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# THE INHIBITION OF SINGLE *N*-METHYL-D-ASPARTATE-ACTIVATED CHANNELS BY ZINC IONS ON CULTURED RAT NEURONES

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SUMMARY

1. Single channels activated by *N*-methyl-D-aspartate (NMDA) were studied in outside-out patches of cultured hippocampal neurones in the presence of glycine and absence of magnesium. The effects of the transition metal ions zinc and cadmium on NMDA channels were tested by placing the membrane patch at the mouth of one of an array of large barrelled flow pipes.

2. Amplitude histograms revealed several conductance levels between 5 and 45 pS with the majority of NMDA-activated openings > 25 pS. Zinc (5–100 $\mu$M) and cadmium (30–100 $\mu$M) reduced the number of large conductance events in a voltage-independent manner. Zinc (30 $\mu$M) reduced the large conductance openings by approximately 70–80%. The small number of events under 20 pS precluded quantitative assessment of the effects of zinc and cadmium on these conductance levels. Zinc inhibition of the calculated macroscopic current due to NMDA-activated channels could be fitted with a single binding site isotherm with an $IC_{50}$ of 12 $\mu$M.

3. Zinc and cadmium also reduced the mean open time of the two largest conductance events of 38 and 43 pS; this reduction was voltage independent. Open-time histograms were fitted with the sum of two exponentials. In the presence of 5 $\mu$M-NMDA at −60 mV, $\tau_{o2} = 10{\cdot}49$ ms and $\tau_{o1} = 1{\cdot}47$ ms; in 30 $\mu$M-zinc, $\tau_{o2} = 3{\cdot}49$ and $\tau_{o1} = 0{\cdot}8$ ms. The 'blocking' rate constant calculated at a membrane potential of +40 mV from the slope of $1/\tau_{o2}$ *vs.* $[zinc]_o$ was $4 \times 10^6$ M$^{-1}$ s$^{-1}$.

4. Closed-time analysis revealed brief ($\tau_c = 0{\cdot}4$–$1{\cdot}0$ ms) zinc-insensitive gaps; longer closed-time intervals were not analysed since all patches contained more than one channel. Both burst duration and the number of bursts were reduced in the presence of zinc.

5. At holding potentials negative to −40 mV in magnesium-free solutions, zinc also induced high-frequency flickering of the open channel which included complete channel closures at 4 kHz filtering. No zinc-induced flickering was seen at positive membrane potentials. The flickering was dose dependent, becoming prominent at zinc concentrations above 30 $\mu$M. Cadmium did not induce flickering at concentrations up to 100 $\mu$M.

6. Increasing the extracellular concentration of either sodium (from 160 to

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500 mM) or calcium (from 2 to 30 mM) had no effect on the mean lifetime of the 43 and 38 pS events. In 30 mM-external calcium or 500 mM-external sodium, zinc (30 $\mu$M) still reduced the number of openings and the mean open time; however, the zinc-induced flickering was much less apparent, suggesting that the flickering was due to zinc binding within the open channel. The difference between zinc and cadmium in evoking high-frequency flicker further supports the correlation between open-channel block of NMDA channels by divalent cations and their water substitution rates.

7. These results demonstrate that the major action of zinc on single NMDA channels is to reduce the mean open time and the frequency of large conductance openings in a voltage-independent manner. This accounts for the inhibition of the macroscopic current and is likely to result from a binding site on the extracellular domain of the receptor–channel complex. We suggest that zinc acts as an allosteric inhibitor at a co-ordination site containing both cysteine and histidine residues, perhaps analogous to the binding site of zinc-dependent DNA-binding proteins and enzymes.

## INTRODUCTION

The behaviour of *N*-methyl-D-aspartate (NMDA) channels is highly dependent on interactions with extracellular divalent cations. These actions can be largely accounted for by divalent cations binding to a site within the pore of the channel, based on the voltage dependence of block by magnesium (Mayer, Westbrook & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984) and the brief closures (flickers) of the channel in the presence of magnesium (Nowak *et al.* 1984). Zinc is also a potent inhibitor of NMDA currents and NMDA receptor-mediated EPSPs (Peters, Koh & Choi, 1987; Westbrook & Mayer, 1987; Forsythe, Westbrook & Mayer, 1988). The inhibition is non-competitive with an $IC_{50}$ of 13 $\mu$M (Mayer, Vyklický & Westbrook, 1989). This is of physiological interest since zinc is contained in synaptic vesicles at nerve terminals using excitatory amino acids as transmitters (Perez-Clausell & Danscher, 1985). With synaptic activity $Zn^{2+}$ is released into the synaptic regions at a concentration thought to be well above the $IC_{50}$ for inhibition of NMDA-activated responses (Assaf & Chung, 1984; Howell, Welch & Frederickson, 1984).

The inhibition of macroscopic NMDA-evoked currents by $Zn^{2+}$ differs markedly from $Mg^{2+}$ since $Zn^{2+}$ blocks outward and inward currents equally well. This lack of voltage dependence suggests that the $Zn^{2+}$ binding site is not within the channel, but on the extracellular surface or possibly within the vestibule (Mayer *et al.* 1989). Furthermore power spectra of current noise evoked by NMDA in the presence of $Zn^{2+}$ or $Cd^{2+}$ can be fitted with a single Lorentzian function, inconsistent with a fast open-channel block mechanism (Mayer, Westbrook & Vyklický, 1988). The zinc binding site appears to be distinct from the glycine modulatory site (Westbrook & Mayer, 1987). It has also been suggested that tricyclic antidepressants act at the zinc site on the NMDA receptor (Reynolds & Miller, 1988; but see Sernagor, Kuhn, Vyklický & Mayer, 1989).

Several mechanisms of action for $Zn^{2+}$ are possible. Zinc could block the open channel or allosterically alter channel gating (e.g. enhanced desensitization, Clapham

& Neher, 1984). In addition, NMDA-activated currents show multiple conductance levels (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987) which may have different mean open times (Cull-Candy & Usowicz, 1987; Ascher, Bregestovski & Nowak, 1988). Thus it is also possible that $Zn^{2+}$ allosterically shifts channel gating to a different open state or conductance level (e.g. see Prod'hom, Pietrobon & Hess, 1989).

To test these possibilities directly, the action of $Zn^{2+}$ was examined on single channels activated by NMDA in outside-out patches of cultured hippocampal neurones. $Zn^{2+}$ reduced both the frequency of channel opening and the mean lifetime of the large conductance states. Our results suggest that $Zn^{2+}$ or $Cd^{2+}$ act as allosteric inhibitors at a site outside the membrane electric field. At $Zn^{2+} > 10$ $\mu$M, voltage-dependent flickering of the channel was also apparent in $Mg^{2+}$-free solutions, but this does not contribute significantly to $Zn^{2+}$ inhibition of the whole-cell current. A preliminary report of these experiments has been presented in abstract (Legendre & Westbrook, 1989).

## METHODS

### *Cell culture*

Primary dissociated hippocampal cultures were prepared from neonatal rats (Sprague–Dawley), and plated at 10000 cells $cm^{-2}$ onto confluent layers of astrocytes. Neonatal rats were anaesthetized with halothane and killed by decapitation. The astrocyte feeder layers were prepared from the hippocampi of newborn rats. Following treatment with 20 U $ml^{-1}$ activated papain (Worthington) for 60 min, the tissue was triturated and plated at 25000 cells $cm^{-2}$ into 35 mm tissue culture dishes or glass cover-slips coated with Vitrogen (Collagen Corp., Palo Alto, USA) and poly-L-lysine (mol. wt 30–70 kDa, $10^{-5}$ M in 0·15 M-borate, pH 8·4, Sigma). The resulting feeder layers were grown in minimal essential medium (MEM) with 10% fetal bovine serum until confluent (12–14 days). When hippocampal neurones were added, the medium was switched to the following composition: 95% MEM, 5% horse serum (Hyclone) and an added nutrient supplement containing insulin, transferrin, putrescine, selenium, corticosterone, progesterone and triiodothyronine (Guthrie, Brenneman & Neale, 1987). Hippocampal cultures were treated 1 day after plating of neurones with a mixture of 5'-fluoro-2-deoxyuridine and uridine (15 and 35 $\mu$g/$ml^{-1}$, respectively) to suppress overgrowth of background cells; half-changes of medium were done weekly thereafter. Recordings were made after 7–14 days in culture.

### *Recording conditions*

Patch-clamp recording of single channels in the outside-out patch configuration was used as described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). Pipettes (5–10 MΩ when filled) were pulled from borosilicate capillary glass (WPI) in two stages on a vertical puller (Sutter Instruments). The pipettes were coated with Sylgard and fire-polished. Pipette solutions contained (in mM): CsCl, 126; CsF, 14; $CaCl_2$, 0·5; $MgCl_2$, 1; EGTA, 5; HEPES, 10; pH was adjusted to 7·2 with CsOH; osmolarity was adjusted to 295 mosM with sucrose.

Experiments were done at room temperature (20–22 °C). The chamber was perfused at 1–2 ml $min^{-1}$ with a solution containing (in mM): NaCl, 162; KCl, 2·4; $CaCl_2$, 2; HEPES, 10; glucose, 10; pH adjusted to 7·3; osmolarity adjusted to 325 mosM with sucrose. Tetrodotoxin (500 nM) was added to block spontaneous electrical activity and picrotoxin (5 $\mu$M) to block GABA channels. Strychnine (2 $\mu$M) and glycine (5 $\mu$M) were added to all NMDA-containing solutions to potentiate NMDA receptor activity and to avoid wash-out effects of endogenous modulators (Johnson & Ascher, 1987). The solutions for drug applications were identical to the bath solution but were prepared with ultrapure cation salts (Aldrich 'gold label') to reduce the residual $Mg^{2+}$ concentration (Mayer *et al.* 1989). Drug solutions were applied by an array of six flow pipes of 300 $\mu$m diameter controlled by solenoid valves (see Mayer *et al.* 1988). To examine NMDA channel activity, the patch was first perfused via a flow pipe containing the extracellular solution and then

moved to an adjacent flow pipe containing NMDA (5 $\mu$M) or NMDA plus either $Zn^{2+}$ (1–100 $\mu$M) or $Cd^{2+}$ (100 $\mu$M). The effect of $Ca^{2+}$ and $Na^{+}$ on zinc inhibition of NMDA-activated channels was tested in solutions containing either 30 mM-$Ca^{2+}$ or 500 mM-$Na^{+}$. The concentrations of divalent cations were not corrected for complex formation with NMDA. All salts and drugs were obtained from Sigma unless otherwise indicated, except for NMDA which was obtained from Cambridge Research Biochemicals.

*Data analysis*

Single-channel currents in outside-out patches were recorded using an Axopatch-1B (Axon Instruments) with the current filtered at 5 kHz. Current records were recorded on a videocassette recorder using an analog–digital converter (VR10, Instrutech Corp.). Single-channel currents were replayed as analog signals, filtered at 2–4 kHz using an 8-pole Bessel filter (Frequency Devices), sampled at a rate five times the cut-off frequency of the filter and analysed using an IBM-AT clone with modified pClamp v.5·5 software (Axon Instruments). Openings and closing of the channels were detected using a 50% threshold criterion (Colquhoun & Sigworth, 1983). Events (openings or closures) briefer than a set duration (usually 200 $\mu$s) were automatically ignored in compiling events lists for amplitude and open-time histograms, depending on the cut-off frequency of the filter. Thus possible transitions to subconductance levels for $< 200$ $\mu$s were ignored. Although transitions between subconductance levels lasting $> 200$ $\mu$s occurred, these were rare and were not systematically excluded from the analysis. For each patch, the threshold was first set at a low level to include all conductance levels > approx. 10 pS and an amplitude histogram was constructed under manual control. Thereafter the threshold was set to a level to include only the large conductance states as verified by a new amplitude histogram.

Due to the occurrence of multiple channels in all patches, closed-time analysis was limited to brief gaps which averaged $\leqslant 1$ ms (see e.g. Ascher *et al.* 1988; Cull-Candy & Usowicz, 1989; Jahr & Stevens, 1990). Overlap of events was avoided by selecting patches with a low density of channels and by removing events containing multiple levels. Open times were defined as events without closures longer than 200 $\mu$s (i.e. the resolution set by the filter frequency). Mean open-time histograms were fitted with the sum of two exponentials; in some cases a third exponential with a time constant of $< 300$ $\mu$s, presumably reflecting brief unresolved openings, was necessary to obtain best fits. The presence of this third component did not alter estimates of $\tau_{o1}$ and $\tau_{o2}$ compared to other patches fitted with two exponentials. Amplitude histograms were fitted with the sum of one or more Gaussian functions, using least-square methods. The single-channel conductance for each level was calculated from the observed reversal potential for each patch.

Results are presented as mean values $\pm$ standard deviation (S.D.).

## RESULTS

Outside-out patches were obtained from hippocampal neurones after 7–15 days in cell culture. Continuous application of NMDA (5 $\mu$M) from a flow pipe evoked channel activity which in all patches contained some overlapping events, suggesting a minimum of two to three channels in each patch (see Fig. 1). The frequency of channel opening remained relatively stable for the 10–30 min recording period used in these experiments.

*Zinc reduces NMDA-induced channel activity*

As shown in Fig. 1, the number of channel openings was markedly decreased in the presence of 30 $\mu$M-zinc. The inhibition by zinc was rapid in onset and was immediately reversed on removal of zinc as was observed in whole-cell recording (Westbrook & Mayer, 1987). The effects of zinc did not appear to be use dependent since prolonged applications did not result in greater inhibition. The reduction in the number of events by $Zn^{2+}$ was dose dependent; 30 $\mu$M reduced the channel activity

to 25–30% of control. In contrast to the effects of other divalent cations such as $Mg^{2+}$, $Zn^{2+}$ reduced single-channel activity over a wide range of membrane potentials from −70 to +50 mV (Fig. 1).

*Multiple conductance levels*

NMDA-activated channels showed multiple conductance levels (Fig. 2*A*), as previously described (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987). Although

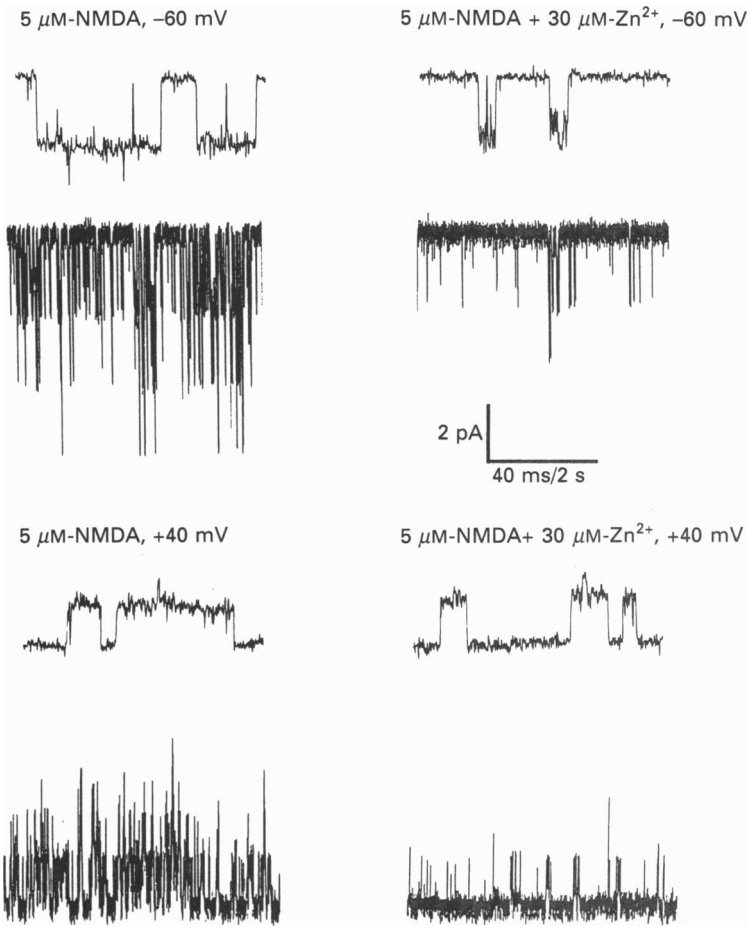


Fig. 1. Zinc reduces NMDA-induced channel activity in a voltage-independent manner. Left, control recording at −60 and +40 mV showing channels activated by 5 μM-NMDA in an outside-out patch at slow and fast time base. Right, in the presence of NMDA plus 30 μM-$Zn^{2+}$, there was a voltage-independent reduction in opening frequency at both holding potentials. The patch contained at least three channels. Filter 2 kHz.

the amplitude of these levels varied slightly between patches, five could usually be defined with amplitude histograms (Fig. 2*B*). For fourteen patches, the large conductance levels of 43 pS (43·6 ± 1·7 pS), 36 pS (36·4 ± 2·2 pS) and 28 pS (27·8 ± 2·4 pS) were much more frequent than small conductance levels of 16 pS

(16·4 ± 2·63 pS) and 10 pS (10·0 ± 2·4 pS). The largest conductance level of 43 pS and the relative frequency of conductance levels is similar to that seen by other investigators (e.g. Jahr & Stevens, 1987; Ascher *et al.* 1988; Cull-Candy & Usowicz, 1989).

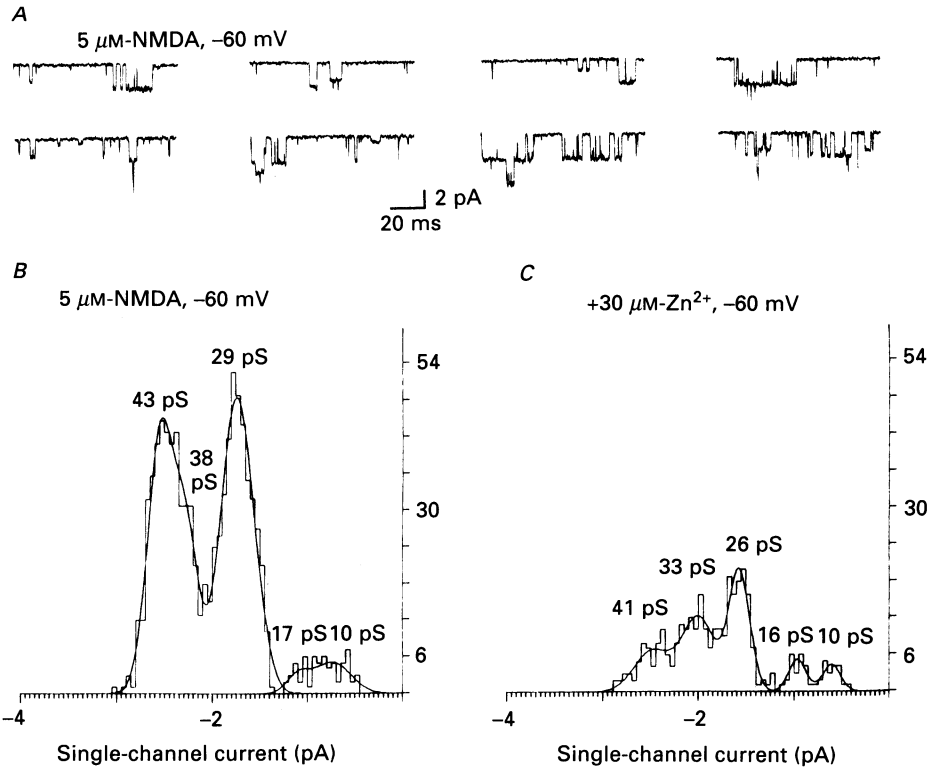


Fig. 2. Zinc reduced the number of large conductance events. *A*, flow pipe applications of 5 μM-NMDA to an outside-out patch evoked openings of several distinct amplitudes. *B*, amplitude histograms for this patch showed three to five conductance levels. *C* (in the same patch), 30 μM-$Zn^{2+}$ reduced only the 43, 38 and 29 pS levels. The conductance of these large openings was slightly reduced. Histograms were compiled from 30 s epochs which had one superposition. The bin width of the two histograms was 0·05 pA. Each histogram was fitted with the sum of three Gaussians for the largest events and the sum of two Gaussians for the smallest events. Single-channel peaks from the Gaussian fits were (in pA, mean ± S.D.): 2·6 ± 0·1, 2·3 ± 0·12, 1·7 ± 0·2, 1·0 ± 0·1 and 0·6 ± 0·2 in NMDA; and 2·5 ± 0·2, 2·2 ± 0·2, 1·6 ± 0·1, 1·0 ± 0·1 and 0·6 ± 0·1 in NMDA + $Zn^{2+}$. Conductance for each level was calculated from the observed reversal potential of 0 mV. Filter 2 kHz, holding potential −60 mV.

Not all conductance levels were equally affected by $Zn^{2+}$. Amplitude histograms compiled from 30 s data epochs in the presence of NMDA or NMDA + $Zn^{2+}$ demonstrated that $Zn^{2+}$ preferentially reduced the number of large conductance levels, i.e. conductance levels > 25 pS. The reduction in the number of 43 and 36 pS events appeared to be greater than for the 28 pS conductance substates (Fig. 2*C*). However, the overlap of the Gaussians prevented clear separation of the current

carried by these closely associated levels. The single-channel conductance of the large openings was slightly reduced (5–15%) in the presence of $Zn^{2+}$ ($\geqslant 30\ \mu M$) at hyperpolarized membrane voltages; this was apparent as open-channel noise during $Zn^{2+}$ application (see below). The number of small conductance levels appeared to be unaffected by $Zn^{2+}$. However, the number of these small openings was often less than seen in the patch of Fig. 2; thus a slight effect of $Zn^{2+}$ on these openings cannot be excluded. It is interesting that $Mg^{2+}$ also preferentially affects the large conductance events (Jahr & Stevens, 1987).

The reduction in NMDA channel activity was first apparent with $Zn^{2+}$ concentrations of 3 $\mu M$ and increased such that there was very little channel activity in the presence of 100 $\mu M$-$Zn^{2+}$. To estimate the $IC_{50}$ for $Zn^{2+}$ action, the amplitude and duration of individual events were obtained from the events list and used to calculate the total patch current as follows:

$$I_{patch} = \Sigma i_{NMDA}\,(t/T),$$

where $I_{patch}$ is the calculated macroscopic current, $i_{NMDA}$ the amplitude of each opening, $t$ its duration and $T$ the total recording duration during which the opening were observed. $I_{patch}$ in the presence and absence of $Zn^{2+}$ was used to compare the $Zn^{2+}$ inhibition on the macroscopic NMDA evoked current.

The concentration–response curve for $Zn^{2+}$ inhibition at $-60$ mV ($n = 5$), normalized to $I_{patch}$ during the control period, is shown in Fig. 3. The data were well fitted with a single binding site isotherm of the form:

$$I_{patch\,(Zn)} = I_{patch}\{1-([Zn^{2+}]/([Zn^{2+}]+IC_{50}))\},$$

where $I_{patch}$ is the control current, $I_{patch\,(Zn)}$ is the total channel current in the presence of NMDA plus zinc and $IC_{50}$ is the concentration of $Zn^{2+}$ that inhibited 50% of the NMDA current. The $IC_{50}$ was 12 $\mu M$ (Fig. 3). Similar results were obtained at a membrane potential of +40 mV (not shown). This closely matches the $IC_{50}$ of 13 $\mu M$ for $Zn^{2+}$ antagonism seen in whole-cell experiments (Mayer *et al.* 1989).

*Zinc reduces the mean open time of the NMDA channel*

The reduction in channel activity described by Fig. 3 results in part from a reduction in the frequency of channel opening, but noise analysis had suggested that $Zn^{2+}$ also affected the mean channel lifetime (Mayer *et al.* 1988). Open times of the large conductance events (> 25 pS) were analysed at positive membrane potentials to minimize the effect of any residual voltage-dependent block by divalent cations including $Mg^{2+}$ (or $Zn^{2+}$). Figure 4*A* illustrates single-channel openings in 5 $\mu M$-NMDA at a holding potential +50 mV. Open-time histograms in the presence of NMDA could be fitted with two exponentials (Fig. 4*C*) with two time constants: $\tau_{o1} = 1{\cdot}5 \pm 0{\cdot}6$ ms and $\tau_{o2} = 10{\cdot}4 \pm 0{\cdot}5$ ms ($n = 13$). Similar values for $\tau_{o1}$ and $\tau_{o2}$ were found over a wide range of membrane potentials from $-70$ to +50 mV in $Mg^{2+}$-free solutions. These values are similar to those previously described for the large conductance levels in cultured neurones (Jahr & Stevens, 1987; Ascher *et al.* 1988). The largest conductance levels of 43 pS and the 36 pS in our experiments were too similar to allow clear separation of these levels for the open-time histograms. Thus $\tau_{o1}$ and $\tau_{o2}$ reflect the kinetic properties of both the 43 and 36 pS levels. However, it

is possible that $\tau_{o2}$ is due to 43 pS openings since both Cull-Candy & Usowicz (1987) and Ascher *et al.* (1988) reported longer mean lifetimes for 45–50 pS events than for 35–40 pS events.

In the presence of 30 $\mu$M-$Zn^{2+}$ the single-channel current remained unchanged at a holding potential of +50 mV, but the open time was markedly shortened (Fig. 4*B*).

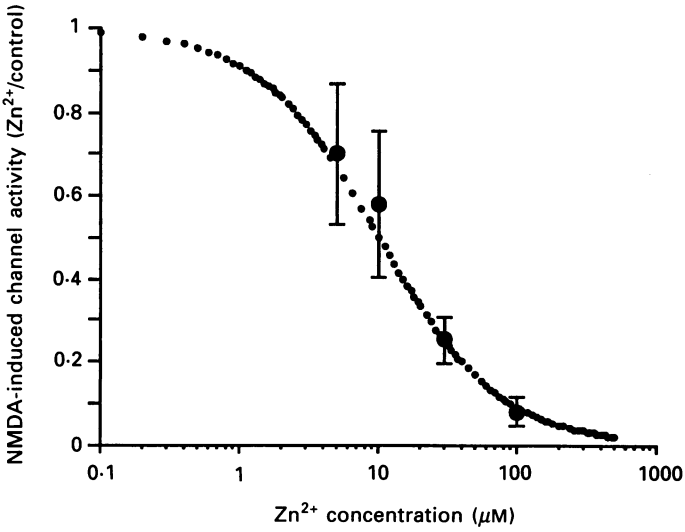


Fig. 3. Zinc reduces NMDA-induced channel activity in a concentration-dependent manner. The $Zn^{2+}$ inhibition curve (dotted line) was fitted with a single binding site adsorption curve with an $IC_{50}$ of 12 $\mu$M. The open time and amplitude of each opening in the events list were multiplied, summed, then divided by the duration of the recording epoch to obtain the channel activity in the presence and absence of $Zn^{2+}$ (see text). Normalized data ($Zn^{2+}$/control) are shown from five different patches recorded at −60 mV.

In the histogram shown in Fig. 4*D*, $\tau_{o1}$ decreased from 1·8 to 0·8 ms and $\tau_{o2}$ from 9·7 to 4·9 ms. The reduction in both open times was dependent on the concentration of $Zn^{2+}$. In 100 $\mu$M-$Zn^{2+}$, the longer open time $\tau_{o2}$ decreased from 10·5±1·3 ms ($V_h$ = −60 mV, $n$ = 9) to 2·1±0·5 ms ($V_h$ = +40 mV, $n$ = 3). At high concentrations of zinc (e.g. 30 $\mu$M), $\tau_{o2}$ was also reduced at a holding potential of −60 mV (3·5±0·5 ms, $n$ = 9) compared to +40 mV (4·7±0·4 ms, $n$ = 3). Since high-frequency flickering occurred at −60 mV in the presence of high concentrations of $Zn^{2+}$, the data points at +40 mV were used for the curve fit shown in Fig. 5. The $IC_{50}$ for $Zn^{2+}$ reduction of $\tau_{o2}$ was 22 $\mu$M which was only slightly different from the value observed (12 $\mu$M) for $Zn^{2+}$ inhibition of the NMDA-induced total patch current activity (see Fig. 3). Zinc caused similar decreases in $\tau_{o1}$; in 30 $\mu$M-$Zn^{2+}$, $\tau_{o1}$ decreased from 1·5±0·6 ms ($n$ = 9) to 0·8±0·3 ms ($n$ = 9) at −60 mV and to 1·1±0·3 ms ($n$ = 3) at +40 mV. No further analysis of $\tau_{o1}$ was performed.

The reduction in the mean open time could suggest a kinetic scheme in which $Zn^{2+}$ induces an additional pathway out of the open state. To obtain an estimate of this rate constant, the relationship between the inverse of the long mean open time ($1/\tau_{o2}$) and $Zn^{2+}$ concentration was plotted as shown in Fig. 6. For a holding potential of

+40 mV, the data points could be fitted with a regression line with a slope of $4{\cdot}1 \times 10^6$ M$^{-1}$ s$^{-1}$. However, this relationship became non-linear for values of $1/\tau_{o2}$ obtained at −60 mV for $Zn^{2+}$ > 30 μM. This suggests that at hyperpolarized membrane potentials zinc has more than one effect on NMDA channel kinetics (see below).

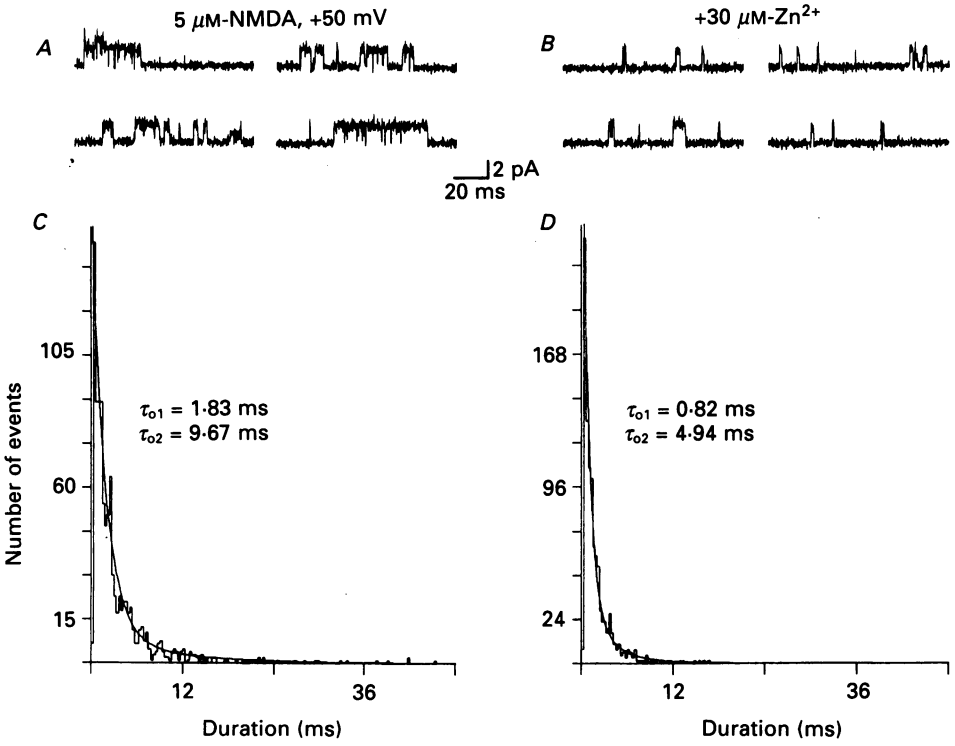


Fig. 4. Zinc decreased the mean open times of the large openings. Large openings are shown from an outside-out patch at +50 mV in the presence of 5 μM-NMDA (*A*) and in the presence of 5 μM-NMDA +30 μM-$Zn^{2+}$ (*B*). Open-time histograms (*C* and *D*) were constructed from the large conductance events using a 50 % threshold criterion to include events > 30–35 pS. Histograms were fitted with two exponentials. Zinc decreased both time constants. A longer recording epoch was used in the presence of $Zn^{2+}$ to construct the open-time histogram. Bin width was 0·3 ms. Filter 4 kHz.

*Closed-time and burst analysis*

Histograms of closed times were fitted by at least three exponentials; however, only the short closed interval of 0·4–1·0 ms was constant between patches (Ascher *et al.* 1988; Cull-Candy & Usowicz, 1989; Jahr & Stevens, 1990). In the presence of NMDA, $\tau_{c1}$ was 0·8±0·1 ms ($n = 4$); in NMDA +30 μM-$Zn^{2+}$, $\tau_{c1}$ was 0·7+0·1 ms ($n = 3$). These short closed intervals have also been reported to be unaffected by $Mg^{2+}$ (Ascher *et al.* 1988; Jahr & Stevens, 1990). The longer closed intervals were not further analysed since all patches contained more than one channel. In order to determine if the burst duration and number of bursts were affected by $Zn^{2+}$, records were analysed by ignoring closed intervals shorter than three times the $\tau_{c1}$ value of

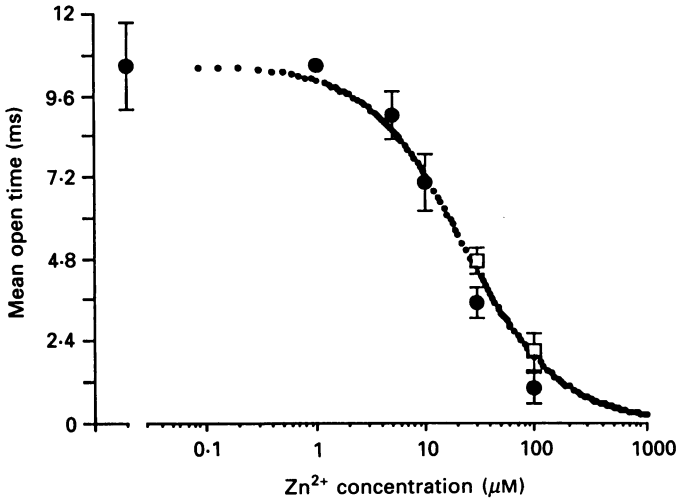


Fig. 5. Zinc reduced $\tau_{o2}$ in a concentration-dependent manner. NMDA concentration was 5 $\mu$M. Concentration–response curve (dotted line) was fitted with a single binding site adsorption curve with $IC_{50} = 22$ $\mu$M. Filled circles are the average ($\pm$S.D.) from nine patches at a holding potential of $-60$ mV. Data point for 1 $\mu$M-$Zn^{2+}$ is the average from two patches. Open squares with error bars are from three patches at a holding potential of $+40$ mV. The curve was fitted using the data obtained at $+40$ mV for $Zn^{2+}$ concentrations of 30 and 100 $\mu$M.

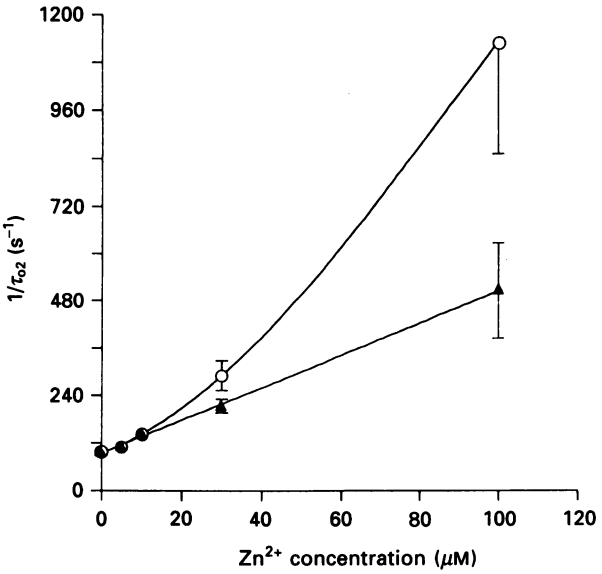


Fig. 6. Relationship between the inverse of the mean open time ($1/\tau_{o2}$) and the $Zn^{2+}$ concentration at membrane potentials of $-60$ mV (○) and $+40$ mV (▲). $1/\tau_{o2}$ was obtained from data shown in Fig. 5. A linear relationship between $1/\tau_{o2}$ and the $[Zn^{2+}]_o$ was seen only at positive membrane potentials with $Zn^{2+}$ concentrations $> 30$ $\mu$M. The slope of the relation between $1/\tau_{o2}$ and $[Zn^{2+}]_o$ was $k_+ = 4{\cdot}1 \times 10^6$ s$^{-1}$ M$^{-1}$. The value of $1/\tau_{o2}$ for $[Zn^{2+}]_o = 0$ was $96{\cdot}5 \pm 10{\cdot}9$ s$^{-1}$ ($n = 9$), corresponding to a mean open time of 10·4 ms.

0·8 ms (e.g. Neher, 1983). The burst duration histograms were fitted with the sum of three exponentials with time constants of 0·6, 3·9 and 27·5 ms ($n = 2$). The longest burst duration was somewhat greater than that reported by Cull-Candy & Usowicz (1989) using a similar critical gap duration. Both the number of bursts and the

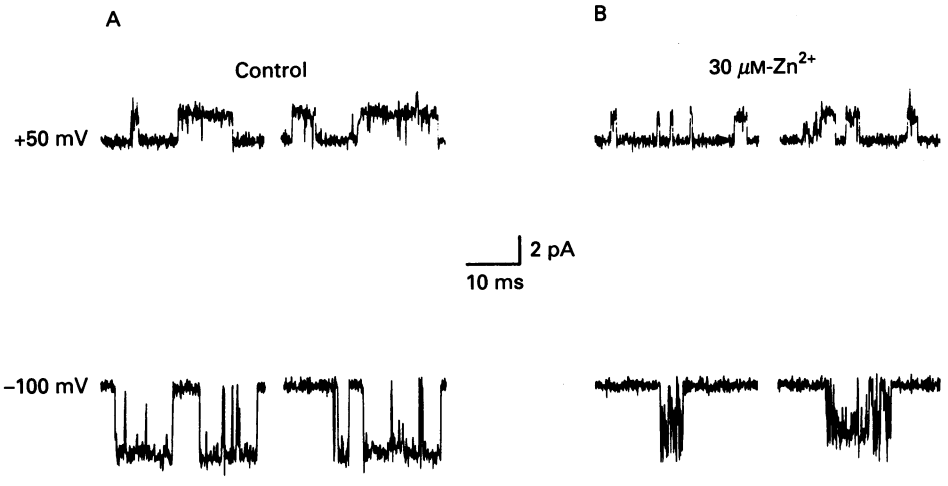


Fig. 7. Zinc-induced flickering of open channels was voltage dependent. In $Mg^{2+}$-free solutions, only a few brief interruptions of the open state were observed at holding potentials of +50 and −100 mV (*A*). In the presence of 30 μM-$Zn^{2+}$ (*B*), single-channel currents induced by NMDA showed flickering at a holding potential of −100 mV. Examples selected to show openings of long duration. At +50 mV no flickering was detected in the presence of $Zn^{2+}$. 5 μM-NMDA. Filter 4 kHz.

duration of the bursts was reduced by zinc ($n = 2$). In the presence of $Zn^{2+}$, the longest time constant was reduced to 17·3 ms (10 μM-$Zn^{2+}$) and 8·5 ms (30 μM-$Zn^{2+}$).

*Zinc evokes flickering in a voltage-dependent manner*

In addition to the voltage-independent reduction in the frequency of channel opening and in mean open times, $Zn^{2+}$ caused an increase in open-channel noise at hyperpolarized holding potentials (see e.g. Fig. 1). At a filter cut-off frequency of 4 kHz, the flickering was prominent and included some complete closures to the baseline (Fig. 7). Except for the occasional brief closures seen even in the absence of $Zn^{2+}$, no flickering was observed at positive holding potentials. The voltage dependence of the flickering suggests that $Zn^{2+}$ can enter the membrane electric field and act as an open-channel blocker similar to other divalent cations (Nowak *et al.* 1984; Mayer & Westbrook, 1987). Higher concentrations of $Zn^{2+}$ were required to cause flickering compared to the voltage-independent effects of $Zn^{2+}$ (Fig. 8). At 100 μM-$Zn^{2+}$, this resulted in a decrease in the apparent single-channel current (Fig. 8). Interruptions of the open channel by $Zn^{2+}$ were less well resolved than with $Mg^{2+}$, but similar to the high-frequency channel noise seen with $Mn^{2+}$ (Ascher & Nowak, 1988). This is consistent with a more rapid open-channel blocking rate for $Zn^{2+}$ than $Mg^{2+}$. Voltage-dependent flickering underlies the small decrease in single-channel current in the presence of $Zn^{2+}$ and the partial voltage dependence of the effect of zinc

on mean open time. Conductance levels < 20 pS appeared not to show zinc-induced flickering (e.g. Fig. 8, 100 $\mu$M-$Zn^{2+}$, middle panel) although the low-conductance events were too infrequent for quantitative analysis.

Since zinc-induced flickering included complete closures, it is unlikely that the open-channel noise reflects switching between different conductance levels, but

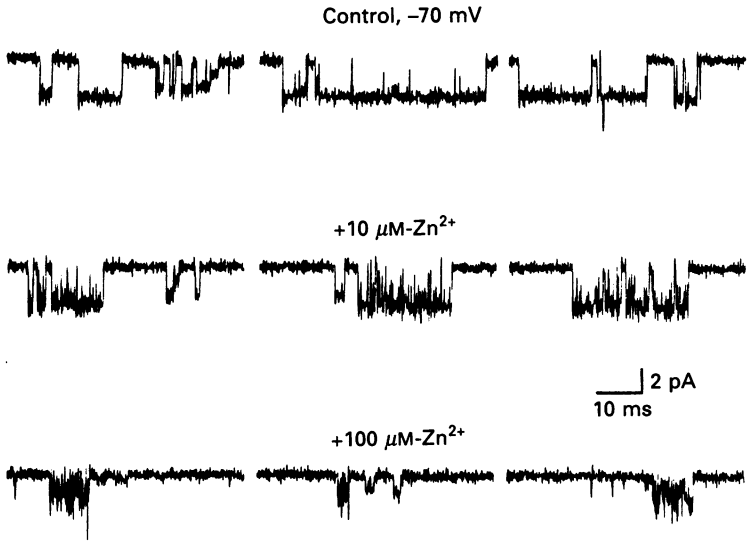


Fig. 8. Zinc-induced flickering of the large conductance NMDA channel was concentration dependent. Single-channel recordings obtained from a single patch in the absence (upper trace) and presence of 10 and 100 $\mu$M-$Zn^{2+}$ (lower traces) are shown. The holding potential was −70 mV. At high concentrations of $Zn^{2+}$ the flicker appeared as unresolved closures with an apparent decrease in the single-channel amplitude. 5 $\mu$M-NMDA. Filter 4 kHz.

rather reflects unresolved closures of the channel. Christine & Choi (1990) have reached similar conclusions using amplitude histogram analysis.

In prior experiments, $Zn^{2+}$ was less effective in reducing whole-cell currents evoked by NMDA at membrane potentials more negative than −60 mV (Westbrook & Mayer, 1987). One explanation for this could be relief of channel block due to $Zn^{2+}$ permeation through the channel (e.g. Mayer & Westbrook, 1987); however, $Zn^{2+}$ was equally effective in causing flickering of the open channel at −100 mV, suggesting that complete relief of $Zn^{2+}$ block does not occur at physiological membrane potentials (Fig. 7).

*Action of cadmium on NMDA-induced channels*

Cadmium and zinc had similar effects on excitatory amino acid responses in whole-cell experiments; the inhibition by $Cd^{2+}$ of NMDA response was voltage independent and was 4 times less potent than $Zn^{2+}$ (Mayer *et al.* 1989). At the single-channel level, $Cd^{2+}$ (100 $\mu$M) caused a reduction in the frequency of NMDA-induced channel opening at a holding potential of −60 mV (Fig. 10 *A* and *B*), and decreased both the long and short open times (Fig. 9*C* and *D*). Cadmium reduced $\tau_{o1}$ from 1·5±0·3 ms ($n = 4$) to 0·9±0·2 ms ($n = 4$) and $\tau_{o2}$ from 10·3±3·8 ms ($n = 4$) to 5·0±0·5 ms ($n =$

4). However, as noted by Ascher & Nowak (1988), 100 $\mu$M-$Cd^{2+}$ did not induce voltage-dependent flickering (Fig. 10*C* and *D*). This provides further evidence that the voltage-independent and voltage-dependent effects of $Zn^{2+}$ represent separate mechanisms.

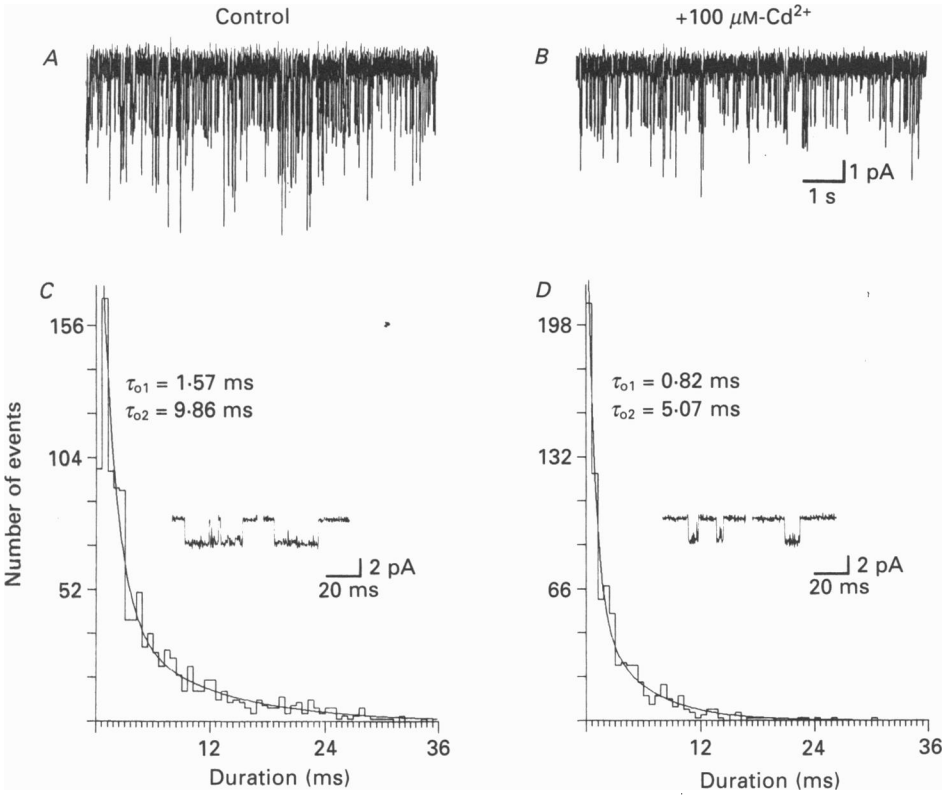


Fig. 9. Cadmium (100 $\mu$M) also reduced NMDA channel activity, but did not cause voltage-dependent flickering. The number of events activated by 5 $\mu$M-NMDA in a multichannel patch (*A*) was reduced in the presence of 100 $\mu$M-$Cd^{2+}$ (*B*). The open-time histogram of the large conductance events from the same patch was fitted with two exponentials. Note that $Cd^{2+}$ reduced both mean open times similar to the action of $Zn^{2+}$. Insets in *C* and *D* show examples of opening (filter 2 kHz) obtained at −60 mV in the absence (*C*) and presence (*D*) of 100 $\mu$M-$Cd^{2+}$. Note the lack of flickering in the presence of $Cd^{2+}$. Bin width 0·6 ms.

*Interaction of permeant ions with zinc*

Competition between permeant ions and a blocking molecule suggests that the blocker acts at a site within a channel (e.g. Moczydlowski, Gardner & Miller, 1984; Miller, 1988). The voltage-dependent flickering induced by high concentrations of $Zn^{2+}$ is likely to result from binding to a site within the NMDA channel similar to $Mg^{2+}$ and non-competitive antagonists such as MK-801 (Mayer *et al.* 1984; Nowak *et al.* 1984; Huettner & Bean, 1988). To test this possibility, the effect of increasing $[Ca^{2+}]_o$ and $[Na^+]_o$ on $Zn^{2+}$ inhibition of NMDA channels was examined.

When $[Ca^{2+}]_o$ was increased from 2 to 30 mM, the single-channel current of the

largest conductance state was reduced to 1·7 pA at a holding potential of −60 mV (Fig. 11*A*). This is consistent with previous reports (Jahr & Stevens, 1987; Ascher & Nowak, 1988). However, in high $[Ca^{2+}]_o$ in the presence of 30 μM-$Zn^{2+}$, voltage-dependent flickering was greatly reduced ($n = 5$) suggesting that calcium competes

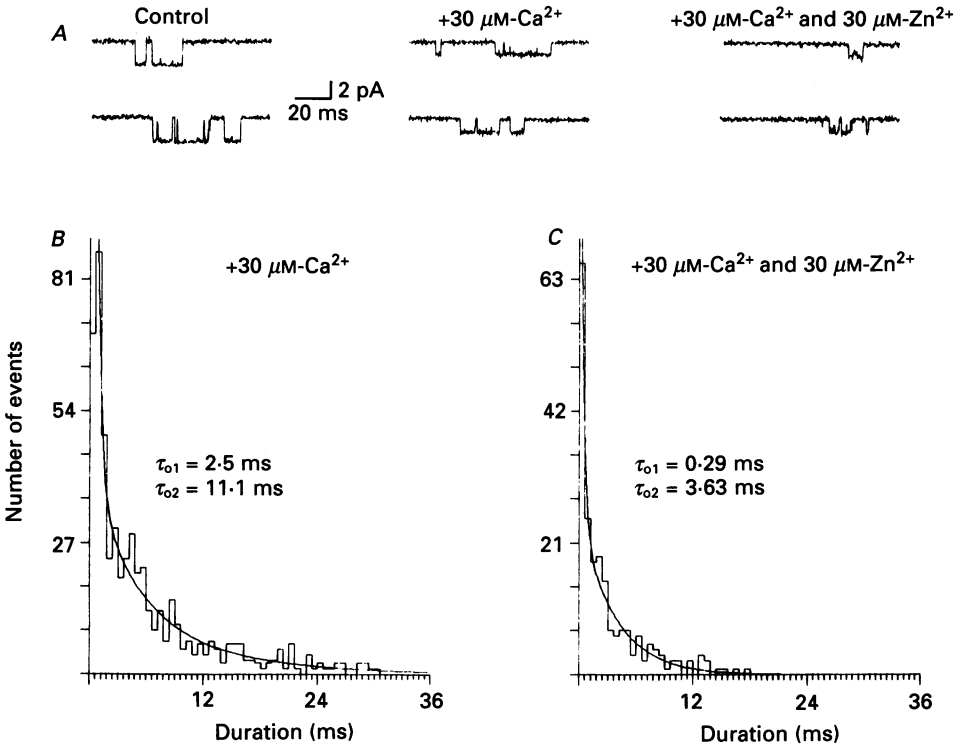


Fig. 10. Calcium (30 mM) prevented flickering induced by $Zn^{2+}$ (30 μM) but did not antagonize zinc-induced reduction in channel activity and mean open time. *A*, single-channel currents were recorded in 2 mM-$Ca^{2+}$ (left column), 30 mM-$Ca^{2+}$ (middle column) and in 30 mM-$Ca^{2+}$ + $Zn^{2+}$ (right column). Holding potential was −60 mV. Note that 30 mM-$Ca^{2+}$ reduces the single-channel current. In the presence of $Zn^{2+}$ (right column) the single-channel amplitude was still reduced but no flickering was observed. Both histograms were fitted with two exponentials. Note that $Zn^{2+}$, even in presence of the high concentrations of $Ca^{2+}$, reduced the mean open times. Bin width 0·6 ms. 5 μM-NMDA. Filter 2 kHz.

with $Zn^{2+}$ in the channel or vestibule (see e.g. Dani, 1986). However the voltage-independent effects of $Zn^{2+}$ were unchanged in the high-calcium solutions (Fig. 10*B* and *C*). In 30 mM $[Ca^{2+}]_o$, the open-time histogram was fitted by two exponentials (Fig. 11*B*) which did not differ significantly from $\tau_{o1}$ and $\tau_{o2}$ in 2 mM $[Ca^{2+}]_o$. Zinc (30 μM) reduced $\tau_{o1}$ from 1·6 ± 0·7 ms ($n = 5$) to 0·4 ± 0·2 ms ($n = 5$) and $\tau_{o2}$ from 10·4 ± 1·3 ms ($n = 5$) to 3·5 ± 0·5 ms ($n = 5$).

Similar effects were seen in high $[Na^+]_o$ (Fig. 11). In 500 mM $[Na^+]_o$, the single-channel current of the largest conductance state increased to 4·5–5 pA at a holding potential of −60 mV (Fig. 11*A* and *B*). In the high $[Na^+]_o$ solutions, $Zn^{2+}$ (30 μM) induced little voltage-dependent flickering, but the voltage-independent effects of

$Zn^{2+}$ were still apparent (Fig. 11*A* and *B*). Zinc (30 $\mu$M) reduced $\tau_{o1}$ from $0{\cdot}9 \pm 0{\cdot}2$ ms ($n = 3$) to $0{\cdot}5 \pm 0{\cdot}1$ ms ($n = 30$ and $\tau_{o2}$ from $9{\cdot}9 \pm 0{\cdot}5$ ms ($n = 3$) to $2{\cdot}7 \pm 0{\cdot}7$ ms ($n = 3$), as shown in Fig. 11*C* and *D*. High $[Na^+]_o$ did not cause voltage-independent changes in the frequency of channel opening or in mean open time, suggesting that

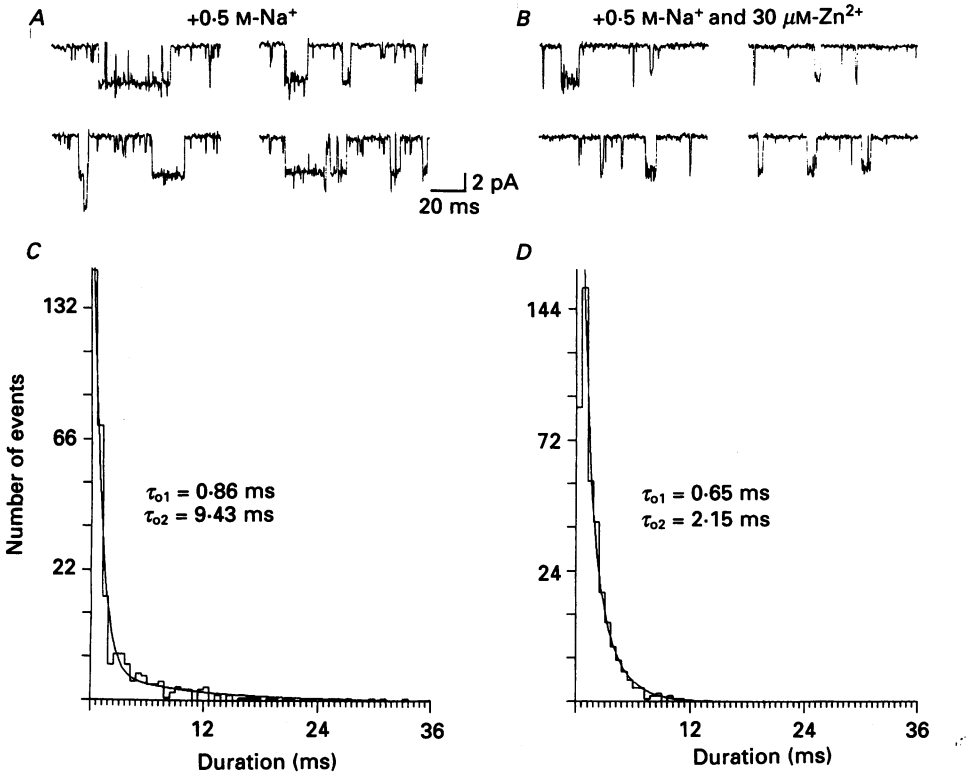


Fig. 11. High concentrations of $Na^+$ (0·5 M) also reduced flickering induced by $Zn^{2+}$ (30 $\mu$M). Single-channel currents were recorded in 0·5 M-$Na^+$ (*A*) and in 0·5 M-$Na^+$ + $Zn^{2+}$ (*B*) at a holding potential of −60 mV. Note that in the presence of 0·5 M-$Na^+$, the single-channel amplitude is increased (*A* and *B*), but in the presence of $Zn^{2+}$ only a few flickerings were observed (*B*). Compare with Fig. 8. Open-time histograms of the large conductance events were constructed from the same patch in 0·5 M-$Na^+$ (*C*) and in 0·5 M-$Na^+$ + $Zn^{2+}$ (*D*). Both histograms were fitted with two exponentials. Note that 30 $\mu$M-$Zn^{2+}$, even in the presence of 0·5 M-$Na^+$, reduced the mean open times. Bin width 0·6 ms. 5 $\mu$M-NMDA. Filter 2 kHz.

$Na^+$ has no zinc-like effect. In high $[Ca^{2+}]_o$, small decreases in the frequency of channel opening were seen, but this is likely to result from complex formation between NMDA and $Ca^{2+}$ (e.g. see Ascher & Nowak, 1988), rather than to a zinc-like action of calcium.

## DISCUSSION

These results demonstrate that the primary effect of $Zn^{2+}$ and $Cd^{2+}$ on NMDA-activated ion channels is a voltage-independent reduction in both the frequency of channel opening as well as the mean lifetime of the large conductance events. This

mechanism appears sufficient to account for the inhibition of the NMDA-activated macroscopic currents and synaptic potentials by group IIB transition metal cations. In $Mg^{2+}$-free saline and at higher $Zn^{2+}$ concentrations, $Zn^{2+}$ induces voltage-dependent flickering of the open channel suggesting that $Zn^{2+}$ also has a weak $Mg^{2+}$-like action. The mechanisms of these two actions are likely to be quite different and will be discussed separately.

*Voltage-independent action of $Zn^{2+}$*

Our earlier studies demonstrated that $Zn^{2+}$ inhibition of macroscopic currents evoked by NMDA is voltage independent and non-competitive (Westbrook & Mayer, 1987). At the single-channel level, both the reduction in summed channel current and the decrease in mean open times of the large conductance events were inhibited to a similar degree ($IC_{50}$s of 12 and 22 $\mu$M) suggesting that the changes in these two parameters are sufficient to account for $Zn^{2+}$ inhibition of macroscopic currents evoked by NMDA. Although the small conductance levels appeared to be unaffected by $Zn^{2+}$, the vast majority of openings were $> 25$ pS; thus the small conductance events contribute little to macroscopic currents. The complete block of macroscopic NMDA currents by high concentrations of $Zn^{2+}$ provides further evidence for preferential opening of NMDA channels to large conductance levels. These observations appear to exclude the possibility that $Zn^{2+}$ increases transitions of NMDA channels to lower conductance levels, e.g. from 45 to 35 pS, since all of the large levels were decreased by $Zn^{2+}$. The small decreases in the single-channel conductance seen in the amplitude histograms in the presence of $Zn^{2+}$ were voltage dependent and could be attributed to unresolved blockages of the channel (see below), rather than to transitions between conductance levels.

Zinc could inhibit the NMDA response by acting at the ligand binding site, at the glycine binding site or at a site within the channel. It is highly unlikely that $Zn^{2+}$ affects ligand binding since the antagonism is non-competitive and much higher concentrations of $Zn^{2+}$ are required to interfere with glutamate in binding assays (Monaghan & Michel, 1987). The co-agonist glycine increases the probability of NMDA channel opening (Johnson & Ascher, 1987). Although $Zn^{2+}$ reduced the frequency of channel opening, several observations make it unlikely that $Zn^{2+}$ is a glycine antagonist. In whole-cell experiments, zinc-induced inhibition was not overcome by raising the glycine concentration from 0·1 to 100 $\mu$M (Mayer *et al.* 1989). Glycine also does not affect the mean channel lifetime (Johnson & Ascher, 1987) and glycine antagonists such as kynurenic acid reduce NMDA currents without affecting the single-channel conductance or mean open time as calculated from current noise (Mayer *et al.* 1988). In contrast, $Zn^{2+}$ does reduce the mean open time. Finally $Zn^{2+}$ is not acting as a classical open-channel blocker, since its action is not voltage and use dependent. This contrasts with channel blocking drugs such as MK-801 (Huettner & Bean, 1988). Overall these data suggest that $Zn^{2+}$ acts on the extracellular domain at a distinct site on the receptor molecule.

*A kinetic description of zinc action*

A description of the action of zinc must account for the decrease in burst duration and mean open time as well as the decrease in the number of events. The limitations of our data and the complexity of NMDA channel gating preclude a definitive kinetic

model. For example, no attempt was made to analyse the two or three conductance levels of 29, 38 and 43 pS separately. Likewise, open-time histograms always contained at least two exponentials (see also Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Ascher *et al.* 1988). However, $Zn^{2+}$ affected each of the large conductance levels and also decreased both the short and long mean open times. Thus as a first approximation, only the simplest case including a single conductance level and open state will be considered.

The reduction of $\tau_{o2}$ suggests that $Zn^{2+}$ reveals a closed (or blocked) state, C(Zn), with a rate constant of $4 \times 10^6$ M$^{-1}$ s$^{-1}$. A single zinc-dependent rate constant could explain the observed effects of $Zn^{2+}$ on NMDA channels if one assumes that return to the open state from C(Zn) is slow, i.e. the number of events seen in a multichannel patch would markedly decrease. Burst duration decreased in the presence of $Zn^{2+}$, thus a sequential kinetic scheme is insufficient since it predicts that total time spent in the open state should remain constant and burst duration should increase in the presence of the inhibitor (e.g. Neher & Steinbach, 1978; Neher, 1983). The open-channel block of NMDA channels by $Mg^{2+}$ is also incompletely described by a sequential model (Nowak *et al.* 1984). For $Zn^{2+}$, a cyclic kinetic scheme incorporating a closed state, C(Zn), accessible from the open configuration could explain both the decreases in burst duration and in the number of bursts. Similar schemes have been proposed for the action of $Mg^{2+}$ by Jahr & Stevens (1990) to account for the presence of brief, $Mg^{2+}$-insensitive gaps.

In a cyclic model, $Zn^{2+}$ affects exit from the open state, but this does not imply that $Zn^{2+}$ acts within the channel like $Mg^{2+}$ or MK-801. Blocking rates for open-channel blockers are quite similar and generally approach the diffusion limit. For $Mg^{2+}$, the blocking rates are $1{\cdot}6$–$1{\cdot}8 \times 10^7$ M$^{-1}$ s$^{-1}$ (Ascher & Nowak, 1988; Jahr & Stevens, 1990) and for MK-801 the blocking rate is $3 \times 10^7$ M$^{-1}$ s$^{-1}$ (Huettner & Bean, 1988). These rates are approximately 10 times faster than the $Zn^{2+}$ blocking rate. The $Zn^{2+}$ blocking rate is similar to that for substance P inhibition of acetylcholine responses ($2{\cdot}7 \times 10^6$ M$^{-1}$ s$^{-1}$, Clapham & Neher, 1984). The $Zn^{2+}$ unblocking rate was $\approx 55$ s$^{-1}$ assuming $K_i = 12$ $\mu$M. Comparable unblocking rates for $Mg^{2+}$ are 1400 s$^{-1}$ at $-60$ mV (Ascher & Nowak, 1988), $0{\cdot}3$ s$^{-1}$ for MK-801 at $-70$ mV, and 2–3 s$^{-1}$ for substance P on acetylcholine responses (Clapham & Neher, 1984). Although this could classify $Zn^{2+}$ as a slow channel blocker, in view of the lack of voltage dependence, it is also consistent with allosteric inhibition.

*Divalent interactions and zinc-induced flickering of the NMDA channel*

Zinc had a second effect on single NMDA channels which was voltage dependent and which has also been analysed recently by Christine & Choi (1990). Open-channel block of NMDA channels by other divalent cations has been shown to correlate with the water substitution rates in the inner co-ordination sphere of the metal ions (Mayer & Westbrook, 1987; Ascher & Nowak, 1988). The voltage-dependent flickering seen with higher concentrations of $Zn^{2+}$, but not with $Cd^{2+}$, appears to fit this hypothesis. The dehydration rate for $Zn^{2+}$ is $\approx 3 \times 10^7$ s$^{-1}$ (Diebler, Eigen, Ilgenfritz, Maas & Winkler, 1969), which is similar to $Mn^{2+}$ and midway between the slow rates for $Mg^{2+}$ ($\approx 10^5$ s$^{-1}$) and the rapid rates for $Ca^{2+}$ and $Cd^{2+}$ ($\approx 3 \times 10^8$ s$^{-1}$). At negative membrane potentials, zinc-induced flickering was much faster than $Mg^{2+}$ and quite similar to $Mn^{2+}$ (e.g. see Fig. 6, Ascher & Nowak, 1988). The reduction of

flicker by high extracellular calcium or sodium also confirms that $Zn^{2+}$ is binding within the open channel.

Since inhibition of the macroscopic current is essentially voltage independent, it is unlikely that zinc-induced flickering plays a major physiological role at the cellular level. Zinc can cause direct neurotoxicity (Choi, Yokoyama & Koh, 1988) and it has been suggested that this is due to $Zn^{2+}$ permeation through NMDA channels. However, relief of $Zn^{2+}$ block of NMDA channels was not seen in our experiments. Although permeation of $Zn^{2+}$ into cultured cortical neurones has been demonstrated with the fluorescent zinc-specific chelator, toluene sulphonamide quinoline (Koh & Choi, 1989), this was only observed in $Ca^{2+}$-free solutions. We suggest that competition by physiological levels of extracellular $Mg^{2+}$ and $Ca^{2+}$ will prevent significant $Zn^{2+}$ permeation through NMDA channels.

*The zinc binding site on the extracellular domain of the NMDA receptor*

Zinc is avidly complexed by several amino acid residues including sulphydryl groups of cysteine residues and the imidazole groups of histidine residues; this might predict the amino acid residues involved in the $Zn^{2+}$ binding site. However, $Zn^{2+}$ inhibition of NMDA responses is rapidly reversible and unaffected by $Hg^{2+}$ (Mayer *et al.* 1989), a much more potent sulphydryl reagent (Li & Manning, 1955; Simpson, 1961). This makes a simple reaction with sulphydryl groups unlikely. In addition, the sulphydryl-reducing agent dithiotreitol potentiates, rather than inhibits, NMDA responses on cultured cortical neurones (Aizenman, Lipton & Loring, 1989). Mercury ($Hg^{2+}$) does irreversibly reduce kainate responses which are restored in the presence of cysteine (Kiskin, Krishtal, Ysyndrenko & Akaike, 1986); and the extracellular domain of the kainate receptor expressed in *Xenopus* oocytes does have multiple cysteine residues (Hollmann, O'Shea-Greenfield, Rogers & Heinemann, 1989). The histidine reagent diethylpyrocarbonate reduces kainate/quisqualate responses in catfish horizontal cells suggesting that imidazole groups of histidine residues may be involved in that response (Christensen & Hida, 1989). This response was also reduced by $H^+$ with a $pK = 6{\cdot}5$, near the $pK$ for the imidazole ring.

Could $Zn^{2+}$ act on a proton binding site? Hydrogen ions can reduce NMDA responses (Dani, Jahr & Stevens, 1988); this effect is voltage insensitive and shifts the $K_d$ of glycine potentiation (Tang, Dichter & Morad, 1989; but see Traynelis & Cull-Candy, 1990). In preliminary experiments we have found that changes in pH from 7·2 to 6·8 reduce NMDA channel activity by approximately 50% without changing the single-channel conductance. Unlike $Zn^{2+}$ inhibition of NMDA channels, there was no change in mean channel lifetime, and the effects of $H^+$ and $Zn^{2+}$ were additive (P. Legendre & G. L. Westbrook, unpublished). This suggests that $Zn^{2+}$ and $H^+$ have different actions.

Since $Zn^{2+}$ appears to act as an allosteric inhibitor, it is conceivable that the binding site is similar to zinc-binding proteins such as metalloenzymes. On alcohol liver dehydrogenase $Zn^{2+}$ is ligated by two cysteines and one histidine in the binding domain for the substrate and coenzyme (Fersht, 1985). In the '$Zn^{2+}$ finger' structure of some DNA binding proteins, the $Zn^{2+}$ co-ordination site involves two cysteine and two histidine residues separated by a loop of twelve amino acids (Evans & Hollenberg, 1988). This is strikingly similar to the cys–cys loop which forms a $\beta$-

structural loop in the extracellular domain of many cloned ligand-gated ion channels including nicotinic acetylcholine receptors (Patrick, Boulter, Goldman, Gardner & Heinemann, 1987) and $GABA_A$ and glycine receptors (Barnard, Darlison & Seeburg, 1987). Only the $\alpha$ and $\gamma$-subunits of the $GABA_A$ receptor have histidine residues within the cys–cys loop (Pritchett, Sontheimer, Shivers, Ymer, Kettenmann, Schofield & Seeburg, 1989). It seems plausible that a similar structure on the NMDA receptor is involved in the $Zn^{2+}$ binding site. Although there is no direct evidence for this at present, the inhibition of $GABA_A$ responses by $Zn^{2+}$ (Westbrook & Mayer, 1987), and the weak effect of $Zn^{2+}$ on kainate receptors which have no cys–cys loop (Hollmann *et al.* 1989), leaves this possibility open.

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