Effect of Zinc on NMDA Receptor-Mediated Channel Currents in Cortical Neurons

Chadwick W. Christine and Dennis W. Choi

Department of Neurology, Stanford University Medical Center, Stanford, California 94305

Recent data have indicated that the divalent cation Zn^{2+} can selectively block central neuronal excitation mediated by *N*-methyl-D-aspartate (NMDA) receptors. The present experiments were conducted to determine the action of Zn^{2+} at the single-channel level. Outside-out membrane patches were prepared from cultured murine cortical neurons. Glutamate, $3\mu M$, in the presence of $5\mu M$ glycine activated channels with a main conductance state of about 50 pS which were blocked in a voltage-dependent manner by Mg^{2+} . Zn^{2+} appeared to have 2 effects on these NMDA receptor-activated channels. First, at concentrations as low as $1\text{--}10\mu M$, Zn^{2+} produced a concentration-dependent reduction in channel open probability, insensitive to membrane voltage between -60 and $+40$ mV; about 50% reduction in open probability was produced by $3\mu M$ Zn^{2+} . This reduction was mostly due to a decrease in opening frequency and only weakly mimicked by Mg^{2+} . Second, at higher concentrations ($10\text{--}100\mu M$) and negative membrane voltages, Zn^{2+} additionally produced an apparent reduction in single-channel amplitude, associated with an increase in channel noise, suggestive of a fast channel block. The amplitude reduction was voltage-dependent, with a δ of 0.51; amplitude distribution analysis suggested that this voltage dependence was primarily contributed by the "on" blocking rate constant, with little contribution from the "off" rate constant. The channel block produced by Zn^{2+} was faster than that of Mg^{2+} , which at $100\mu M$ and negative membrane voltages induces flickering of the NMDA receptor-activated channel without changing apparent channel amplitude.

Thus, Zn^{2+} may reduce NMDA receptor-activated channel currents on cortical neurons by acting at 2 different sites, one outside the membrane field and affecting opening frequency, and the other inside the channel and interfering directly with the passage of ions. In this manner, Zn^{2+} co-released with glutamate from presynaptic terminals may dynamically modulate NMDA receptor-activated currents.

The mammalian CNS contains large amounts of chelatable Zn^{2+} (Maske, 1955; Frederickson et al., 1983; Danscher et al., 1985). Much of this Zn^{2+} is localized to synaptic vesicles in

excitatory boutons throughout the telencephalon and is likely released into the synaptic cleft during excitation (Assaf and Chung, 1984; Howell et al., 1984), especially at higher rates of neuronal firing (Aniksztejn et al., 1987). The extracellular accumulation of synaptically released Zn^{2+} may be substantial. Assaf and Chung (1984) found that exposure to high K^+ induced the release of 18% of the Zn^{2+} contained in rat hippocampal slices, an amount of Zn^{2+} that distributed evenly in tissue extracellular space would produce a concentration of $300\mu M$.

Based on the observations that Zn^{2+} selectively attenuates the neuroexcitatory and neurotoxic effects of *N*-methyl-D-aspartate (NMDA) receptor agonists on cortical neurons, while slightly increasing the effects of AMPA or quisqualate (Peters et al., 1987), we proposed that Zn^{2+} might be an important endogenous modulator of the postsynaptic response to co-released transmitter glutamate. A similar conclusion was reached independently by Westbrook and Mayer (1987), who found that Zn^{2+} could noncompetitively reduce NMDA-evoked currents in hippocampal neurons. In addition, Forsythe et al. (1988) showed that Zn^{2+} could block slow NMDA receptor-mediated excitatory postsynaptic potentials in hippocampal neurons.

The mechanisms by which Zn^{2+} might produce this physiologically relevant antagonism of NMDA receptor activation has not yet been defined. Another divalent cation, Mg^{2+} , has been established to produce voltage-dependent brief interruptions (flickering) of current through the NMDA receptor-linked channel (Nowak et al., 1984; Ascher and Nowak, 1988). The simple possibility that Zn^{2+} might act exactly like Mg^{2+} seems not to be the case, as Zn^{2+} antagonism of cellular NMDA responses appears to show less voltage dependence (Peters et al., 1987; Westbrook and Mayer, 1987). The purpose of the present study was to investigate the nature of Zn^{2+} antagonism at the single-channel level. Abstracts have appeared (Christine and Choi, 1988, 1989).

Materials and Methods

Cortical cell culture. Mixed cortical cell cultures, containing both neuronal and glial elements, were prepared as previously described (Choi et al., 1987) from fetal mice at 14–18 d gestation. Dissociated cortical cells were plated in Primaria (Falcon) 35 mm dishes (10^6 cells/dish) in Eagle's minimal essential medium (MEM—Earle's salts, supplied glutamine-free) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 mM), and glucose (total, 21 mM). Cultures were kept at $37^\circ C$ in a humidified CO_2 -containing atmosphere. After 5–12 d *in vitro*, non-neuronal cell division was halted by exposure to 10^{-5} M cytosine arabinoside, and the cells were shifted into a maintenance medium identical to the plating media, but lacking fetal serum. Subsequent media replacement was carried out twice per week. Cultures from 8–20 d *in vitro* were used.

Patch-clamp methods. Patch pipettes were pulled from borosilicate glass tubing in 3 or 4 stages using a Sutter Instrument P-80 puller. Pipette

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Correspondence should be addressed to Dennis W. Choi, Department of Neurology, C-338, Stanford University Medical Center, Stanford, CA 94305.

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resistance was 8–15 mΩ when filled with the below pipette solution and placed in the bath. A silver–silver chloride wire was used as the electrode within the patch pipette and, through an agar/saline bridge, as the bath ground. A List EPC-7 patch-clamp amplifier with a 10 kHz filter setting was used to measure membrane currents and control transmembrane potential. The electrode balancing circuit of the amplifier was adjusted to give zero current just prior to each experiment and was routinely checked after a patch had been lost. Offset potentials after an experiment seldom exceeded 1 mV.

Recordings were made at room temperature using the outside-out patch configuration (Hamill et al., 1981). Only stable recordings lacking spontaneous channel activity were studied. Patch current and pipette potential were recorded continuously and stored on magnetic tape for subsequent analysis. Patch seal resistance was determined by measuring the change in membrane current when holding potential was changed (usually in 10 mV steps).

Solutions. The patch pipette was filled with an “intracellular” solution containing (in mM) 140 CsCl, 4 NaCl, 0.5 CaCl₂, 5 EGTA (pCa ≈ 7.3), and 10 HEPES. Prior to an experiment, the maintenance medium was rinsed from the culture dish and replaced by a defined solution containing (in mM): 140 NaCl, 2.8 KCl, 1 CaCl₂, 10 HEPES, and 5 glucose. The pH of all solutions was adjusted to 7.3 using NaOH (final pipette [Na⁺] = 18 mM; final extracellular [Na⁺] = 146.6 mM).

After an outside-out patch was obtained, the pipette tip was inserted into a constant-flow fluid-exchange pipette (Yellen, 1982) mounted distal to a rotary valve allowing rapid selection of experimental perfusion solutions. Full replacement of solution from the dead space between the valve and the exchange pipette took approximately 30 sec (this was confirmed with junction potential shifts after a change to solution with a reduced salt concentration). All experimental solutions were composed of the bathing solution (see above) plus 5 μM glycine to increase the opening of NMDA receptor-activated channels (Johnson and Ascher, 1987); glucose was omitted. Most experimental solutions also contained 3 μM glutamate, along with the desired concentration of either Zn²⁺ or Mg²⁺. Glutamate was the selected agonist for the NMDA receptor because its dissociation constant with divalent cations is known. Glutamate and glycine concentrations were increased when Zn²⁺ was present in excess of 1 μM to correct for their complexing (Perrin, 1986) and maintain free concentrations of 3 and 5 μM, respectively. For example, in the presence of 100 μM Zn²⁺, added glutamate was 3.4 μM and added glycine was 5.3 μM.

Single-channel analysis. Data selected for analysis was played back from magnetic tape and converted to digital format. In general, recordings with frequent double-channel openings or substate transitions were excluded from quantitative analysis; thus, analysis was based largely on patches with only a single active channel. Patch current was normally filtered at 1–2 kHz (–3 dB corner frequency, 8-pole Bessel) and digitally sampled at 5 times the filtering frequency.

Two types of amplitude histograms were constructed. The first type, representing mean channel amplitudes, was based on analysis by a commercially available software package (P-Clamp 4.0, Axon Instruments). Mean amplitude values were calculated for each event that exceeded a threshold of one-half the average channel amplitude. Events shorter than 2 msec were considered to be attenuated by the high-frequency filter and hence not included in these mean amplitude histograms, but they were included in dwell-time analysis. Care was taken to reset the baseline when shifts occurred.

A second type of amplitude histogram was constructed for rate-constant analysis of the channel flicker. A routine written in PASCAL was used to construct point by point amplitude histograms directly from digitized current records. These histograms therefore reflected the full range of channel activity, rather than the means of defined events.

In the 2-state fast channel block model (Neher and Steinbach, 1978), an open channel alternates rapidly between an open and blocked state, with the block “off” rate, α , and the “on” rate, β .

If channel current is filtered with a first-order filter of time constant τ , FitzHugh (1983) and Yellen (1984) have demonstrated that the amplitude distribution will be a β -distribution with 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

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

Since practical filters are not first order, an empirical conversion for an 8-pole Bessel filter is given by Yellen (1984) as $\tau = 0.228/f$, where τ is the time constant of the equivalent single-pole filter. We found that filtering at 1 kHz provided the best resolution of the amplitude distribution, yielding $\tau = 228 \mu\text{sec}$.

For this analysis we formed histograms of current amplitude from portions of experiments selected for a single active channel with no substate activity along with some baseline current. Care was taken to avoid data with a changing baseline. A Gaussian curve was fitted to the peak corresponding to the baseline and was subtracted from the histogram to remove the closed channel contribution; this procedure produced little change in the histogram fits since analysis was not attempted when the baseline overlapped the open channel level. The mean of the Gaussian fit was used as the zero-amplitude bin of the amplitude histogram; in each experiment, histograms were normalized to full channel amplitude obtained when no blocking ion was present.

Computer-generated β -distributions were fitted by eye to each normalized amplitude histogram by adjusting the 2 parameters a and b . The fit was quite sensitive to small changes in both parameters, so optimization to within 0.3 generally involved little ambiguity. In order to compensate for the broadening of the experimental distribution by unrelated noise, the theoretical distribution was also broadened by convolving it with the Gaussian used to fit the baseline.

Dwell times were determined using a half-amplitude definition of transitions. Three measurements were determined from the dwell-time analysis: the open probability (P_o), the frequency of opening (f_o), and mean channel open time (τ_o). To compare data from different experiments, we scaled raw values of f_o and P_o with a control period when no Zn²⁺ or Mg²⁺ was present. Open probability and opening frequency were then expressed as a percentage of this control period.

Open time histograms were constructed from dwell-time analysis, and these were fitted with single- and double-exponential fits to determine the mean channel open time. In all cases, the first bin was ignored in the fitting process. While some experiments required a double exponential for a good fit ($\tau_1 \approx 1$ –2 msec, $\tau_2 \approx 9$ –10 msec (see Jahr and Stevens, 1987; Ascher et al., 1988), we found that most were adequately fitted with a single exponential ($\tau \approx 5$ msec). We used the values of single-exponential fits to compare different experimental conditions.

Drugs and reagents. Glutamate and glycine were purchased from Sigma. ZnCl₂ and MgCl₂ were analyzed reagent grade from Baker.

Results

Recordings were obtained from outside-out patches that showed no spontaneous channel activity. Perfusion of these patches with the “extracellular” solution plus 5 μM glycine alone did not activate channels, but the addition of 3 μM glutamate to the perfusate often elicited prominent channel activity (Figs. 1, 2). These channels had a main conductance state of about 50 pS, with infrequent transitions to subconductance states ranging from 15 to 50 pS. Most recordings did not have frequent substate activity, and those which did were excluded from quantitative analysis. Based on activation by 3 μM glutamate, sensitivity to Mg²⁺ block, mean channel open time, and I – V relationship (see below), these channels can be clearly identified as linked to the NMDA receptor.

Figure 1A shows the overall qualitative effect of Zn²⁺ on these NMDA channels at a holding potential of –50 mV. Zn²⁺, 10 μM, produced a clear-cut reduction in the number of channel openings; 100 μM Zn²⁺ reduced channel amplitude as well (Fig. 1A). Both of these effects were reversible. In addition, Zn²⁺ application was reproducibly associated with an upward shift in the patch current baseline, reflecting an increase in patch resistance. The magnitude of this increase varied among different patches; in a given patch, it increased with membrane hy-

Figure 1. Zn^{2+} curtails NMDA channel activity. **A**, Pen writer traces of activity elicited by $3\ \mu\text{M}$ glutamate in a patch containing multiple active channels. Arrows mark changes in perfusion solutions; some mixing of solutions is inevitable and is most apparent during the washout period with control solution. Membrane potential = $-50\ \text{mV}$; channel openings are downward. Addition of Zn^{2+} caused an increase in apparent seal resistance; values were $7.1\ \text{G}\Omega$ (control), $7.2\ (1\ \mu\text{M}\ \text{Zn}^{2+})$, $10.5\ \text{G}\Omega$ ($10\ \mu\text{M}\ \text{Zn}^{2+}$), $65\ \text{G}\Omega$ ($100\ \mu\text{M}\ \text{Zn}^{2+}$), and $6.5\ \text{G}\Omega$ (second control). **B**, In this experiment, divalent cations increased seal resistance from $3.0\ \text{G}\Omega$ (control) to $8.9\ \text{G}\Omega$ ($10\ \mu\text{M}\ \text{Zn}^{2+}$) and $3.4\ \text{G}\Omega$ ($10\ \mu\text{M}\ \text{Mg}^{2+}$). Zn^{2+} was more effective in reducing opening frequency than Mg^{2+} ; membrane potential = $-50\ \text{mV}$, same time and current scale as in **A**.

perpolarization and raised Zn^{2+} concentrations. For example, in one recording $100\ \mu\text{M}\ \text{Zn}^{2+}$ increased patch resistance by a factor of 4.5 at a holding potential of $-60\ \text{mV}$, but only a factor of 1.2 at $+30\ \text{mV}$. This action of Zn^{2+} did not depend upon the presence of active NMDA channels in the patch. Mg^{2+} at similar concentrations did not have such a pronounced effect on patch resistance (Fig. 1B).

Further examination of the effect of Zn^{2+} on channel amplitude revealed that increasing Zn^{2+} over the range $1\text{--}100\ \mu\text{M}$ produced a graded reduction in apparent mean single-channel amplitude, associated with an increase in open-channel noise (Figs. 2, 3). In comparison, $100\ \mu\text{M}\ \text{Mg}^{2+}$ at negative membrane potentials produced resolvable flickering between the full open and the closed states (Fig. 3A). Neither Zn^{2+} nor Mg^{2+} had an effect on channel amplitude or mean channel open time at pos-

itive holding potentials (Figs. 3B, 4A), although both of these divalent cations did reduce opening frequency at all tested membrane potentials (see below).

The effect of $1\text{--}100\ \mu\text{M}\ \text{Zn}^{2+}$ on the channel $I\text{--}V$ relationship, constructed from amplitude histograms, is shown in Figure 4A. At Zn^{2+} concentrations greater than $10\ \mu\text{M}$ and holding potentials below $-50\ \text{mV}$, channel amplitude leveled off consistent with a voltage-dependent block. Unfortunately, membrane potentials more negative than $-80\ \text{mV}$ were poorly tolerated by patch membranes. It was thus not possible to determine whether the Zn^{2+} block could be relieved with strong hyperpolarization as has been reported with other divalent cations, most notably Mg^{2+} (Mayer and Westbrook, 1987).

Of note, the $I\text{--}V$ curves did not pass through the origin but instead inverted at about $-6\ \text{mV}$. Part of this offset was likely

Figure 2. Zn^{2+} reduces single-channel amplitude in a concentration-dependent manner. These data, all from the same patch, were elicited by application of $3\ \mu\text{M}$ glutamate; membrane potential = $-60\ \text{mV}$ and openings are downward. Panel **A** shows sample traces, while panel **B** shows the corresponding amplitude histograms. Histograms represent the frequency of specified mean channel amplitude (see Materials and Methods); the bin width was $0.1\ \text{pA}$. The first trace shows a control record (no Zn^{2+}). In subsequent traces and their corresponding histograms, increasing concentrations of Zn^{2+} (10 , 50 , and $100\ \mu\text{M}$) were included in the experimental solution. Data were filtered at $2\ \text{kHz}$ and sampled at $10\ \text{kHz}$ both for display in **A** and for analysis in **B**.

Figure 3. Zn^{2+} block is voltage-dependent but qualitatively different from Mg^{2+} block. Activity from a single patch perfused with $3\text{ }\mu\text{M}$ glutamate without (control) or with the indicated divalent cations. *A*, Traces obtained at -60 mV ; *B*, traces obtained at $+40\text{ mV}$. The Zn^{2+} and Mg^{2+} traces at $+40\text{ mV}$ reveal a second active channel. Data was filtered at 2 kHz and sampled at 10 kHz .

Figure 4. Zn^{2+} reduces single-channel amplitude in a voltage-dependent manner. *A*, Current-voltage relations of NMDA channels in experimental solutions containing different concentrations of Zn^{2+} . Results from different experiments have been pooled for $100\text{ }\mu\text{M Zn}^{2+}$ ($n = 4$), for $10\text{ }\mu\text{M Zn}^{2+}$ ($n = 3$), and for control ($n = 5$). The data for $1\text{ }\mu\text{M Zn}^{2+}$ and $50\text{ }\mu\text{M Zn}^{2+}$ are from single experiments. *B*, Data from above are plotted as $i_b/(i_o - i_b)$ using points at $100\text{ }\mu\text{M Zn}^{2+}$ as i_o . The line denotes the fit of equation 1 (see text) to the data: $K_B(0) = 909\text{ }\mu\text{M}$ and $\delta = 0.51 \pm 0.014$.

Figure 5. Zn^{2+} concentration dependence of blocking rates. *A*, Examples of β -distribution fits to full data amplitude histograms. Proceeding from right to left, $[\text{Zn}^{2+}]$ increases as indicated. Increasing $[\text{Zn}^{2+}]$ shifted the amplitude distribution toward zero, and the fits reflect this shift as an increase on the on rate; the off rates were relatively unaffected. Values for the on rates at 10 , 50 and $100\text{ }\mu\text{M Zn}^{2+}$ are 5.0×10^3 , 1.0×10^4 , and $1.7 \times 10^4\text{ sec}^{-1}$; for the off rates they are 2.7×10^4 , 2.6×10^4 , and $2.7 \times 10^4\text{ sec}^{-1}$. Bin width was 0.06 pA ; membrane potential was -50 mV . *B*, Concentration dependence of blocking rates. The on and off rates are plotted as a function of $[\text{Zn}^{2+}]$. Points represent means ($\pm\text{SEM}$) of 3 experiments. The line plotted through the on rate constants has a slope of $2.25 \times 10^6\text{ M}^{-1}\text{ sec}^{-1}$; the line represents the mean value ($2.9 \times 10^4\text{ sec}^{-1}$).

Figure 6. Voltage-dependence of blocking rate constants. *A*, β -distribution fits at indicated membrane potentials. In this experiment, $[Zn^{2+}]$ was held constant at $100 \mu M$ and the membrane potential varied from -30 to -70 mV. As the membrane is hyperpolarized, the amplitude distribution shifts toward zero, reflecting an increase in the on rate but not the off rate. Values for the on rates at -30 , -50 , and -70 mV are 1.5×10^4 , 3.5×10^4 , and 5.9×10^4 ; for the off rates, they are 2.9×10^4 , 2.7×10^4 , and 2.7×10^4 . Bin width was 0.06 pA at each voltage. *B*, Voltage dependence of blocking rate constants. On and off rate constants are plotted as a function of membrane potential. Points represent the mean ($\pm$ SEM) of fits from 5 experiments. Linear regression of the on rate constants yields a line corresponding to $\delta = 0.54$. The line drawn through the off rate constant points is horizontal and represents the mean value over the voltage range (2.78×10^4 sec $^{-1}$). A plot of the ratio off rate constant/on rate constant ($=K_d$) yields a similar slope and yields a value of $990 \mu M$ for K_d at 0 mV. The Zn^{2+} concentration was $100 \mu M$.

due to the slightly higher activity of monovalent ions in the patch pipette vs the bath (119.7 vs 113.9 mM), which would account for a -1.3 mV offset if Cs^+ and Na^+ permeate the channel equally well. The balance of the offset may indicate a slightly greater permeability to Cs^+ over Na^+ . Such an offset in the I - V relationship has been noted previously (Cull-Candy and Usowicz, 1987).

The voltage dependence of the Zn^{2+} block could be described using the relation (Woodhull, 1973)

$$i_b/(i_o - i_b) = \{K_d(0)/[B]\} \exp(z\delta V_e/kT), \quad (1)$$

where i_b is the channel current in the presence of the blocker, i_o is the current in the absence of blocker, $K_d(0)$ is the dissociation constant for the blocker at 0 mV, $[B]$ is the concentration of the blocking ion, z is its valence, δ is the fraction of the membrane potential that is affecting block, V is the membrane potential difference, e is the elementary charge, k is Boltzmann's constant, and T is the temperature in Kelvin. At $100 \mu M$ Zn^{2+} ,

Figure 7. Effect of Zn^{2+} and Mg^{2+} on open probability and opening frequency. Open probability and opening frequency at -50 mV is plotted as a function of divalent cation concentration. All data points represent the mean values from 4–6 experiments ($\pm$ SEM).

this equation yielded values of 0.51 for δ and $909 \mu M$ for $K_d(0)$ (Fig. 4*B*).

The most likely explanation for the observed voltage-dependent effect of Zn^{2+} on channel amplitude is that it produced a flicker block qualitatively similar to that produced by Mg^{2+} , but much faster. Frequent brief interruptions of channel current too rapid to be resolved as individual events would lead to an apparent reduction in the level of open-channel current and would account for the observed increased noise in this current. Such a poorly resolved rapid flickering block has been shown to characterize the block of the Ca^{2+} -activated K^+ channel by Cs^+ (Yellen, 1984), and may also characterize the block of the NMDA channel by Mn^{2+} (Ascher and Nowak, 1988).

Attempts to resolve Zn^{2+} flickering by raising the high-frequency filter cutoff to 2 kHz were unsuccessful; higher settings were deemed unsatisfactory due to excessive high-frequency artifact noise. As an alternative test of the flicker hypothesis (and a method for estimating block on and off rates), we analyzed amplitude histograms of channel currents in the presence of Zn^{2+} according to a simple model of open-channel block (see Materials and Methods, as well as Neher and Steinbach, 1978; Yellen, 1984). In this model, the forward reaction rate, β , depends on the concentration of Zn^{2+} , while the rate of unblocking, α , is independent of Zn^{2+} concentration. Such a mechanism has been proposed for the block by Mg^{2+} of the NMDA receptor-activated channel (Ascher and Nowak, 1988).

Increasing Zn^{2+} concentration from 10 to $100 \mu M$ at a constant holding potential of -50 mV produced a progressive shift in the channel amplitude histograms toward smaller values; these histograms could be closely approximated by theoretical β -distributions convoluted with baseline noise (Fig. 5*A*). Resultant calculated values for the block on rate increased linearly from 5.0×10^3 to 1.7×10^4 sec $^{-1}$ with increasing Zn^{2+} concentration (Fig. 5*B*). In contrast, the block off rate remained nearly constant at 2.7×10^4 sec $^{-1}$ with changing Zn^{2+} concentration (Fig. 5*B*). The finding that only the on rate was dependent on Zn^{2+} concentration is thus consistent with the simple blocking scheme mentioned above.

Similarly, increasing membrane hyperpolarization from -30 to -70 mV also produced a downward shift in the channel amplitude histograms, which were well fit by the theoretical β -distributions (Fig. 6*A*). Over this membrane potential range,

Figure 8. Effect of Zn^{2+} on mean channel burst duration. Open-time histograms were prepared from a single recording where the patch (held at -50 mV) was first exposed to a control solution with $3 \mu\text{M}$ glutamate and then an experimental solution containing $100 \mu\text{M}$ Zn^{2+} . Single-exponential fit to the control histogram (1078 events) gives $\tau = 4.3$ msec and that to the $100 \mu\text{M}$ Zn^{2+} histogram (175 events) gives $\tau = 1.5$ msec. Intermediate concentrations of Zn^{2+} produced a graded effect on mean channel burst duration; $1 \mu\text{M}$ Zn^{2+} gave $\tau = 4.1$ msec, $10 \mu\text{M}$ Zn^{2+} gave $\tau = 3.2$ msec.

Figure 9. Voltage independence of Zn^{2+} effect on channel open probability and mean channel burst duration. *A*, Open probability P_o as a function of membrane potential. Either with or without $10 \mu\text{M}$ Zn^{2+} , P_o shows minimal voltage dependence. Values for P_o represent means from 4 experiments $\pm$ SEM. Linear regression yields slopes of $0.076\%/mV$ for control and $0.13\%/mV$ for $10 \mu\text{M}$ Zn^{2+} . *B*, Mean channel open time or burst duration, τ , as a function of membrane potential. Either with or without Zn^{2+} , τ shows minimal voltage dependence. Each value for τ represent the mean of 3 experiments $\pm$ SEM. Linear regression through the data yields slopes of 0.0003 msec/mV for control and 0.006 msec/mV for $10 \mu\text{M}$ Zn^{2+} .

the on rate constant increased from 1.5×10^8 to $5.9 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$, while the off rate constant remained steady at about $2.8 \times 10^4 \text{ sec}^{-1}$ (Fig. 6*B*). The voltage dependence of the on rate constant line fitted by regression would account for a δ of 0.54 in Equation 1 above. A plot of K_d (not shown) yielded a value of $990 \mu\text{M}$ by extrapolation to 0 mV, which agrees with the value of $909 \mu\text{M}$ calculated from the Woodhull model (Fig. 4*B*). Thus, the voltage dependence of the Zn^{2+} block is fully accounted for by the voltage dependence of the forward blocking reaction.

The next experiments were designed to characterize the ability of Zn^{2+} to reduce channel opening. First, quantitative examination of the ability of Zn^{2+} to reduce channel open probability (P_o) revealed a potent, concentration-dependent action (Fig. 7). Only about $3 \mu\text{M}$ Zn^{2+} was needed to reduce P_o by half. Mg^{2+} shared this effect with Zn^{2+} but was about 100 times less potent.

When there is only one active channel, open probability can be broken down as follows:

$$P_o = f_o \tau$$

where f_o is the opening frequency and τ is the mean channel open time. Therefore, a reduction in P_o could be due to a reduction in opening frequency, f_o , or a reduction in mean channel open time, τ . The effect of Zn^{2+} on P_o was largely accounted for by reduction in f_o , for Zn^{2+} concentrations below $10 \mu\text{M}$ (Fig. 7). Above $10 \mu\text{M}$, P_o was reduced further than f_o , suggesting the existence of a concurrent reduction in τ (Fig. 7). This was confirmed by direct examination of open-time histograms; for example in one experiment, $100 \mu\text{M}$ Zn^{2+} reduced τ from 4.30 to 1.56 msec (Fig. 8). These open-time histograms were constructed from data filtered at 1 kHz; since brief interruptions in channel current were not resolved, the estimate of τ in the presence

of Zn^{2+} is best considered to reflect the burst duration of the channel.

Neither the effect of Zn^{2+} on P_o (Fig. 9A) nor the effect of Zn^{2+} on τ (Fig. 9B) showed much voltage dependence. For these voltage studies, P_o was studied rather than f_o because the latter is confounded by reduced signal to noise resolution at depolarized membrane potentials.

Discussion

The channels studied here in outside-out membrane patches removed from murine neocortical neurons appear similar to those described in previous studies of NMDA receptor-activated channels on central neurons. Others have found NMDA channels to be selectively activated by low micromolar concentrations of glutamate (Nowak et al., 1984; Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987), to have a main conductance state of 50 pS, and to be blocked in a voltage-dependent fashion by Mg^{2+} (Nowak et al., 1984; Ascher and Nowak, 1988).

The central finding of the present study is that 1–100 μM Zn^{2+} could powerfully attenuate these NMDA channel currents, consistent with previous cell-level experiments showing that the same concentrations of Zn^{2+} could selectively block NMDA-induced neuroexcitation (Peters et al., 1987; Westbrook and Mayer, 1987) or neurotoxicity (Peters et al., 1987; Koh and Choi, 1988). Three main effects of Zn^{2+} were observed: (1) an apparent increase in patch seal resistance; (2) a reduction in channel open probability, for the most part due to reduction in channel opening frequency; and (3) a voltage-dependent reduction in apparent channel amplitude, most likely due to interruptions of channel current too rapid to be resolved by the recording techniques employed.

Zinc increases apparent patch resistance

Zn^{2+} consistently decreased baseline pipette current, evident as a steady upward shift in the patch current record. The effect was Zn^{2+} concentration dependent and increased with membrane hyperpolarization. It varied in magnitude from patch to patch, was not dependent on the presence of NMDA channel activity, and was not mimicked by equimolar Mg^{2+} . Its basis remains to be defined. One possible explanation is an interaction of Zn^{2+} with charged moieties in the membrane or the pipette glass surface, leading to a stabilizing action on the glass/membrane interface (see Corey and Stevens, 1983). Another possibility might be a reduction of streaming potential currents between the pipette and the membrane. Alternatively, it is possible that Zn^{2+} might produce a progressive blockade of unresolved small membrane channels, leading to an increase in average membrane resistance. In any case, the baseline shift induced by a given concentration of Zn^{2+} was relatively stable and did not interfere with the analysis of NMDA channels.

Zinc reduces NMDA channel open probability

Zn^{2+} reduced the probability of opening of NMDA channels, largely due to a reduction in the frequency of channel opening. At a membrane potential of -50 mV, 3 μM Zn^{2+} reduced both open probability and channel opening frequency by about 50%. This potent effect was probably not explained by reduction of free agonist concentration secondary to metal ion complex formation since the concentration of applied glutamate and glycine was increased to compensate for complexing according to published stability constants (Perrin, 1986). Such a correction assumes that Zn^{2+} -glutamate complexes are unavailable to activate the NMDA receptor.

If this assumption is incorrect, then the actual reduction of opening frequency by Zn^{2+} would be even higher.

Another possible explanation for the ability of Zn^{2+} to reduce channel-opening frequency would be competitive antagonism at the agonist-recognition site. However, 1 mM Zn^{2+} was observed to reduce 3H -glutamate binding to rat synaptic plasma membranes to only 76% of control (Monahan and Michel, 1987), and it is possible that some of this reduction might be explained by the formation of Zn -glutamate complexes. Also, to the extent that reduction of channel openings is a major component of Zn^{2+} antagonism of NMDA receptor-activated currents, simple competition with agonist-receptor binding would be inconsistent with the observations that Zn^{2+} block of neuroexcitation (Peters et al., 1987; Westbrook and Mayer, 1987) and neurotoxicity (Koh and Choi, 1988) persists in the face of high agonist concentrations.

More likely, Zn^{2+} interacts with the NMDA receptor-channel complex to reduce the coupling between agonist binding and channel gating or to enhance desensitization of the NMDA receptor (Mayer et al., 1989). The observation that the effect of Zn^{2+} on open probability was largely voltage-independent suggests that it may result from the binding of Zn^{2+} to a relatively superficial site on the NMDA receptor-channel complex. Reduction of opening frequency is not unique to Zn^{2+} ; Nowak et al. (1984) discovered that Mg^{2+} also reduced NMDA channel opening. However, we found Mg^{2+} was much weaker in this regard than Zn^{2+} . The ability of low micromolar concentrations of Zn^{2+} to reduce NMDA channel-opening frequency in a voltage-independent fashion probably accounts for the finding that 5 μM Zn^{2+} could reduce NMDA-induced whole-cell currents in hippocampal neurons even at positive membrane potentials (Westbrook and Mayer, 1987). On the other hand, prior observations suggesting some voltage dependence to Zn^{2+} block of NMDA-induced neuronal responses at negative membrane potentials (Peters et al., 1987, figure 3C; Westbrook and Mayer, 1987, figure 3A) probably reflect the superposition of a contribution from the voltage-dependent channel block discussed below.

In addition to reducing channel-opening frequency, higher concentrations of Zn^{2+} ($> 10 \mu M$) produced a reduction in burst duration, also with little sensitivity to membrane voltage (Fig. 9). The channel open time observed here in the absence of Zn^{2+} , 5.2 msec, is similar to the 5.8 msec observed by Ascher et al. (1988) on murine central neurons.

Zinc reduces single-channel amplitude

At negative membrane potentials, Zn^{2+} also reduced single-channel amplitude. This voltage-dependent reduction was Zn^{2+} concentration dependent between 1–100 μM . The smoothly progressive nature of the reduction with increasing Zn^{2+} concentration argues against the idea that Zn^{2+} potentiates a lower conductance state of the NMDA channel while simultaneously curtailing the activity of the full conductance state (Mayer et al., 1988). Another possible mechanism for the observed reduction in channel amplitude is that Zn^{2+} might screen a negative surface potential near the channel entrance, thereby reducing cation access to the channel. Indeed, Ascher and Nowak (1988) have postulated such a surface potential screening mechanism to account for the shift of the outward current-voltage relation produced by high concentrations of extracellular Ca^{2+} . However, while the progressive reduction of single-channel cur-

rent seen with Zn^{2+} is consistent with surface potential screening, Zn^{2+} did not produce the predicted shift in the outward current-voltage relation. Furthermore, the observed increase in single-channel noise, as well as the much higher potency of Zn^{2+} over Ca^{2+} (nearly 1000-fold at -50 mV) are difficult to reconcile with a simple surface-potential model. Instead, as discussed earlier, present observations better fit the hypothesis that Zn^{2+} causes very brief interruptions of current through the channel.

Supporting this flicker hypothesis was the finding that the effect of Zn^{2+} on channel-amplitude histograms could be closely approximated by theoretical β -distributions predicted by a simple model of open-channel block. In particular, the model's prediction that only the block on rate would vary with changes in Zn^{2+} concentration was consistent with experimental data. The additional finding that the voltage dependence of the on rate was sufficient to account entirely for the voltage dependence of the overall block was in line with rate constant data derived from dwell-time analysis of the Mg^{2+} block, which suggested greater voltage dependence of the on rate (e -fold/17 mV of hyperpolarization) compared with the off rate (e -fold/47 mV of depolarization) (Ascher and Nowak, 1988). Of note, the on and off rate constant values for Zn^{2+} block are more than an order of magnitude faster than the corresponding values for Mg^{2+} block, consistent with greater experimental difficulty in resolving full channel flicker with Zn^{2+} compared with Mg^{2+} . Furthermore, at depolarized potentials, Zn^{2+} may have considerably greater affinity for its channel binding site [calculated $K_d(0) = 0.9$ mM] than Mg^{2+} [$K_d(0)$ calculated by Ascher and Nowak (1988) to be 8.8 mM].

Two sites of action?

The data collected here are most consistent with the idea that there may be 2 major sites of action for Zn^{2+} on the NMDA receptor-channel complex, distinguishable by differences in effect on channel currents, required Zn^{2+} concentration, and voltage dependence (Fig. 10). We recognize that other models can be considered.

One major site of action may be a high-affinity, voltage-independent binding site near the external membrane surface, responsible for reducing channel-opening frequency. This site could modulate receptor-channel interaction or could gate the channel from a very superficial channel site. Certain other divalent cations also may be able to interact to varying degrees with this voltage-independent site. For example, Cd^{2+} reduces whole-cell currents in a voltage-insensitive manner (Mayer and Westbrook, 1987), and binding studies have shown that both Cd^{2+} and Zn^{2+} decrease the association and dissociation rates of ^3H -MK-801 (Reynolds and Miller, 1988). The effect of Zn^{2+} to reduce channel burst duration could be mediated by the same binding site as reduction in opening frequency or by yet another binding site.

The second major site may be a lower-affinity, voltage-dependent site within the NMDA channel itself, responsible for a rapid flicker block of the open channel. The voltage dependence of this site is characterized by a δ of 0.51, which suggests that it senses approximately half the transmembrane potential field, and thus may be distinct from the voltage-dependent Mg^{2+} site, which senses most of the transmembrane field ($\delta \approx 1$) (Ascher and Nowak, 1988). It is reasonable to postulate that the channel Zn^{2+} site is superficial to the channel Mg^{2+} site, although unknown factors such as interactions with other ions within the channel pore preclude simple translation of apparent δ into a

Figure 10. Model for Zn^{2+} action on the NMDA receptor-channel complex. Speculative model shows 2 sites of action, one influencing the coupling of the NMDA receptor to its channel and the other within the channel itself, perhaps shallow to the Mg^{2+} site. Some of the Zn^{2+} released by presynaptic terminals might permeate the open NMDA channel, perhaps triggering changes in the postsynaptic cell. The PCP site is illustrated as a separate site for convenience; it could be the same as the Zn^{2+} or the Mg^{2+} site. Modulatory sites for glycine (Johnson and Ascher, 1987) and for polyamines (Ransom and Stec, 1988) are also included.

physical location within the channel (Ascher and Nowak, 1988). The exact relationships between the Zn^{2+} site, the Mg^{2+} site, and the phencyclidine (PCP) site within the NMDA channel will require further studies for clarification. MacDonald et al. (1987) found that ketamine had an apparent δ of 0.53, while our calculations from the data published by Huettner and Bean (1988) suggest that the PCP ligand MK-801 could have a steeper voltage dependence, with δ near 1.

Beyond its intrinsic significance as an antagonist of NMDA receptor-activated currents, we speculate that the interaction of Zn^{2+} with a site in the NMDA channel might herald a subsequent event of potential physiological importance—the permeation of Zn^{2+} through the channel. Ascher and Nowak (1988) as well as Mayer and Westbrook (1987) have discussed the correlation between a divalent cation's rate constant for substitution of the inner sphere of H_2O (Diebler et al., 1969) and its permeability through the NMDA channel. Fast permeators of the NMDA channel, such as Ca^{2+} , Sr^{2+} , and Ba^{2+} exchange their water shells nearly 1000 times faster than voltage-dependent blockers, Co^{2+} and Mg^{2+} . Interestingly, rates for both Zn^{2+} and Mn^{2+} fall between those of fast permeators and the blockers. Zn^{2+} exchanges its inner hydration shell more than 100 times faster than Mg^{2+} and about 10 times faster than Mn^{2+} . Since 20 mM Mn^{2+} may carry an inward current (Mayer and Westbrook, 1987), it does not seem unreasonable to suppose that Zn^{2+} might permeate the channel. Indeed, permeation of a Zn^{2+} ion through the channel, followed by replacement of the channel block by another Zn^{2+} ion, could be the molecular substrate for rapid flicker block.

Zn^{2+} is capable of interacting with many biological molecules; it is intriguing to imagine that the Zn^{2+} released from neuronal presynaptic terminals might be a "transsynaptic messenger," entering postsynaptic neurons through NMDA channels and triggering metabolic alterations (Weiss et al., 1989). The neu-

rotoxicity produced by intense Zn^{2+} exposure (Choi et al., 1988) could be in part due to such Zn^{2+} entry, as it is competitively attenuated by MK-801 (Choi and Koh, 1988).

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