Spermine and Arcaine Block and Permeate *N*-Methyl-D-Aspartate Receptor Channels

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ABSTRACT Polyamines such as spermine are thought to be endogenous regulators of NMDA (*N*-methyl-D-aspartate)-type glutamate receptors. Polyamine block of NMDA receptors was studied in excised outside-out patches from rat hippocampal neurons and *Xenopus* oocytes expressing recombinant receptors. Extracellular spermine and arcaine reduced NMDA single-channel conductance in a voltage-dependent manner, with partial relief of block evident at large inside negative membrane potentials. Reducing extracellular Na^+ concentration increased the apparent affinities for spermine and arcaine, indicating strong interaction between spermine and permeant ions. Internal spermine also blocked NMDA channels in a voltage-dependent manner, with relief of block evident at large inside positive potentials. The Woodhull model of channel block by an impermeant ion adequately described the actions of external spermine from −60 to +60 mV, but failed for more negative potentials. Eyring rate theory for a permeable blocker with two barriers and one binding site adequately described the voltage-dependent block and relief from block by both external and internal spermine over the range of −120 to +60 mV. These findings indicate that polyamines block and permeate neuronal NMDA receptor channels from the extracellular and intracellular sides, although sensitivity to internal spermine is probably too low to be physiologically relevant.

INTRODUCTION

Polyamines are ubiquitous organic polycations that interact with diverse cellular targets such as nucleic acids and proteins. Intracellular polyamines block a number of ion channels, including inwardly rectifying K^+ channels (Lopatin et al., 1994, 1995; Yang et al., 1995), ryanodine receptors of the sarcoplasmic reticulum (Uehara et al., 1996), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and kainate-type glutamate receptors (Bahring et al., 1997; Donevan and Rogawski, 1995). Modulation by endogenous polyamines is implicated in the inward rectification exhibited by each of these channels. External polyamines modulate *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, (Araneda et al., 1993; Benveniste and Mayer, 1993; Chao et al., 1997; Kashiwagi et al., 1996; McGurk et al., 1990; Williams, 1997). It is not yet known whether polyamine block is an important mode of NMDA receptor regulation in vivo. Spermine, spermidine, and putrescine are present in micromolar concentrations in the brain and are released from presynaptic terminals in the hippocampus under physiological conditions (Harman and Shaw, 1981; Seiler and Deckardt, 1978; Shaw and Pateman, 1973). Thus polyamines may influence a wide range of physiological processes mediated by NMDA receptors, including synaptic plasticity, synaptogenesis, and the formation of neuronal circuitry (for a review, see Williams, 1997).

Spermine potentiates responses of neuronal and recombinant NMDA receptors in the presence of saturating concentrations of glutamate and glycine (Araneda et al., 1993; Benveniste and Mayer, 1993; Lerma, 1992; Rock and Macdonald, 1995; Zhang et al., 1994), an effect that involves an increase in the frequency of channel opening and burst duration (Araneda et al., 1993; Rock and Macdonald, 1995) and may involve a reduction in desensitization (Lerma, 1992). Spermine potentiation at high glycine is voltage-independent (Araneda et al., 1993; Durand et al., 1993; Williams et al., 1994), subunit-specific (Williams et al., 1994; Zhang et al., 1994), and associated with a change in pH sensitivity (Traynelis et al., 1995). Heteromeric receptors containing NR1 subunits without the amino-terminal insert and the NR2B subunit exhibit spermine potentiation at high glycine (Zhang et al., 1994), whereas receptors containing NR1 subunits with the amino-terminal insert or NR2A or NR2C subunits do not exhibit this form of potentiation (Durand et al., 1993; Zhang et al., 1994). Spermine potentiation is thought to be mediated at an external site formed by regions within the extracellular amino-terminal domain and the extracellular loop between the M3 and M4 domains of the NR1 subunit (Kashiwagi et al., 1996; Williams et al., 1995). Extracellular spermine may compete with Mg^{2+} for this common stimulatory site (Paoletti et al., 1995). In addition, spermine increases the affinity of the NMDA receptor for glycine, which is responsible for spermine potentiation at low glycine (Benveniste and Mayer, 1993; McGurk et al., 1990) and is thought to be mediated at a distinct site (Kashiwagi et al., 1996).

Spermine also blocks responses of neuronal and recombinant NMDA receptors. Spermine reduces apparent

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NMDA single-channel conductance in a voltage-dependent manner when applied from the extracellular side (Araneda et al., 1993; Rock and Macdonald, 1995). Spermine block is affected by the NR2 subunit, but not by the NR1 splice variant (Williams, 1997). Thus NR1/NR2C and NR1/NR2D receptors exhibit reduced voltage-dependent block by spermine, relative to that of NR1/NR2A or NR1/NR2B receptors (Williams, 1997). Because spermine is a polyvalent cation at physiological pH (Morris and Harada, 1980), it could reduce putative negative charges in the vicinity of the permeation pathway, thereby reducing cationic flux through the channel ("charge screening"; Rock and Macdonald, 1995).

The present study was undertaken to examine molecular mechanisms underlying polyamine block of NMDA receptors and to examine the possibility that spermine blocks and permeates the channel from both the extracellular and intracellular sides. We used patch-clamp techniques to record NMDA single channels in excised outside-out patches from rat hippocampal neurons and from *Xenopus* oocytes expressing recombinant NMDA receptors. Single-channel recording is particularly valuable in the analysis of polyamine action at NMDA receptors in that it enables dissection of fast channel blocking from other actions, which is not possible for whole-cell recording of neuronal NMDA responses. The present study shows that the polyamines spermine and arcaine reduce NMDA single-channel currents in a voltage-dependent manner when added from either side of the membrane. Analysis of the voltage dependence indicates relief of block at high inside negativity for external spermine or high inside positivity for internal spermine. Erying rate theory analysis of the voltage dependence of polyamine block is consistent with the action of spermine and arcaine as permeant cationic blockers of NMDA receptor channels.

MATERIALS AND METHODS

Cell culture

Dissociated cell cultures of hippocampus were prepared from rat embryos (17–19 days old) as described (Araneda et al., 1993). Briefly, hippocampi were rapidly dissected and transferred to a solution containing 0.025% trypsin at 37°C for 15 min. Cells were dispersed by trituration with a fire-polished Pasteur pipette and plated onto polylysine-coated coverslips. Cells were maintained in minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and N2 supplement (50 units/ml penicillin and 25 μg/ml streptomycin). Cells were incubated in a humidified 5% CO_2/95% O_2 atmosphere at 37°C and fed once a week. Neurons were used after 1–3 weeks in culture.

Single-channel recording from hippocampal neurons

Electrophysiological recording from hippocampal neurons was performed on the stage of an inverted phase-contrast microscope at room temperature. NMDA single-channel currents were recorded from excised outside-out patches as described (Hamill et al., 1981). Pipette resistance ranged from 5 to 10 MΩ with an internal solution consisting of (in mM) 150 CsCl, 0.5 $CaCl_2$, 5.0 EGTA, 10 HEPES-Cs (pH 7.2). Cells were bathed in an external solution containing (in mM) 160 NaCl, 2.5 KCl, 1 $CaCl_2$, 10 HEPES-Na^+ (pH 7.2). In some experiments the concentration of NaCl in the external solution was reduced to 80 mM and sucrose was added to maintain the osmolarity, or the concentration of $CaCl_2$ was raised from 1 to 160 mM and NaCl was omitted.

For recording, tetrodotoxin (TTX) (500 nM) and bicuculline (10 μM) were added to the external solution to block Na^+ and $GABA_A$ receptor channels, respectively. Test solutions containing NMDA (10 μM with 1 μM glycine) in the presence or absence of polyamines were delivered to the patch from a multibarrel array fed by gravity; the array was moved laterally to apply the different solutions to the patch.

Single-channel recording from recombinant NMDA receptors expressed in *Xenopus* oocytes

Rat $NR1_{100}$ cDNA was cloned in this laboratory (Durand et al., 1992). Mouse ϵ2 (corresponding to rat NR2B) cDNA was a gift of Dr. M. Mishina (Niigata, Japan). cDNAs were subcloned into the pBluescript SK(−) vector for oocyte expression. To generate templates for transcription, circular plasmid cDNAs were linearized with *Bam*HI ($NR1_{100}$) or *Not*I (ϵ2). Capped mRNAs were synthesized as run-off transcripts from linearized plasmid cDNAs with T3 or T7 polymerase (Ambion mMessage mMachine transcription kit, 2 h at 37°C) and examined electrophoretically on a denaturing gel. Concentration and integrity of mRNAs were assessed after staining with ethidium bromide by direct comparison of sample mRNAs with an RNA standard ladder (Gibco BRL).

To express recombinant NMDA receptors, selected stage V and VI oocytes from adult female *Xenopus laevis* were injected with a mixture of in vitro transcribed RNAs (20 ng/cell; NR1:NR2 = 1:3), using a Nanoject injector (Drummond Scientific Co., Broomall, PA). Oocytes were maintained at 18°C in culture buffer (103 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.5). Two to seven days after injection, single-channel currents were recorded from outside-out patches excised from devitellinized oocytes at ambient temperature under voltage clamp, using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). Single-channel activity was elicited by the application of NMDA (100 μM with 10 μM glycine). Pipette resistance and internal and external solutions were as described above for patch recording from hippocampal neurons (with the exception that TTX and bicuculline were omitted from the external solution). These conditions were used to permit direct comparisons between neuronal and recombinant NMDA receptors. To examine spermine block from the intracellular side, spermine at indicated concentrations was added to the internal solution, and the pH was adjusted to 7.2 by titration with HCl.

Molecular modeling

Corey-Pauling-Koltum (CPK) space-filling models for spermine and arcaine were generated using Chem3D Pro software (CambridgeSoft Corp., Cambridge).

Data acquisition and analysis

Single-channel recording and data processing were performed using an Axon TL-1 interface (Axon Instruments, Burlingame, CA) connected to a PC and PCLAMP 6.0 software for single-channel analysis (Axon Instruments). Records were filtered at 2–5 kHz, digitized at 10 kHz, and stored by means of a video recorder. Single-channel current values were obtained from all point amplitude histograms. For each patch the currents were measured at potentials between −100 and +60 mV (10-mV steps) in control conditions and in the presence of increasing concentrations of the polyamines. Because it was not possible to obtain the full concentration-response relations in the same patch, each data point is the average ± SEM

from three to nine patches in the absence or presence of one or more concentrations of the drugs. Averaged data were used to calculate apparent affinities according to the logistic equation:

$$\text{fractional current} = I_B/I_0 = (I_0 - I_{min})/(1 + ([B]/IC_{50})^n) + I_{min} \quad (1)$$

where I_0 and I_B correspond to the current in the absence and in the presence of the blocker, respectively, I_{min} is the residual current at a saturating concentration of blocker, [B] is the blocker concentration, IC_{50} is the concentration that produces half-maximum block (apparent affinity), and n is a slope factor equivalent to the Hill coefficient.

To characterize the voltage dependence of block, we first applied the Woodhull model for voltage-dependent channel block by an impermeant ion (Woodhull, 1973). This is a two-barrier, one-site model, i.e., there is a single binding site in the channel with single barriers between the external and internal solutions. The model assumes that the blocking ion crosses a barrier from the external side to reach the binding site, that the inner barrier prevents permeation of the blocking ion, that occupancy of the binding site by permeant ions is negligible, and that when the site is occupied by the blocking ion, it cannot be occupied by permeant ions. In addition, the model assumes that 1) the blocking ion is a single point charge; 2) the peak of the outer barrier is half-way between the external surface of the membrane and the binding site; and 3) the blocking ion does not alter the electrical field across the membrane. Because spermine and arcaine are likely to assume extended conformations in solution, the charges associated with them will be spatially separated; thus the first of these assumptions is unlikely to apply. Nevertheless, we used this equation to obtain a first approximation of the electrical distance of the polyamine-binding site from the surface and its affinity for the polyamine. The model predicts that the currents in the presence of polyamine will be fit by the following equation:

$$I_B/I_0 = 1/(1 + [B]/(K_D(V_m)) \quad (2)$$

This equation differs from Eq. 1 in that the minimum current is zero, the Hill coefficient is unity, and the IC_{50} is replaced by the binding affinity of the blocker, $K_D(V_m)$, which is explicitly voltage dependent. In this model, the voltage dependence is given by

$$K_D(V_m) = K_D(0)\exp[-z\delta FV_m/RT] \quad (3)$$

where $K_D(V_m)$ is the affinity at membrane potential, V_m; $K_D(0)$ is the affinity at 0 membrane potential; z is the charge on the polyamine; F, R, and T have their usual meanings ($RT/F \approx 25$ mV at 25°C); and δ is the fraction of the membrane electric field (measured from the extracellular face of the membrane) that the blocker senses at its binding site.

Because this Woodhull analysis suggested that the NMDA channel is somewhat permeable to spermine and arcaine, we extended the analysis by applying a two-barrier, one-site model with a finite internal barrier. Rather than use the Woodhull two-barrier, one-site model, we applied Eyring rate theory, which goes beyond the Woodhull model in that it explicitly includes competition between permeant ions for occupancy of the binding site and permits adjustment of the electrical distances of the barriers as well as of the binding site. This approach has been used to explain divalent ion permeation in the NMDA channel (Premkumar and Auerbach, 1996; Schneggenburger, 1998). The rate constants for transitions of each ion out of the binding site are of the form

$$k = (kT/h)\exp[(-\Delta G - \delta^* z_i V_m F)/kT] \quad (4)$$

where ΔG is the ion's energy at the peak of the barrier being crossed minus that in the binding site (at $V_m = 0$), δ^* is the fraction of the membrane potential developed between the binding site and peak of the barrier, z_i is the effective charge of the ith ion, V_m is the membrane potential, and k, h, F, and T have their usual meanings. Equations describing the rate constants for the transition of each ion from the external or internal solution into the binding site are of the same form, but the ΔG's are the energies at the peaks of the barriers (the external and internal energies being defined as zero in the absence of membrane potential), and the right sides of the equations are multiplied by the mole fraction of that ion in the external or internal solution.

For this model there are six adjustable parameters for each ion, the energies in the absence of applied voltage at the binding site, U, and at the peaks of the barriers, G_2 and G_3, and the electrical distances (fraction of the applied voltage) at the three locations, D_1 for the binding site, D_2 for the outer barrier, and D_3 for the inner barrier (defined as fractions of the distance from the outer membrane surface). The energies were allowed to differ for Na^+ and for the applied polyamine, but the electrical distances of the three locations were assumed to be the same. The equations were fit to the data using AJUSTE, a nonlinear curve-fitting program based on the Gauss-Newton method (Alvarez et al., 1992). This procedure adjusts the parameters by minimizing summed squared errors between experimental I-V data and the simulated curves.

All data are presented as means ± SEM unless otherwise stated. Statistical tests were performed by means of Student's unpaired t-test.

RESULTS

Extracellular spermine and arcaine reduce NMDA-induced currents

To characterize the actions of spermine on neuronal NMDA channels, we recorded from outside-out patches excised from cultured rat hippocampal neurons. At −60 mV, NMDA (10 μM with 1 μM glycine) activated single channels with a main unitary conductance of 51 ± 1 pS, which did not vary with voltage ($n = 13$; Figs. 1 and 2). Transitions from the main conductance state to sublevels were observed occasionally (dwell times of less than 1% of that of the main state; data not illustrated) but were not included in the analysis.

At −60 mV, application of spermine or arcaine to the outside of the patch attenuated the apparent single-channel conductance (Fig. 1, *top row*); this action was readily reversible. At −60 mV, spermine (300 μM) reduced unitary conductance by 47% to 27 ± 1 pS (n = 4), and arcaine (100 μM) reduced unitary conductance by 57% to 22 ± 1 pS ($n = 4$). Visual inspection of the records of Fig. 1 suggests that both polyamines increased open channel noise of individual excised patches, measured as the standard deviation of the signal during periods with no apparent closures. An increase in open channel noise associated with reduced single-channel current amplitude is indicative of a mechanism whereby polyamines attenuate current by entering and leaving a block site in the channel very rapidly relative to the time constant of the recording system.

At +60 mV, neither spermine (300 μM) nor arcaine (100 μM) affected NMDA single-channel current amplitude or open channel noise (Fig. 1, *bottom row*). However, at +60 mV, spermine at a 10-fold higher concentration (3 mM) reduced NMDA single-channel current amplitude by 21% ($p < 0.02$; Fig. 2 *A*). We did not evaluate potentiation in these experiments, but we previously showed that in some patches, spermine increased the frequency of channel open-

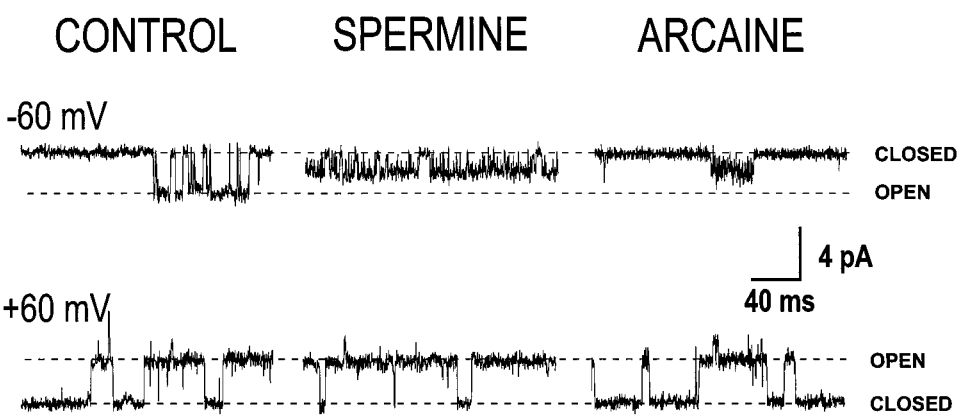

FIGURE 1 Extracellular spermine and arcaine reduce NMDA single-channel conductance. Representative single-channel records at −60 mV and +60 mV are from outside-out patches excised from hippocampal neurons. NMDA (10 μM with 1 μM glycine) activated single-channel currents with a main unitary conductance of 51 ± 1 pS at −60 mV ($E_{rev} \approx 0$; $n = 13$). Spermine (300 μM) and arcaine (100 μM) reduced unitary conductance by ~50%. At +60 mV, spermine (300 μM) and arcaine (100 μM) produced little or no reduction of current amplitude (*lower traces*). Both polyamines increased open channel noise. The dotted line indicates the current amplitude of the open state of the channel.

ing without affecting the mean duration of openings (Araneda et al., 1993).

Block by external polyamine is voltage- and concentration-dependent

In the absence of external polyamine, the current-voltage (*I-V*) relation for single NMDA receptors was nearly linear over the range of −100 mV to +60 mV, with a reversal potential (E_{rev}) close to zero (Fig. 2). In the presence of polyamine, the slope of the *I-V* relation for NMDA channels decreased and then increased again with increasing inside negativity, exhibiting an inflection point in the range of −40 to −60 mV. The nonlinearity was more prominent at higher concentrations of polyamine. This deviation from linearity is consistent with increased entry of polyamine into the

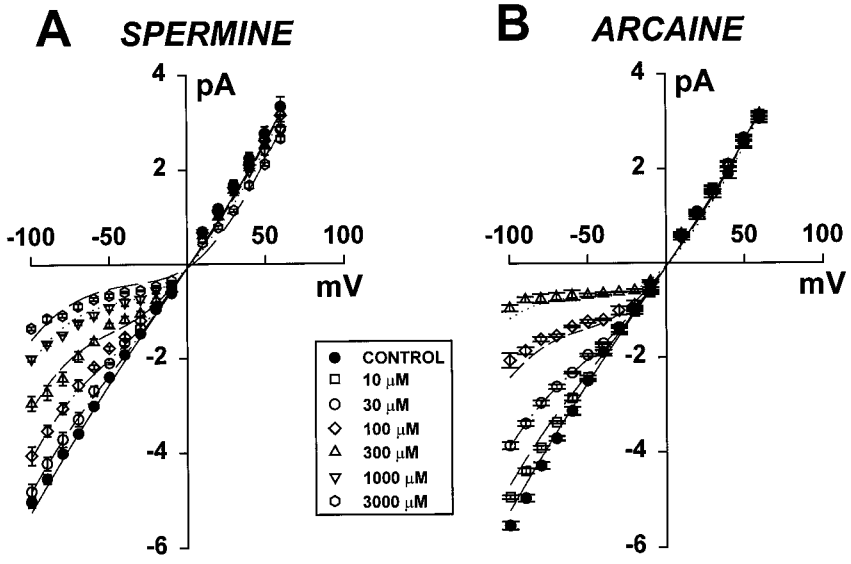

FIGURE 2 Block by extracellular polyamines is partially relieved at more negative membrane potentials. NMDA single-channel current-voltage relations were observed in the presence of (*A*) spermine or (*B*) arcaine. Single-channel recording was performed from outside-out patches excised from hippocampal neurons. NMDA single-channel currents were elicited by the application of NMDA (10 μM in the presence of 1 μM glycine) in the absence and presence of spermine (30 μM to 3 mM) or arcaine (10–300 μM) at varying holding potentials. Single-channel current-voltage (*I-V*) relations were generated by recording NMDA single-channel currents at holding potentials in the range of −100 mV to +60 mV. Data points are means ± SEM of single-channel current amplitudes recorded from a minimum of three patches. (*A*) Single-channel *I-V* curves in the presence of increasing concentrations of extracellular spermine (30–3000 μM). Spermine block from the extracellular side increased with inside negativity from 0 mV to −60 mV, with relief of block evident at potentials negative to −60 mV. There is a small reduction in current amplitude at positive holding potentials by 3000 μM spermine. (*B*) *I-V* curves in the presence of increasing concentrations of extracellular arcaine (10–300 mM). At higher concentrations of arcaine, the single-channel currents were very small and could not be measured accurately. Arcaine block from the extracellular side increased with inside negativity from 0 mV to −60 mV, with slight relief of block evident at potentials negative to −60 mV. At the same concentrations, arcaine produced a greater fractional block of NMDA single-channel currents than did spermine, consistent with a somewhat higher affinity for arcaine. Smooth curves are calculations according to Eyring rate theory, using parameters in Table 1.

channel with increasing inside negativity and with permeation of spermine and partial relief of block at still larger negative potentials (see below). The decrease in slope at negative potentials represents a decrease in apparent single-channel chord conductance (Fig. 2). The decrease in conductance was greater at higher concentrations of polyamine; at −60 mV 3 mM spermine decreased the conductance by ~70% (Fig. 3 *A*). At positive holding potentials (up to +60 mV), high concentrations of spermine (but not arcaine) produced a small but significant reduction in single-channel conductance (Fig. 2 *A*). This action may have been due to binding by spermine to a second, more superficial site, as has been suggested for block of NMDA channels by Ca^{2+} (Premkumar and Auerbach, 1996; Sharma and Stevens, 1996). Neither polyamine detectably altered the reversal potential E_{rev} for NMDA currents.

The concentration dependence of polyamine block of NMDA single channels (Fig. 2) was analyzed by fitting the logistic equation (Eq. 1) to the fractional block as a function of polyamine concentration at each potential (Fig. 3 *A*). At −60 mV, the spermine concentration that produced half-maximum block (the IC_{50}) was 278 μM for spermine, with a Hill coefficient of 0.8, and 81 μM for arcaine, with a Hill coefficient of 1. Thus arcaine was ~3.5 times more potent than was spermine in blocking NMDA single-channel currents. At −60 mV, I_{min} at saturating spermine determined by extrapolation was 0.11. At this potential, I_{min} for arcaine was near zero.

The concentration dependence of polyamine block was also determined at reduced ionic strength, 80 mM NaCl instead of 160 mM NaCl (Fig. 3 *B*). The curves were of the same form, but the apparent affinity was increased, e.g., at

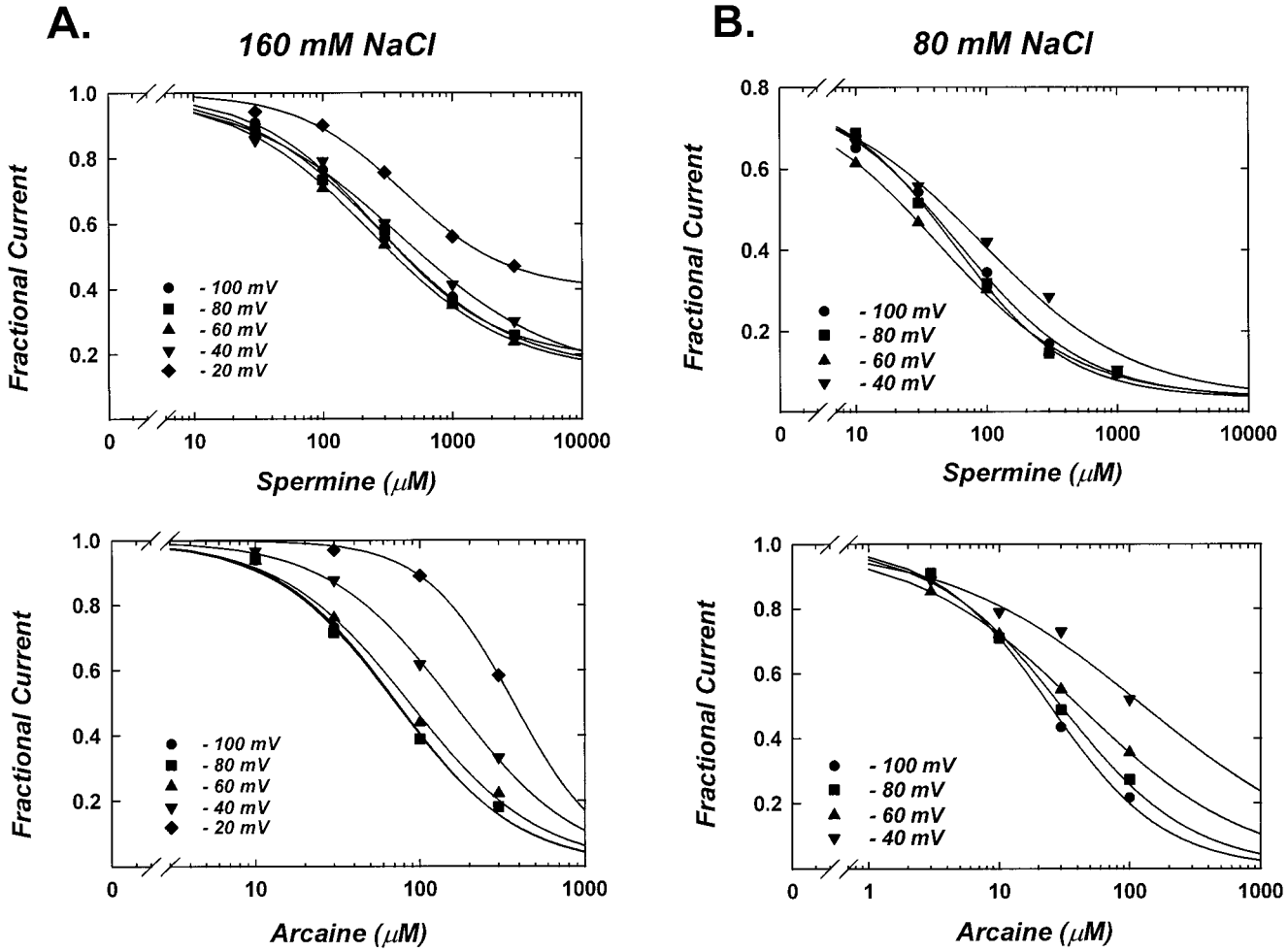

FIGURE 3 Reducing extracellular Na^+ concentration increases affinity for spermine and arcaine block from the extracellular side. Concentration-response curves are for the block of NMDA single-channel conductance by spermine (*top panel*) and arcaine (*bottom panel*) at (*A*) 160 mM and (*B*) 80 mM extracellular NaCl. Single-channel records were obtained from outside-out patches excised from hippocampal neurons at holding potentials ranging from −20 to −100 mV. NMDA single-channel currents were elicited by the application of NMDA (10 μM in the presence of 1 μM glycine) in the absence and presence of spermine (30–1000 μM) or arcaine (10–300 μM). Under these conditions, I_{min}/I_0 was 0.4 for spermine and 0.0 for arcaine. Data at 160 mM NaCl are from Fig. 2. Data points are means ± SEM from a minimum of three different patches and are normalized to the control NMDA conductance observed in the absence of polyamine. Continuous lines represent best fits of the logistic equation (Eq. 1). The concentration-response curves were shifted to the left at more negative voltages and in 80 mM NaCl.

−60 mV the K_D was decreased to 45 μM for spermine and 30 μM for arcaine. Thus decreasing ionic strength increased spermine affinity by about sixfold and arcaine affinity by about threefold. As discussed below, the increased affinity is ascribable to competition between Na^+ and spermine at the block site.

Woodhull analysis of polyamine block

To examine the mechanism of polyamine block of NMDA channels, we applied the Woodhull analysis for voltage-dependent channel block by an impermeant ion (Woodhull, 1973). This treatment assumes that occupancy of the channel by the blocker prevents entry of other ions, that occupancy of that site by other ions is negligible, and that the outer energy barrier encountered by the blocker is located at a fixed electrical distance half-way between the external membrane surface and the block site. Equilibrium constants (K_D's) calculated from the concentration-response curves in Fig. 3 according to Eq. 1 were plotted on a logarithmic scale as a function of membrane potential (V_m) (Fig. 4 *A*). Plots of ln K_D as a function of V_m for both spermine and arcaine were essentially linear over the range of membrane potentials from −50 mV to −10 mV, indicative of open channel block. However, for spermine, ln(K_D) was nearly constant between −50 and −70 mV and increased somewhat between −80 and −100 mV. Similarly for arcaine, ln K_D increased very little between −60 and −100 mV. These findings indicate that the Woodhull analysis for block by impermeant ions accounts for the action of spermine and arcaine at moderate but not at large negative potentials. Extrapolation of the linear region of the ln K_D versus V_m relation gave zero voltage affinity values, $K_D(0)$, of 3.33 mM for spermine and 0.78 mM for arcaine. From the slope of the linear region, we calculated that the product of the charge on the blocker and the electrical distance of the binding site, $z\delta$, was 1.42 for spermine and 0.98 for arcaine. If it is assumed that each polyamine has an effective charge of about +2 (see below), the Woodhull analysis predicts that the binding sites for spermine and arcaine are at approximately one-half to two-thirds the electrical distance across the membrane.

Using the values of $K_D(0)$ and $z\delta$ that we determined from the linear regions of the ln K_D versus V_m relations and the Woodhull equations, we calculated current-voltage relations for NMDA responses in the presence of blocker over the entire range of applied voltages (smooth curves in Fig. 4 *B*) and compared them to the observed responses (illustrated for 1 mM spermine and 300 μM arcaine). The observed currents were markedly greater than the calculated

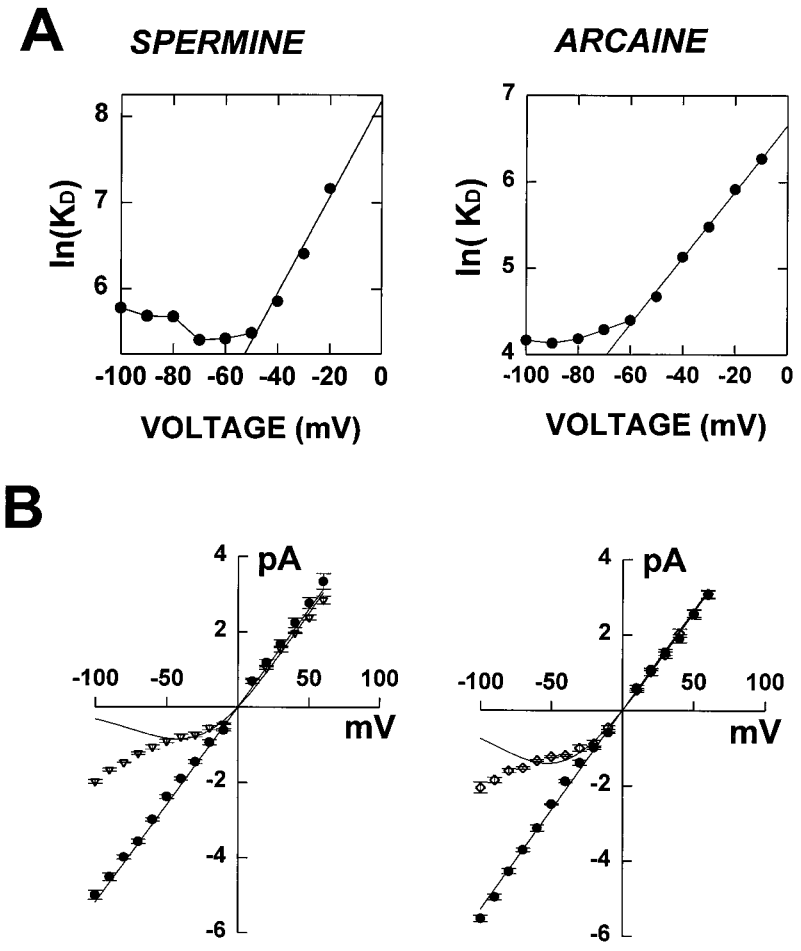

FIGURE 4 Woodhull analysis of voltage dependence of spermine and arcaine block. Reduction in NMDA single-channel currents by spermine and arcaine deviates from predictions of the Woodhull model for an impermeant blocker. (*A*) Plots of ln K_D versus V_m generated from the data in Fig. 3. Extrapolation of the linear regions to zero voltage gives values of binding affinity $K_D(0)$; the slope of this line is used to determine values of $z\delta$. (*B*) The current-voltage relations predicted by the Woodhull model for block by spermine at 1000 μM (*left*) and by arcaine at 300 μM (*right*) over the range −100 mV to +60 mV are superimposed on the experimental data. Current-voltage relations for both drugs fit the theoretical curves from ~ −50 to +60 mV, but at more negative potentials currents exceeded predicted values, indicative of relief of block.

values at holding potentials negative to about −50 mV. This deviation from the Woodhull model for block by an impermeant ion is consistent with permeation of the channel at sufficiently negative potentials.

Analysis using Eyring rate theory

To examine polyamine action further we applied Eyring rate theory, which explicitly includes competition at the binding site(s) between the blocking ion and other ions in the solution (see Hille, 1992; Woodbury, 1971). We fit the equations for a two-barrier, one-binding-site model to the *I-V* relations for NMDA single channels in the presence of spermine. In this model the binding site may be unoccupied or occupied by either Na^+ or spermine. The electrical distances of the barriers and binding site were assumed to be the same for Na^+ and spermine. Additional assumptions were as follows: 1) Na^+ (160 mM) and spermine (30–3000 μM) were the only permeant cations present in the external solution and 2) $P_{Na}/P_{Cs} \approx 1$, so that the internal Cs^+ ions (150 mM) are equivalent to Na^+ ions (Mayer and Westbrook, 1987). The first assumption raises some concern, because the contribution of Ca^{2+} to the NMDA current is significant (Schneggenburger, 1998) and Ca^{2+} ions reduce unitary conductance of NMDA receptors (Jahr and Stevens, 1993). Although the charge on spermine is ~4 at physiological pH, the molecule is likely to have an extended conformation in solution, so that the effective charge at the binding site would be less. Moreover, the molecule is ~1.9 nm long (Fig. 9), which is ~2.4 times the Debye length in mammalian saline (0.8 nm), and the charges at either end of the molecule would be significantly screened from each other. We found that calculations with a charge on spermine of +2 gave a better fit to the experimental data than those with a charge of +4.

Calculations from these assumptions fit the observations quite well, including the decreased block due to permeation at more negative potentials (*smooth curves* in Fig. 2, *A* and *B*). To obtain the fits we fixed the three electrical distances at different values and allowed the AJUSTE program to find the energies of the barriers and binding site. We fixed the binding site at electrical distances of 0.25, 0.5, and 0.75 and for each position varied the location of the barrier peaks between 0.25, 0.5, and 0.75 times the distance between the surface and the binding site, e.g., the barrier closest to either surface was at 0.0625. For each distance set, we determined the energies and examined the fit of the resulting *I-V* relations to the experimental curves. The calculations for Na^+ alone were insensitive to barrier location, but inclusion of the spermine data (with independent barrier and binding energies) restricted the distance parameters. Best fit to the data was achieved by setting the external and internal energy barriers at electrical distances of $D_2 = 0.125$ and $D_3 = 0.875$, respectively, and the binding site at $D_1 = 0.5$. The electrical distances and energies of the barriers and well in units of *kT* are shown in Table 1. The energy of the binding site corresponds to a K_D for spermine of 0.8 mM at 0 mV and 0.04 mM at − 70 mV. These K_D's, which represent the values in the absence of other permeant ions, are smaller than those obtained with the Woodhull analysis, in which the apparent K_D's are increased by competition from other permeant ions. The energy well for spermine was deeper than that for Na^+, indicative of lower conductance for spermine than for Na^+. The maximum barrier height was about the same for spermine and for Na^+, predicting that permeabilities of spermine and Na^+ are about the same (see Discussion). The analysis also predicts that permeation of the blocker contributes to transmembrane currents, more so at large negative potentials and at higher concentrations of blocker and lower concentrations of Na^+. The predicted current at 100 mM external and internal spermine, zero Na^+, and −60 mV is ~1 pA. We could not test this point, because at high external spermine concentrations (>30 mM) the stability of the patches was compromised. Predicted arcaine currents will be smaller than spermine currents.

TABLE 1 Parameters of the two-site, one-barrier models

	Neuronal receptors: 160 mM/80 mM NaCl			$NR1_{100}$/NR2B receptors	
	Na^+	Spermine	Arcaine	Na^+	Spermine
U	−6.43	−11.2/−13.1	−12.2/−12.6	−5.61	−11.8
G_2	6.34	2.38/2.06	8.20/3.80	6.27	4.25
G_3	5.53	6.01/4.71	12.0/8.19	6.39	4.21
D_1	0.5	0.5	0.5	0.6	0.6
D_2	0.125	0.125	0.125	0.45	0.45
D_3	0.875	0.875	0.875	0.7	0.7
SUMSQ	—	2.27/2.07	1.68/1.62	2.10	6.88
$K_D(0)$ (mM)	89	0.77/0.12	0.28/0.19	203	0.42
$K_D(-75)$ (mM)	20	0.040/0.006	0.014/0.010	46	0.02

U is the energy of the binding site, and G_2 and G_3 are the energies of the inner and outer barriers, respectively, all expressed in units of *kT*. D_1, D_2, and D_3 are the electrical distances of the binding site, outer barrier, and inner barrier from the outer membrane surface. SUMSQ is a measure of the goodness of fit achieved by the AJUSTE program. $K_D(0)$ and $K_D(-75)$ are the binding site affinities at zero and −75 mV. For neuronal receptors calculations were made for 160 mM Na^+ and spermine, then for 160 mM Na^+ and arcaine, using the same electrical distances and energies for Na^+. Calculations for 80 mM Na^+ were made with the same values for Na^+, but allowing new energy values for spermine and arcaine. The procedure was repeated with independent parameter values for $NR1_{100}$/NR2B receptors.

We also applied the two-barrier, one-site model to the action of spermine and arcaine in 80 mM Na^+. The current-voltage relations at 80 mM NaCl (Fig. 5) were reasonably well fit by the Eyring treatment with the same parameters as for 160 mM Na^+. A better fit was obtained with the same electrical distances but somewhat different values for the energies (*smooth curves* in Fig. 5). Modest changes in the blocking parameters may result from an additional action of the external ions, such as charge screening, from increased Debye length, or from deviations of the competition between spermine and Na^+ from the simple mechanism assumed (see Discussion).

Blocking actions of polyamines on recombinant NMDA receptors

Because hippocampal neurons express a heterogeneous population of NMDA receptor subtypes, which differ in their sensitivities to spermine potentiation and possibly to block, we examined polyamine action on recombinant NMDA receptors expressed in *Xenopus* oocytes. We used excised, outside-out patches from oocytes expressing the $NR1_{100}$/NR2B subtype. This subtype exhibits block by spermine but little or no potentiation (Zhang et al., 1994). NMDA evoked single-channel responses with unitary conductance of 51 ± 3 pS ($E_{rev} \approx 0$; $n = 10$; Fig. 6 *A*). Current-voltage relations were determined for the main open state during polyamine application from the internal as well as the external side of the membrane. As was observed with neuronal NMDA receptors, application of spermine to the external side of an outside-out patch reduced unitary conductance of recombinant $NR1_{100}$/NR2B receptors at negative potentials but had little effect at positive potentials (Figs. 6 *B* and 7, *left graph*, sample records at ±60 mV). The *I-V* relation for large negative potentials did not show an inflection, unlike that for neuronal receptors, but the flattening of the curve is consistent with a degree of relief of block. Application of spermine (1 mM) to the inside of the patch (by inclusion in the pipette solution) reduced unitary conductance at positive potentials but had little effect at negative potentials other than at the highest concentration (Figs. 6 *C* and 7, *right graph*). For large positive potentials there appeared to be a degree of relief of block. At the highest concentration (10 mM) spermine caused a small reduction in current at negative potentials, in addition to the greater block at positive potentials

Concentration-response curves for inhibition of NMDA responses by external and internal spermine are shown for different voltages in Fig. 8. The curves are fits of Eq. 1. The Hill coefficients were close to 1. I_{min} ranged between ~0.08 and 0.2 for external spermine and was ~0.2 for internal spermine. As for neuronal receptors (Fig. 3), the apparent K_D of the blocker was shifted to the left at more negative voltages for extracellular spermine. Correspondingly, the K_D for block by internal spermine was shifted to the left at more positive voltages.

The NMDA responses of $NR1_{100}$/NR2B receptors under all of these conditions were reasonably well fit by a two-barrier, one-site model (*smooth curves* in Fig. 7, fitted as for Fig. 2, parameters in Table 1). An effect of high concentrations of spermine at potentials tending to prevent it from

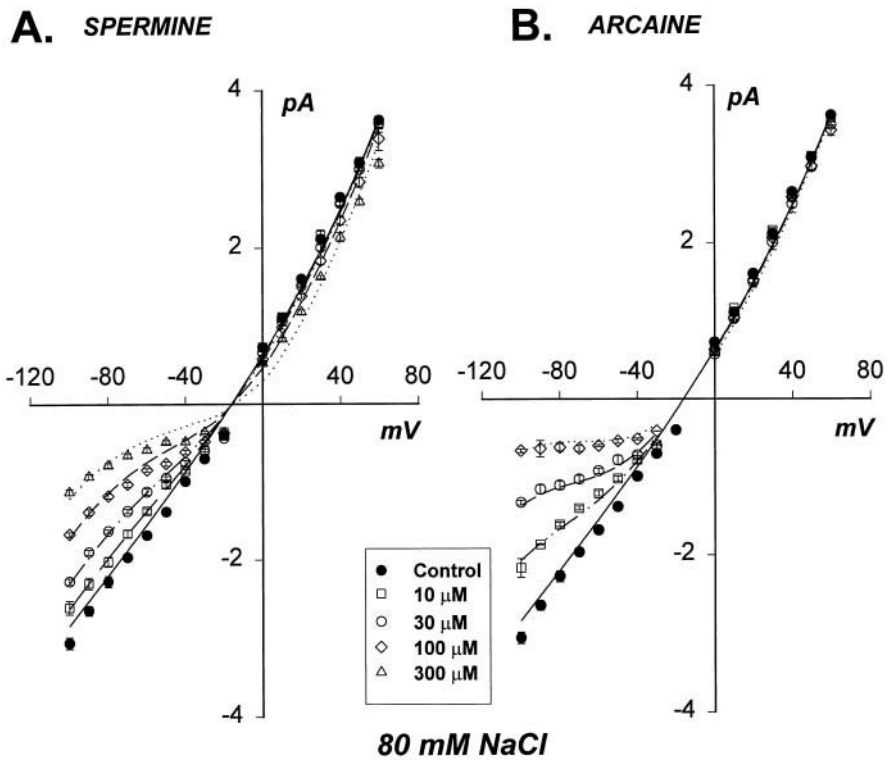

FIGURE 5 Single-channel current-voltage relations in the presence of spermine and arcaine at reduced NaCl. Plots are as in Fig. 2. Sensitivity to the polyamines is increased. Smooth curves are calculations according to Eyring rate theory, using parameters in Table 1.

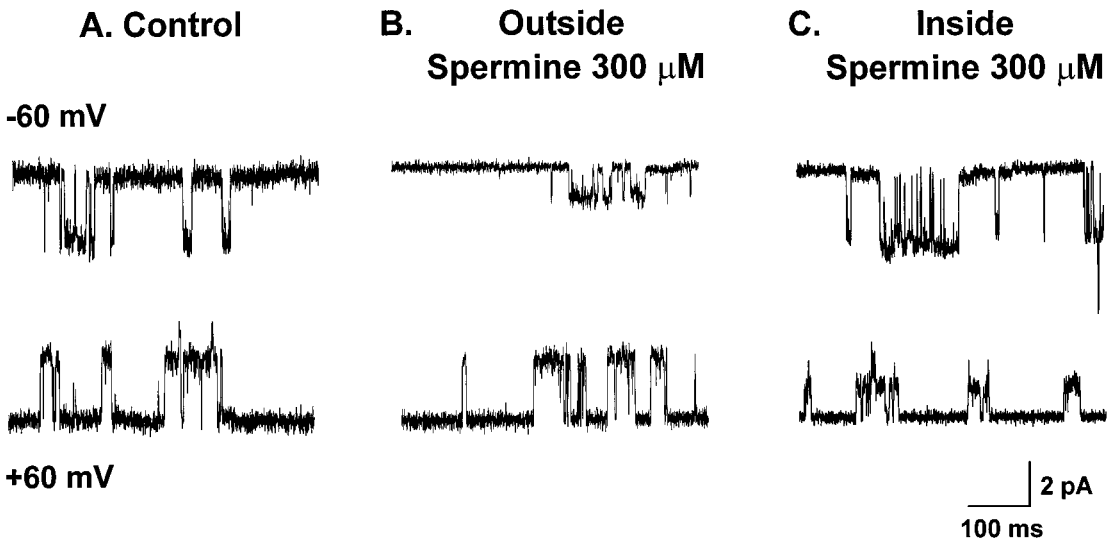

FIGURE 6 Intracellular as well as extracellular spermine reduces single-channel conductance of recombinant NMDA receptors. Representative single-channel records at −60 mV and +60 mV are from outside-out patches excised from *Xenopus* oocytes expressing $NR1_{100}$/NR2B receptors. (*A*) Control responses to NMDA (100 mM with 10 μM glycine). (*B*) At −60 mV spermine (300 μM) applied from the external side reduced current amplitude by ~50%. At +60 mV, external spermine (300 μM) produced little or no reduction in response. Same patch as for *A*. (*C*) At −60 mV internal spermine (300 μM in the pipette solution) had no effect on current amplitude, but at +60 mV, amplitude was reduced by ~50%.

entering the channel is predicted by the Eyring analysis and results from flux of spermine down a very large concentration gradient. The overall results are consistent with polyamine block by binding to a single site within the channel, which the blocker reaches from both sides of the membrane. The location parameters are quite different from those for neuronal receptors, and the predicted spermine permeability is higher than that for Na^+. The differences from the neu-

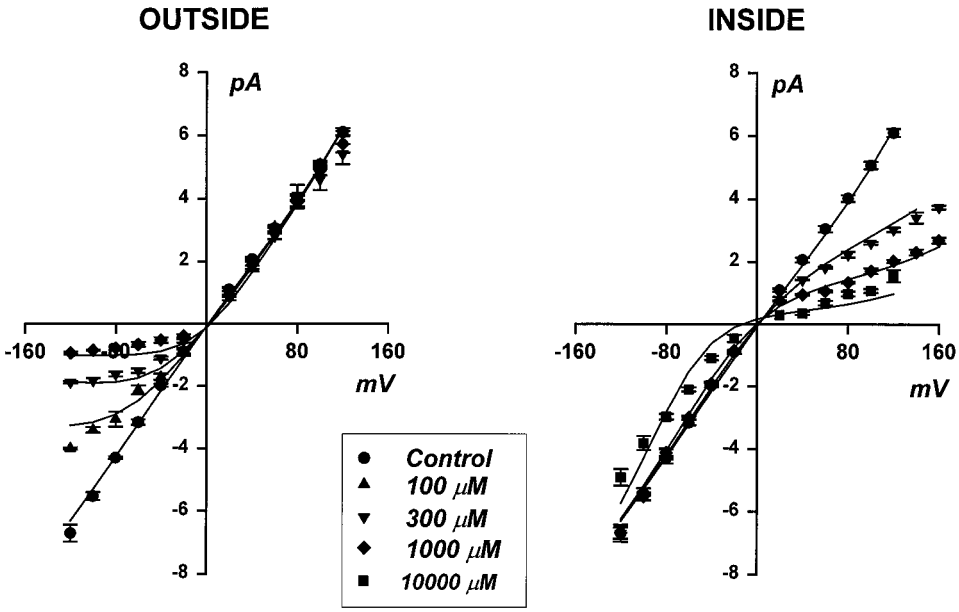

FIGURE 7 Block of $NR1_{100}$/NR2B receptors by spermine applied from either side is relieved at large potentials, tending to drive spermine through the channel. *Left graph:* Current-voltage relations of responses to NMDA (100 μM with 10 μM glycine) in the absence or presence of extracellular (outside) spermine (0.1, 0.3, and 1 mM). Spermine block increased with intracellular negativity, but at a reduced rate negative to ~ −80 mV, suggestive of permeation as in Fig. 2. *Right graph:* Current-voltage relations of single-channel responses to (extracellular) NMDA in the absence or presence of intracellular (inside) spermine (0.3, 1, and 10 mM in the pipette solution, different patches for different solutions). Spermine block increased with increasing intracellular positivity, but at a reduced rate positive to ~ +80 mV, suggestive of permeation. There was a small reduction in the current amplitude by 10 mM intracellular spermine at negative potentials. Data are means ± SEM of currents from a minimum of three patches. Smooth curves are fits of an Eyring two-barrier, one-site model with parameter values indicated in Table 1.

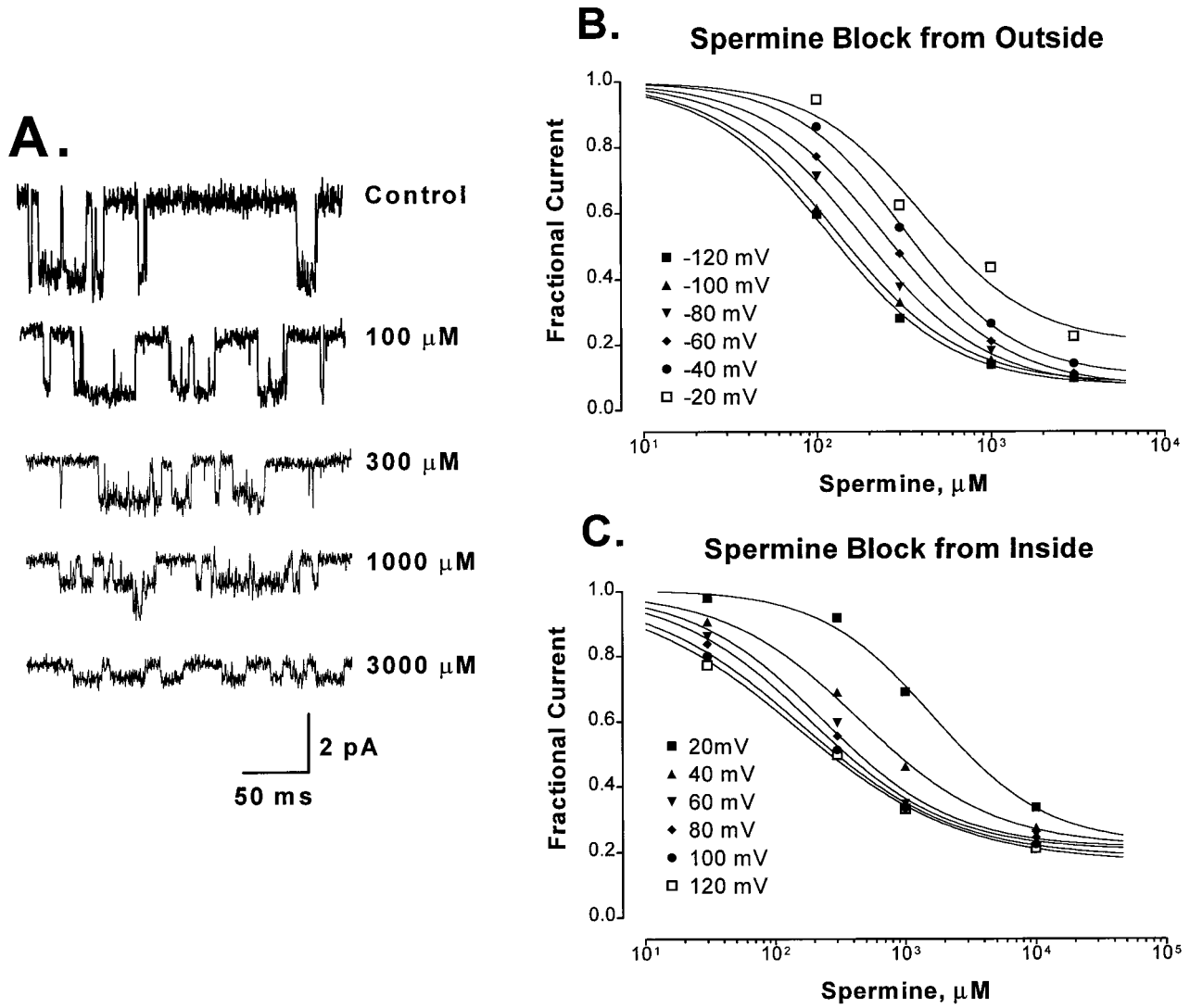

FIGURE 8 Concentration-response curves for the reduction of unitary conductance of $NR1_{100}$/NR2B receptors by external and internal spermine at different voltages. (*A*) Sample records of single-channel responses at −60 mV and different concentrations of external spermine. Single-channel records were obtained from outside-out patches excised from oocytes expressing $NR1_{100}$/NR2B receptors at holding potentials ranging from −20 to −120 mV as in Fig. 6. NMDA single-channel currents were elicited by the application of NMDA (10 μM in the presence of 1 μM glycine) in the absence and presence of (*B*) external spermine (30–3000 μM) or (*C*) internal spermine (30–1000 μM) at holding potentials ranging from −20 to −120 mV. Data points are means ± SEM from a minimum of three different patches and are normalized to the control NMDA current observed in the absence of polyamine. Continuous lines represent best fits of the logistic equation (Eq. 1). The concentration-inhibition curves were shifted to the left at more negative voltages for external spermine and at more positive voltages for internal spermine.

ronal receptors may represent differences in the receptor subtypes as well as the cellular environment.

DISCUSSION

NMDA channel block by external spermine and arcaine

The present study provides evidence for voltage-dependent block and permeation of neuronal and recombinant NMDA receptor channels by the polyamines spermine and arcaine. Cationic channel blockers are thought to enter the open channel and attenuate current by preventing current-carrying ions from permeating. Block by external polyamine increased with increasing negativity up to −60 mV, evidence for direct interaction of the blocker with the electric field. However, at potentials negative to −60 mV, the block did not increase as much as would be predicted for simple block by an impermeant ion and, for spermine application to neuronal receptors, actually decreased at the most negative potentials tested. This deviation is most readily explained by permeation of the polyamine through the channel at large negativities and distinguishes polyamines from other "classical" blockers of NMDA channels such as phencyclidine and MK-801. Internal spermine also blocked NMDA channels. The finding of relief of block by external spermine at extreme inside negativity is in agreement with whole-cell recording of NMDA responses in the presence of external

spermine (Benveniste and Mayer, 1993; Chao et al., 1997). Work on other channel types has demonstrated an inflection in the *I-V* relation as a characteristic of block induced by a permeable channel blocking ion (Bezanilla and Armstrong, 1972; French and Wells, 1977). Examples of block by permeable cations include block of squid axon potassium channels by Na^+ (Bezanilla and Armstrong, 1972; French and Shoukimas, 1985; French and Wells, 1977) and block of cardiac ryanodine receptors (Uehara et al., 1996) and kainate GluR6 receptors (Bahring et al., 1997) by polyamines. In contrast, polyamines block Kir channels, a mechanism that may underlie the so-called intrinsic gating, but do not permeate them (Lopatin et al., 1994).

The modest reduction in single-channel conductance at positive potentials and high concentrations of spermine may be a result of permeation of spermine against the voltage gradient but down its concentration gradient (cf. Fig. 6). The observed reduction is less voltage dependent than predicted for this mechanism, and spermine may have an additional action similar to that of Ca^{2+}, which is thought to reduce NMDA responses at positive potentials by binding to a second, extracellular site away from the voltage gradient (Sharma and Stevens, 1996).

The energy barriers were considerably higher for arcaine than for spermine, which is consistent with the smaller diameter of spermine (~0.44 nm) compared with that of arcaine (~0.55 nm) (Fig. 9).

For neuronal receptors the barrier heights for spermine and Na^+ were quite similar, indicating that the permeabilities, as opposed to the conductances, would not be very different. For $NR1_{100}$/NR2B receptors the predicted permeability for spermine was greater than that for Na^+. Because recordings were unstable at high polyamine concentrations, we did not determine the reversal potentials at these concentrations, which would have permitted us to establish the permeability ratio directly. Spermine permeability of GluR6 channels is relatively low (Bahring et al., 1997), and these authors used a three-barrier, two-site model to describe spermine potentiation, although fit by a two-barrier, one-site model was adequate, except for the high spermine permeability. The NMDA channel may actually be more permeable to spermine, and a single binding site model may be appropriate. For Mg^{2+} permeation, separate internal and external binding sites are indicated from mutational and biophysical studies, but the sites are located physically close to each other (Wollmuth et al., 1998a,b). The extended structure of spermine suggests that treatment of its binding as a single event is unrealistic, and more complex models may be required.

The site of polyamine block

Recent studies identify amino acid residues whose mutation affects polyamine block of NMDA channels. The asparagine within the second transmembrane domain (TM2) of the NR1 subunit (N598 and N619 in subunits without and with the N1 splice cassette) and the corresponding two asparagines in NR2A, which affect Ca^{2+} permeability and block of NMDA channels by Mg^{2+}, also influence voltage-dependent block of NMDA channels by spermine, N^1-dansylspermine, and the polyamine-conjugated spider toxin argiotoxin (Chao et al., 1997; Raditsch et al., 1993; Zheng et al., 1998). These findings are consistent with overlap of the sites at which Mg^{2+} and spermine bind within the NMDA channel. The critical residues are at the tip of the reentrant loop formed by the TM2 domains of the receptor subunits (Kuner et al., 1996; Wollmuth et al., 1998a,b), which might appear to argue for a single binding site. However, mutational and studies indicate that single asparagine mutations differentially affect block by internal and external Mg^{2+} and that blocking sites are different (Kohler et al., 1993; Wollmuth et al., 1998a,b). Block of GluR6 channels by internal spermine could be treated by a two-barrier, one-site model but required greater spermine permeability than was consistent with biionic measurements against Na^+ (Bahring et al., 1997). A three-barrier, two-site model gave a satisfactory fit with lower spermine permeability. Although our two-bar-

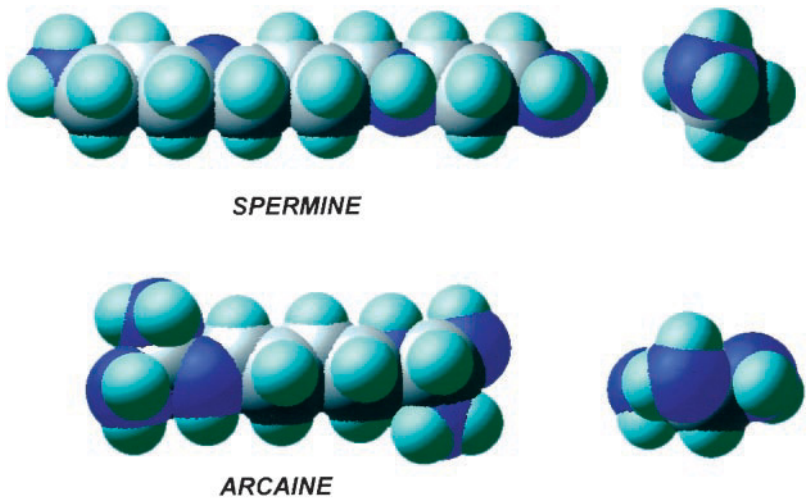

FIGURE 9 CPK models of spermine and arcaine, showing extended conformations from side and end-on views. Spermine has a diameter of ~0.4 nm and a length of ~1.9 nm. Arcaine has a diameter of ~0.5 nm and a length of ~1.3 nm. Because the Debye length in mammalian saline is ~0.8 nm, these molecules are ~2.4 and ~1.7 Debye units in length in the solutions employed in our experiments.

rier, one-site treatment also predicted a high spermine permeability, we did not evaluate a three-barrier, two-site model, because we did not have biionic data.

A contribution from neutralization of surface charge

Because the polyamines are polyvalent cations, they could reduce conductance through neutralization of surface charge. Because maximum block by arcaine is nearly complete, arcaine has been thought to be a channel blocker (Donevan et al., 1992). Reduction in surface charge does not produce complete block, and lack of complete block by high concentrations of spermine might be considered an indication that spermine acted by neutralization of surface charge. The increased effectiveness of block at lowered ionic strength might also be considered evidence for surface charge neutralization. However, both of these actions of spermine can be explained by the permeation model. Apparent blocking affinity is increased at lowered ionic strength because of lack of competition for the binding site, and block is incomplete because spermine can permeate. Calculations from the Eyring model showed concentration inhibition curves comparable to those observed (Fig. 3, calculations not illustrated)

Physiological significance of polyamine block

The polyamines spermine, spermidine, and putrescine are present in neurons and, upon depolarization, are released from presynaptic terminals in the hippocampus (Harman and Shaw, 1981; Seiler and Deckardt, 1978; Shaw and Pateman, 1973). Free cytoplasmic concentrations of polyamines appear to be too low ($\sim$50 μM spermine), at least in HEK293 cells (Bowie and Mayer, 1995), to have much effect on NMDA receptors directly, certainly not at inside negative potentials (see Fig. 7), but if polyamines were concentrated in and released from vesicles, extracellular concentrations might reach levels sufficient to modulate NMDA responses. Early in neonatal life, the major form of the NR2 subunit is NR2B, which assembles with NR1 subunits lacking the N-terminal insert to form channels that are potentiated by spermine at saturating concentrations of glycine. All subtypes of NR1/NR2A subunits show potentiation at low concentrations of glycine, which is mediated by an increase in glycine affinity, but none show potentiation at saturating glycine. In more mature neurons, NR2A is more prevalent and is likely to form channels that are more susceptible to the blocking effects of polyamines. It is not known whether changes in intracellular or extracellular levels of polyamines alter their modulation of glutamate receptors. Residues in kainate and AMPA receptors homologous to the NMDA receptor TM2 asparagines affect block by polyamines (Bowie and Mayer, 1995; Verdoorn et al., 1991). Intracellular polyamines also mediate rectification of neuronal nicotinic receptors (Haghighi and Cooper, 1998), inwardly rectifying K^+ channels (Lopatin and Nichols, 1996) and ryanodine receptors (Uehara et al., 1996); thus, a variety of channels are regulated by this mechanism.

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