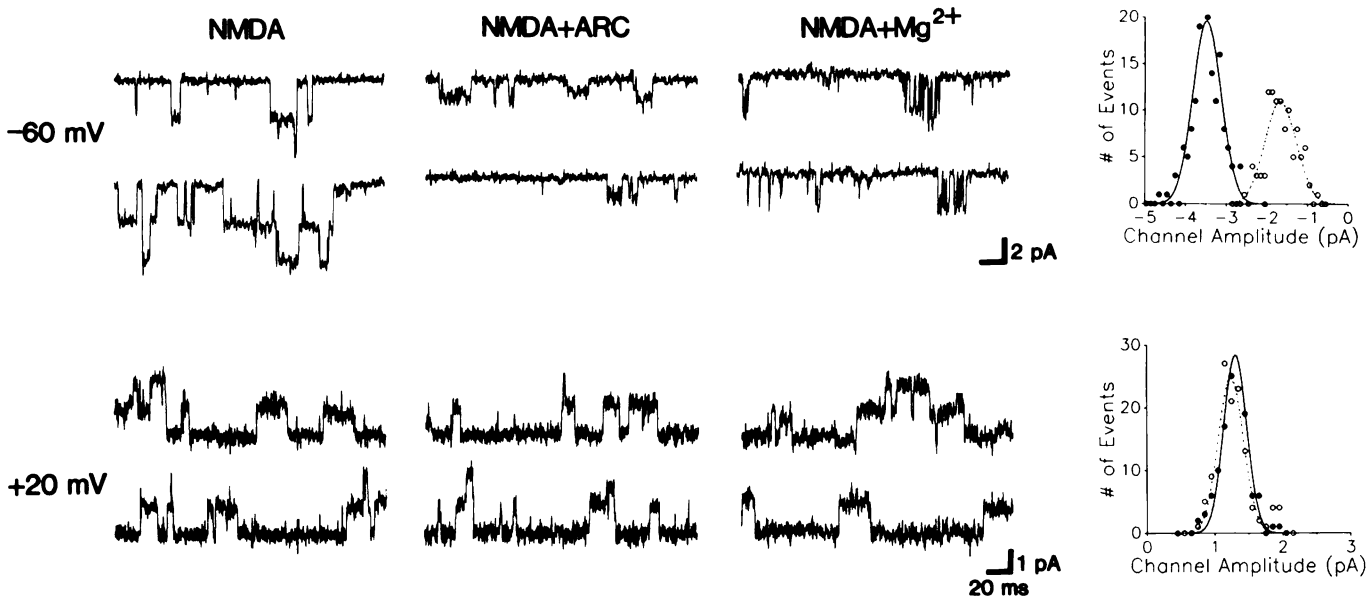

Fig. 7. Single-channel currents activated by 2 μM NMDA in the presence of 50 μM arcaine (*ARC*) or 30 μM Mg^{2+}, at −60 mV and +20 mV, demonstrating the voltage-dependent reduction in apparent channel amplitude produced by arcaine and the flickery block produced by Mg^{2+}. The histograms plot the distribution of channel amplitudes in the absence (●) and presence (○) of arcaine (bin width = 0.1 pA); double openings were ignored.

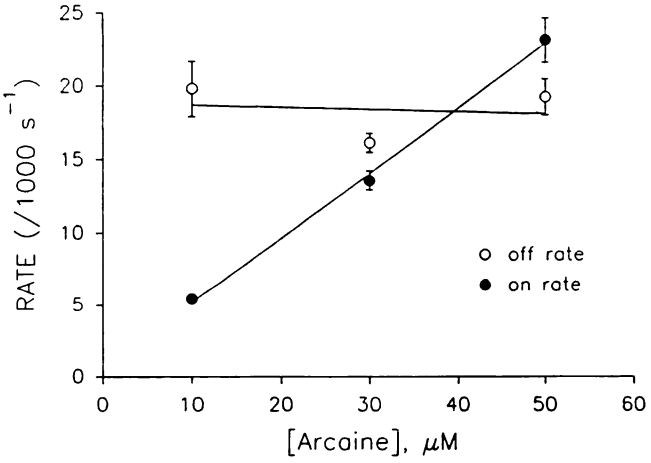

Fig. 8. Arcaine binding (on) and unbinding (off) rates determined from single-channel recordings in the presence of various concentrations of arcaine. Apparent rates were as determined from the β distribution fit to all-points amplitude histograms, as described in Materials and Methods. Values shown are the means ± standard errors of data from four to six patches. Increasing the concentration of arcaine caused an increase in the apparent rate of channel block, whereas the unblocking rate was relatively concentration independent. The *straight lines* were fit using a linear least-squares method. The second-order rate constant determined from the slope of the best fit line to the apparent on-rate data is 4.4×10^8 M^{-1} sec^{-1}. The mean off rate is 1.9×10^3 sec^{-1}.

lyamine site was inactive or not present under most circumstances, it is unlikely that arcaine acts as a polyamine inverse agonist, as has been proposed for DA10 (19, 22).

Our observations at the single-channel level provide additional support for the open channel blocking mechanism. These studies demonstrated that arcaine caused a concentration-dependent decrease in the apparent amplitude of the single-channel currents activated by NMDA. The reduction in channel amplitude was strongly voltage dependent, as expected from the voltage dependence of block of the whole-cell currents, and there was an associated increase in the open channel noise. Hence, the apparent decrease in channel amplitude with arcaine likely results from high frequency flickering of the channel between open and blocked states that is not resolved by our recording equipment. Similar reductions in apparent channel amplitude have been observed with Mn^{2+} (7) and high concentrations (10–100 μM) of Zn^{2+} (11, 34). Mg^{2+}, on the other hand, usually causes flickering of the NMDA channel without a reduction in amplitude (as shown in Fig. 7A) (5, 7), although at high concentrations it can also produce a decrease in open channel amplitude (e.g., Fig. 1 in Ref. 7). The rate of block determined according to the method of Yellen (28) was concentration dependent, whereas the rate of unblock was independent of concentration, consistent with the simple model of open channel block described by Neher and Steinbach (30). We note that these results are inconsistent with an effect of arcaine on the permeation properties of the channel (e.g., a charge-screening effect). The rates for arcaine were very similar to those derived, using similar methods, for the voltage-dependent block of NMDA channels by Zn^{2+} (11) and were substantially faster than those for Mg^{2+} (7).

In conclusion, in the present study we have demonstrated that arcaine antagonizes the actions of NMDA by binding to a site within the channel of the NMDA receptor complex. Although the IC_{50} of the arcaine block of NMDA-evoked currents is 10-fold higher than that described for the block of NMDA-induced [^{3}H]dizocilpine binding (24, 33), it is similar to that observed in the more physiological studies examining the effect of arcaine on NMDA-evoked norepinephrine release from rat brain slices (29). Open channel block provides a likely mechanism to explain the action of arcaine on [^{3}H]dizocilpine binding in the absence of exogenous polyamines. Moreover, it raises obvious difficulties in the use of arcaine as a selective polyamine site antagonist.

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