High-Affinity Zn Block in Recombinant *N*-Methyl-D-Aspartate Receptors with Cysteine Substitutions at the Q/R/N Site

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ABSTRACT In ionotropic glutamate receptors, many channel properties (e.g., selectivity, ion permeation, and ion block) depend on the residue (glutamine, arginine, or asparagine) located at the tip of the pore loop (the Q/R/N site). We substituted a cysteine for the asparagine present at that position in both NR1 and NR2 *N*-methyl-D-aspartate (NMDA) receptor subunits. Under control conditions, receptors containing mutated NR1 and NR2 subunits show much smaller glutamate responses than wild-type receptors. However, this difference disappears upon addition of heavy metal chelators in the extracellular bath. The presence of cysteines at the Q/R/N site in both subunits of NR1/NR2C receptors results in a 220,000-fold increase in sensitivity of the inhibition by extracellular Zn. In contrast with the high-affinity Zn inhibition of wild-type NR1/NR2A receptors, the high-affinity Zn inhibition of mutated NR1/NR2C receptors shows a voltage dependence, which resembles very much that of the block by extracellular Mg. This indicates that the Zn inhibition of the mutated receptors results from a channel block involving Zn binding to the thiol groups introduced into the selectivity filter. Taking advantage of the slow kinetics of the Zn block, we show that both blocking and unblocking reactions require prior opening of the channel.

INTRODUCTION

The current interpretation of ionic channel selectivity assumes that it is controlled both by the size of the smallest constriction through which permeating ions must pass and by the arrangement of coordinating groups that are needed to replace water molecules around the partially dehydrated permeating ion (Hille, 1992). The molecular structure of a selectivity filter has been obtained by an x-ray crystallographic study of KcsA, a bacterial potassium channel (Doyle et al., 1998). KcsA is a homotetramer in which the selectivity filter is build around the central symmetry axis of the protein by four identical re-entrant pore-loops, each contributed by a separate subunit. The pore-loop motif consists of a short α-helix bent from the outer membrane surface toward the center of the channel, followed by a stretch of amino acids that returns to the outer surface. The functional groups lining the pore are all backbone carbonyl oxygens belonging to the five amino acids (T, V,G, Y, and G) of the extended stretch. The remarkable potassium selectivity of these channels is proposed to result from a perfect fit of the carbonyl rings around dehydrated permeating potassium ions.

In comparison, much less is known about the selectivity filters of vertebrate ionotropic glutamate receptors. These channels are multimeric membrane proteins made of an unknown number of subunits sharing a common membrane topology with three presumed transmembrane segments (TM1, TM3, andTM4) and a pore-loop (called TM2 and located between TM1 and TM3) (see Dingledine et al., 1999, for a review). In contrast with voltage-dependent channels, the pore-loop of glutamate receptors dips into the membrane from the cytoplasmic side (Wo and Oswald, 1994; Kuner et al., 1996; Kupper et al., 1996). The selectivity filter of these ionotropic receptors involves particular residues located half-way through the pore-loop structure at a locus called the Q/R/N site because it contains either a glutamine (Q) or an arginine (R) in both α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptor subunits, whereas an asparagine (N) is present at the homologous position in all *N*-methyl-D-aspartate (NMDA) receptor subunits. In AMPA and kainate receptors, the presence of an R at the Q/R/N site suppresses both Ca permeability and block by intracellular polyamines (see Hollmann and Heinemann, 1994, for a review). In NMDA receptors, substituting Q for N decreases both the Ca selectivity and the voltage-dependent block by extracellular Mg^{2+} ions (Burnashev et al., 1992; Mori et al., 1993; Sakurada et al., 1993), and enlarges the maximal constriction diameter of the selectivity filter (Wollmuth et al., 1996). Based on the accessibility of substituted cysteines to methanethiosulfonate reagents applied in either the extracellular or the intracellular compartments, Kuner et al. (1996) proposed that the Q/R/N locus is positioned at the top of the pore loop, i.e., in a position that would be similar to that of the first residue (T75) of the extended stretch of the KscA pore-loop.

Despite the abundance of data pointing to the involvement of the Q/R/N site in the function of glutamate receptor selectivity filters, a precise mechanistic role for the residue occupying this site (linked to the nature of its lateral chain) is still lacking. An interesting proposal was recently made by Tikhonov et al. (1999). Having observed that in the KcsA structure the hydroxyl group of T75 might be posi-

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tioned so as to make a hydrogen bond with the backbone carbonyl oxygen of the same residue in the neighboring subunit, they made the hypothesis that in NMDA receptors the amide group of the Q/R/N site asparagines could similarly make a hydrogen bond with the oxygen of the backbone carbonyl of the homologous residue in the neighboring subunit. In the oligomeric receptor, the ring of homologous asparagines will form a diaphragm, which may determine the selectivity filter size.

We undertook the present study to test a similar hypothesis. In AMPA and kainate subunits, two glutamines are systematically found in tandem, one at the Q/R/N site and one at the next position [(Q/R/N)+1]. At the homologous positions in NMDA subunits, there are either two consecutive asparagines (in NR2 subunits) or an asparagine followed by a serine (in NR1 subunits). Several studies have shown that the (Q/R/N)+1 asparagine of NR2 subunits also contributes to the selectivity filter of NMDA receptors (Kuner et al., 1996; Wollmuth et al., 1996) and controls block by external Mg (Kupper et al., 1996, 1998; Wollmuth et al., 1998). We made the hypothesis that the amide groups of the residues at position Q/R/N could form hydrogen bond(s) with either the amide or the hydroxyl group of the residues at position (Q/R/N)+1 in the neighboring subunit, building a complete diaphragm around the pore through lateral chain interactions between subunits. This diaphragm hypothesis was tested using NMDA receptors. If a ring of hydrogen-bonded asparagines and serines does form in wild-type receptors, systematically substituting cysteines for the neighboring asparagines and serines might lead to a cysteine ring that in turn might form a tight disulfide-bridged diaphragm under oxidizing conditions. Cysteines were therefore introduced at both the Q/R/N and the (Q/R/N)+1 positions of NR1 and NR2A subunits, and indeed glutamate responses of NR1(NCSC)/NR2A(NCNC) receptors were 1) of much smaller amplitude than that of wild-type receptors and 2) very strongly potentiated (over 10-fold) by the reducing agent DTE. However, we realized that the effects of DTE resulted from its heavy metal chelating properties (Cornell and Crivaro, 1972; see Paoletti et al., 1997) rather than from its reducing action, suggesting that cysteine substitutions at (or next to) the Q/R/N site may create an inhibitory heavy metal binding site of high affinity. Wild-type NR1/NR2A receptors are known to possess a high-affinity Zn inhibitory site (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998). Such receptors were therefore not suited for the characterization of a cysteine-engineered site for heavy metals. We used instead NR1/NR2C receptors that, when of wild-type genotype, are only slightly sensitive to extracellular Zn (inhibition with a 30 μM IC_{50}). We show that substituting a cysteine at the Q/R/N site of both subunits of NR1/NR2C receptors creates a high-affinity Zn blocking site within the pore. We further show that this effect is specific to the Q/R/N position, that Zn access to the blocking site requires prior opening of the NMDA channel, and that Zn can be trapped in the closed channel. These observations add new constraints on both the structure of the selectivity filter and the gating mechanism.

MATERIALS AND METHODS

NMDA receptor subunit constructs and heterologous expression

Wild-type and mutant constructs

The pcDNA3 expression plasmids for Rat NR1a (simply referred to as NR1 in what follows) and NR2A subunits have been described in Paoletti et al. (1997). The "wild-type" NR2C used in our experiments is $NR2C_{M1}$, a chimerical construct that carries the NR2A TM1 segment and has a 275-amino-acid deletion at the C-terminus (gift of Peter Seeburg, Max-Planck-Institut für Medizinische Forschung, Heidelberg, Germany). $NR2C_{M1}$ was chosen because it expresses better than wtNR2C while retaining its pore properties (Kuner and Schoepfer, 1996) and its low-affinity voltage-independent Zn inhibition (M. Amar and J. Neyton, unpublished observation). $NR2C_{M1}$ was subcloned in a modified version of the pcDNA3 expression vector. Point mutations were produced according to the method of Kunkel (1985). For each mutation, at least two independent clones were isolated, in which the presence of the mutation was verified by sequencing across the mutated region (~50 bp on either side of the mutation). The two independent clones were functionally characterized to confirm that the modified phenotype resulted from the planned mutation rather than from a stray mutation produced during the mutagenesis process. The different mutants constructed for this study, together with their simplified nomenclature are NR1(N598C) = R1(NC), NR1(N598C-S599C) = NR1(NCSC), NR2A(N595C-N596C) = NR2A(NCNC), $NR2C_{M1}$(N593C) = NR2C(N_1C), and $NR2C_{M1}$(N594C) = NR2C(N_2C).

Heterologous expression of NMDA receptors in Xenopus *oocytes*

Oocytes were prepared and kept as described in Paoletti et al. (1995) and recorded 1–5 days after either DNA or cRNA injection. Capped cRNAs were transcribed from linearized expression plasmids with T7 RNA polymerase (Ambion, Montrouge, France). For DNA injections, 20 nl of a mixture of NR1 and NR2 plasmids (ratio 1:2) at a final concentration of 10 ng/μl were injected into the oocyte nuclei. For cRNA injection, 50 nl of a similar mixture at a final concentration of 100 ng/μl were injected. Experiments were performed on oocytes that responded to saturating doses of glutamate and glycine (100 μM each) with currents within the 0.5–5-μA range.

Buffering solutions for Zn and chemicals

In this study, we measured Zn inhibition of NMDA receptors over a large concentration range (0.05 nM to 100 μM). Previous work in the laboratory indicated that 20–50 nM Zn contaminates our control solutions (Paoletti et al., 1997). Zn-buffered solutions were used to control the actual free Zn concentration in the submicromolar range. Tricine (*N*-tris[hydroxymethyl-]methylglycine), a Zn chelator of moderate affinity with constants $K_1 = 10^{-5}$ M for the equilibrium M + L $\leftrightarrow$ ML and pKa = 8.15 (see Paoletti et al., 1997) was added at 10 mM to buffer Zn in the range 3 nM to 1 μM. *N*-[2-acetamido]iminodiacetic acid (ADA) with $K_1 = 10^{-7.3}$ M and pKa = 6.52 (Martell and Smith, 1989) was added at 1 mM to buffer Zn in the 0.05–3 nM range. At pH 7.3 and with 10 mM tricine, calculations performed with the MaxChel program (Bers et al., 1994) show that there is a linear relation: $[Zn]_{free} = [Zn]_t/100$ for $[Zn]_{free} < 1$ μM. A linear relation,

$[Zn]_{free} = [Zn]_t/17{,}000$, was also found at pH 7.3 with 1 mM ADA for $[Zn]_{free} \leq 3$ nM. For all Zn-inhibition dose-response curves, a Zn-free reference solution was made by adding 10 μM diethylenetriamine-pentaacetic acid (DTPA), a strong Zn chelator ($K_D = 10^{-15.6}$ M) to the zero-added Zn buffered solution.

All chemicals were purchased from Sigma (Saint-Quentin Fallavier, France). Zn was added as chloride salts ($ZnCl_2$, ACS reagent quality) by dilution from 1 M stock solutions prepared in 0.1 M HCl.

Recording and data analysis

Recording conditions

The two-electrode voltage-clamp and superfusion system have been described previously (Paoletti et al., 1997). Bath solutions always contained (in mM): 100 NaCl, 2.8 KCl, 5 HEPES, and 0.3 $BaCl_2$, with pH adjusted to 7.3 with NaOH. The low Ba concentration was chosen as a compromise between the necessity of having divalent cations in the recording solutions to reduce endogenous currents through cationic channels activated in divalent cation-free solution and the minimization of chloride currents through endogenous Ca-activated channels (see Weber, 1999).

Currents activated by application of agonists (glutamate and glycine both applied at a saturating dose of 100 μM) were recorded either at a steady-state voltage (−60 mV unless specified), or during 4-s-long −100/+50-mV voltage ramps (in this latter case, both capacitive and leakage currents were recorded before each application of agonists and subtracted from the traces recorded in the presence of the agonists).

Spontaneous run-down of the responses to agonists was systematically observed with NR1-NR2C_{M1} receptors. To circumvent this difficulty, each response under test conditions was generally bracketed by two responses under reference conditions (for example, Zn-free solution for Zn inhibition curves). The relative current under a particular test condition was calculated as the ratio between the current measured in the test condition and the mean of the reference currents recorded just before and after the test current.

All experiments were performed at room temperature (18–25°C).

Data analysis

Full dose-response curves for Zn voltage-dependent inhibition at −60 mV were obtained in a minimum of three different oocytes for each construct. For the constructs with a moderate or low sensitivity to Zn (NR1(NC)/NR2Cwt, NR1wt/NR2C(N_1C), and NR1wt/NR2Cwt), voltage-dependent and voltage-independent Zn inhibition occurs in the same concentration range. To separate them, Zn inhibition of the response to agonists was measured during voltage ramps. The voltage-independent inhibition was directly measured at +50 mV (relative current = $R_{+50\ mV}$). The voltage-dependent inhibition at −60 mV was calculated as the measured inhibition corrected for the voltage-independent inhibition (corrected relative current = $R_{-60\ mV}/R_{+50\ mV}$). Experimental points of dose-response curves shown in Fig. 3 correspond to the means of the relative currents (corrected when necessary). Error bars represent standard deviations. Lines correspond to the best fit performed with the Sigmaplot fitting procedure using the following equation:

$$f(x) = 1 - 1/[1 + (IC_{50}/x)^n], \quad (1)$$

where x is the Zn concentration and IC_{50} and n (Hill coefficient) are the free parameters.

RESULTS

Heavy metal chelation markedly potentiates glutamate responses of NR1NCSC/NR2ANCNC receptors.

We substituted cysteines for the Q/R/N asparagine and the next residue in NR1 and NR2A subunits, expecting that, under oxidizing conditions, inter-subunit disulfide bridges would form, leading to a decreased pore diameter. In oocytes expressing wild-type receptors, saturating doses of glutamate and glycine induce robust responses that usually exceeds 1 μA in the day after DNA injection (see Fig. 1 *A*). In contrast, responses of oocytes expressing NR1(NCSC)/NR2A(NCNC) receptors recorded under control conditions had a much lower amplitude, even a few days after DNA injection (Fig. 1 *B*, prior DTE addition). Bath application of the reducing agent DTE (3 mM) results in a fast but moderate potentiation of the response of wild-type receptors (1.5 ± 0.1-fold, $n = 3$; see also Fig. 1 *A*). In mutant receptors, application of DTE induced a much more pronounced potentiation (17 ± 3-fold, $n = 3$, measured at the end of the 2-min DTE application where DTE effects had not reached equilibrium; see Fig. 1 B_1). A possible interpretation of these results was that in mutated receptors additional disulfide bridges would spontaneously form under control conditions, leading to low-amplitude responses. However, additional experiments did not support this conclusion.

Some of the effects of DTE can result from the chelation of heavy metals present as contaminating traces in the solutions. In wild-type NR1/NR2A receptors, such an effect seems to account fully for the fast DTE potentiation shown in Fig. 1 *A* (see Paoletti et al., 1997). We suspected that a similar chelating effect could explain the marked DTE potentiation seen with mutated receptors in Fig. 1 B_1 and thus repeated the experiments with DTPA (a strong heavy metal chelator without reducing properties). The recording in Fig. 1 B_2 was obtained in the same oocyte as that in Fig. 1 B_1 using DTPA instead of DTE; 10 μM DTPA induces a slowly developing potentiation that resembles that of DTE (9.5 ± 2.5-fold, $n = 3$, measured at the end of a 2-min DTPA application). This result indicated that cysteines at or next to the Q/R/N site could form a high-affinity heavy metal inhibitory site. We decided to characterize this engineered heavy metal binding site and did not further address our initial hypothesis of a P-loop inter-subunit hydrogen bond diaphragm.

Low nanomolar Zn concentrations inhibit NR1(NC)/NR2C(N_1C) receptors

Among heavy metals, Zn was a possible candidate for the strong inhibition of NR1(NCSC)/NR2A(NCNC) receptors observed under control conditions. Zn is often coordinated by thiol groups in protein high-affinity binding sites (see Glusker, 1991), and it is present in our control solutions at 20–50 nM (see Paoletti et al., 1997). Zn is known to inhibit NMDA receptors via two mechanisms: a voltage-dependent block of the pore and a voltage-independent inhibition. For both inhibitions, the affinity depends on the type of NR2

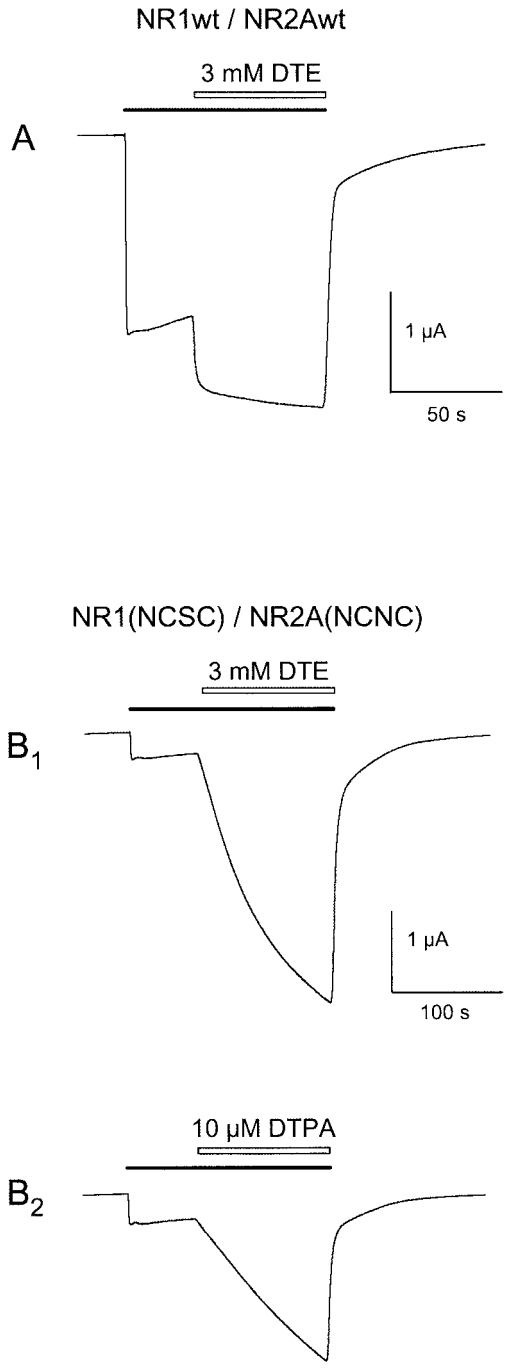

FIGURE 1 The strong potentiation of NR1(NCSC)/NR2A(NCNC) receptors induced by DTE involves heavy metal chelation. (*A*) Current response to the application of agonists (glutamate and glycine; *solid bar*) recorded 1 day after cDNA nuclear injection in an oocyte-expressing wild-type NR1/NR2A receptor; 3 mM DTE added during the agonist application (*open bar*) induced a fast but moderate potentiation (1.4-fold). (B_1) Current response to the agonist application recorded 1 day after cDNA nuclear injection in an oocyte-expressing NR1(NCSC)/NR2A(NCNC) receptor. The response is much smaller. Addition of DTE induces a slow but marked potentiation of the response (15-fold). (B_2) Response to the agonist recorded in the same oocyte as in B_1. The DTE potentiation has been washed by a 2-min application of 0.5 mM DTNB. Addition of DTPA during the response to agonists also induced a marked potentiation (7-fold). Note that the potentiation develops more slowly than that in B_1 and that it has not reached equilibrium at the end of the DTPA application. All recordings were obtained at −60 mV. In the experiments shown in this and the following figures, the agonists glutamate and glycine were applied at the saturating concentration of 100 μM.

subunit. The voltage-dependent block has an apparent affinity (measured at −60 mV) ranging from 30 μM for receptors containing either NR2A or NR2B (see Paoletti et al., 1997) to 200 μM for receptors containing NR2C subunits (see Fig. 3). The voltage-independent inhibition IC_{50} is in the 10–20 nM range for NR2A-containing receptors, whereas it is in the micromolar range for receptors containing NR2B (~0.5 μM), NR2C (~30 μM), or NR2D (~2 μM) (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998). NR1/NR2C receptors have the lowest sensitivity to Zn and thus seemed to be more appropriate than NR1/NR2A receptors to test the possibility that cysteines at or next to the Q/R/N site may induce an additional high-affinity Zn inhibition.

Fig. 2 *A* shows the effects of 1 and 5 nM Zn on the response to glutamate and glycine of NR1(NC)/NR2C(N_1C) receptors. Each recording, obtained at −60 mV, started in a Zn-free solution (10 μM DTPA). Glutamate and glycine were applied at a saturating concentration in the Zn-free solution. Once the response had reached a steady-state amplitude, the bath solution was quickly changed to a solution containing the agonists plus either 1 or 5 nM free Zn. The addition of Zn induced a marked decrease in the response amplitude to ~50% and ~10% of the Zn-free response, respectively. This high-affinity Zn inhibition developed slowly after Zn application with a time constant inversely related to the Zn concentration (see also Fig. 2 *B*). Upon Zn wash, the response increased also slowly but with similar time constants for both Zn applications. The possibility that these slow kinetics reflect the slow time constant of our perfusion system was tested in the same experiment by applying 1 mM Mg instead of Zn (Fig. 2 *A*, dotted line). External Mg is known to produce a fast flickery block of NMDA receptors (kinetics in the milliseconds range; Ascher and Nowak, 1988). The time constants of the Mg inhibition measured at both the application and wash of Mg were at least fourfold faster than those for Zn, indicating that the slow Zn inhibition kinetics can be used to estimate the Zn binding and dissociation reaction rates. The fact that the Zn wash-in time constant is linearly related to the Zn concentration, whereas Zn washout shows no dependence upon this parameter (Fig. 2 *B*) suggests that Zn inhibition is governed by a bimolecular reaction mechanism. Assuming that this is the case, we obtain $k_{off} = 2.4 \pm 0.3\ 10^{-2}\ s^{-1}$ and $k_{on} = 1.4 \pm 0.3\ 10^{7}\ s^{-1}M^{-1}$ (eight independent experiments performed at −60 mV).

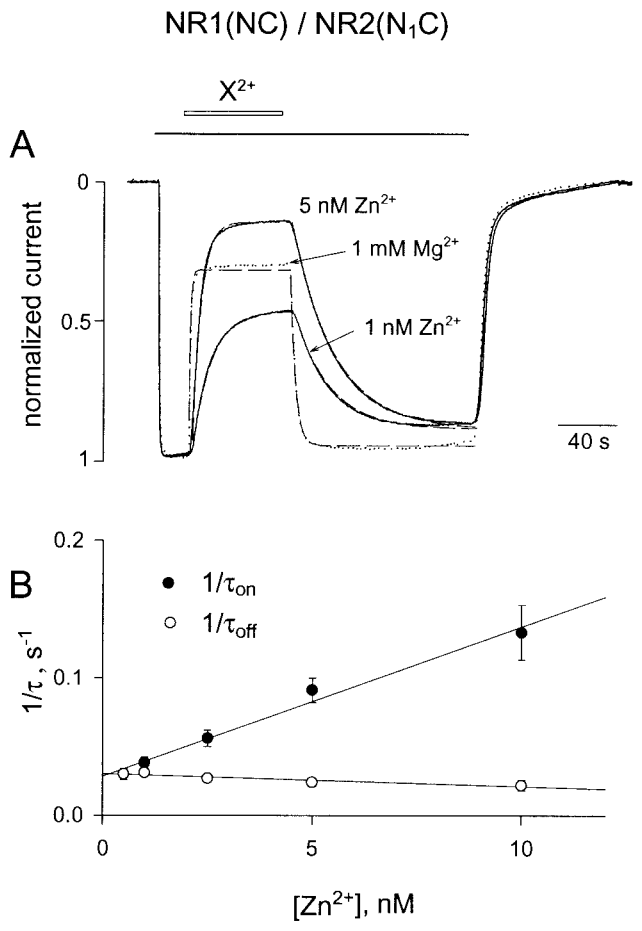

FIGURE 2 Low nanomolar Zn concentrations inhibit NR1(NC)/NR2C(N_1C) receptors. (*A*) Three superimposed responses to agonists recorded at −60 mV in the same oocyte. Divalent cations, either Mg (···) or Zn (——), were added 20 s after the beginning of the agonist application. The resulting response inhibition develops (and washes out) much faster with Mg than with Zn. Exponential fits of the response decay and recovery during and after Zn or Mg application are superimposed as dashed lines on each trace. τ_{on} and τ_{off} are, respectively, 22 s and 42 s for 1 nM Zn, 9 s and 50 s for 5 nM Zn, and 1.4 s and 6.1 s for 1 mM Mg. All solutions contained 1 mM ADA, and the indicated Zn concentrations are free Zn concentrations. Responses have been normalized to the maximum response before divalent cation application. (*B*) Zn wash-in time constant increases linearly with the Zn concentration, whereas Zn washout is independent of the Zn concentration. Each data point corresponds to the mean of three independent experiments. The lines are linear regression through data points.

The presence of a cysteine at the Q/R/N site of both NR1 and NR2 subunits is required for high-affinity Zn inhibition of NR2C-containing receptors

The stoichiometry of NMDA receptors is not certain, though growing evidence suggests that functional receptors are made of two NR1 plus two NR2 subunits (see Dingledine et al., 1999). The nanomolar affinity in NR1(NC)/NR2(CN_1C) suggested that the Zn^{2+} cation is coordinated by more than a single thiol group on the inhibitory site, but is it necessary for the thiol groups to be contributed by both types of subunits? Full dose-response curves of Zn inhibition were obtained at a steady-state voltage of −60 mV for the mutant receptors NR1(NC)/NR2C(N_1C), NR1(NC)/NR2Cwt, and NR1wt/NR2C(N_1C) and for wild-type NR1-NR2C receptors (Fig. 3). The presence of a cysteine at all Q/R/N positions (NR1(NC)/NR2C(N_1C) receptors) induces an impressive decrease in Zn IC_{50} from 190 μM (wild-type receptors) to 0.9 nM, i.e., a 220,000-fold increase in the apparent Zn affinity. In contrast, cysteine substitution in only one type of subunit moderately increases Zn affinity (300-fold and 30-fold for NR1(NC)/NR2Cwt and NR1wt/NR2C(N_1C), respectively). The high affinity for Zn, which is observed in fully mutated receptors, thus requires the presence of a cysteine at the Q/R/N site in both types of subunit.

We next tested whether cysteines at position (Q/R/N)+1 in the NR2C subunit have a similar effect on Zn inhibition as cysteines at Q/R/N. Zn inhibition curves were obtained

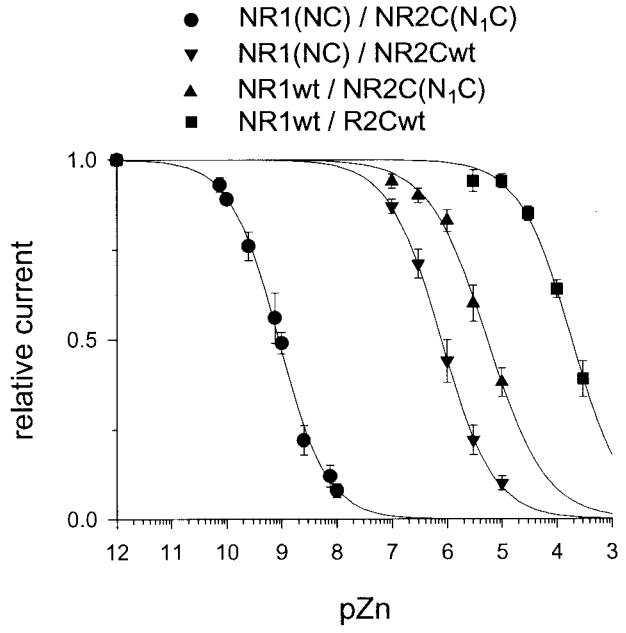

FIGURE 3 Dose-response curves of Zn inhibition in wild-type and mutant NR1/NR2C receptors. Oocytes were voltage clamped at −60 mV. Relative current at a given Zn concentration was calculated as the ratio of the response measured in the presence of Zn over the mean of two responses obtained under Zn-free condition just before and after the response in the presence of Zn. Each experimental point represents the mean and standard deviation of at least three experiments performed on different oocytes. In the case of wild-type and NR1wt/NR2C(N_1C) receptors, the relative current has been corrected for voltage-independent Zn inhibition (see Materials and Methods). Zn was buffered using either 1 mM ADA or 10 mM tricine (see Materials and Methods). Curves have been fitted with a Hill equation (see Materials and Methods) resulting in the following values for IC_{50} and Hill coefficient, respectively: 190 μM and 0.91 for NR1wt/NR2Cwt, 5.4 μM and 0.81 for NR1wt/NR2C(N_1C), 0.78 μM and 0.92 for NR1(NC)/NR2Cwt, and 0.88 nM and 1.01 for NR1(NC)/NR2C(N_1C).

for a series of receptors incorporating NR2C(N_2C) and either NR1wt or NR1(NC). They showed no difference compared with those obtained with NR2Cwt (data not shown). Thus, the presence of a cysteine at the NR2C (Q/R/N)+1 position has no obvious effect on Zn apparent affinity.

The high-affinity Zn inhibition of NR1(NC)/NR2C(N_1C) receptors is voltage dependent

In glutamate receptors, the Q/R/N site controls several properties of the pore selectivity filter (see Introduction). Binding of the divalent Zn^{2+} cation to a residue located at this position, i.e., within the pore, should depend on the transmembrane voltage. To address this point, Zn inhibition curves were obtained for NR1(NC)/NR2C(N_1C) at different voltages. Fig. 4 plots the measured Zn IC_{50} as a function of voltage. Below −80 mV, the Zn apparent affinity varies very little with voltage, but above −40 mV there is a marked increase in Zn IC_{50} with voltage. The limiting slope measured at depolarized potentials (e-fold per 14 mV) is close to that of external Mg block (Ascher and Nowak, 1988). This observation suggested that Zn might bind in the pore at the Mg blocking site. In this case Mg should compete with Zn for blocking the channel. We tested this prediction by measuring the effect of external Mg on Zn block of NR1(NC)/NR2C(N1C) channels. Under Zn-free conditions (10 μM DTPA in the bath solution), 3 mM Mg induces a 80 ± 8% reduction of the current at −60 mV (n = 3). This corresponds to an apparent Mg dissociation constant (K_{Mg}) of 0.75 mM. Zn inhibition was measured in the presence of 3 mM Mg in the bath and the resulting Zn $IC_{50(Mg)}$ was 5.3 ± 0.7 nM (n = 3, data not shown). Therefore, 3 mM Mg induced a 5.9-fold shift in the observed Zn IC_{50}, which is close to the 5-fold shift predicted by a purely competitive effect ($IC_{50(Mg)} = IC_{50(0Mg)}$ (1 + [Mg]/K_{Mg}).

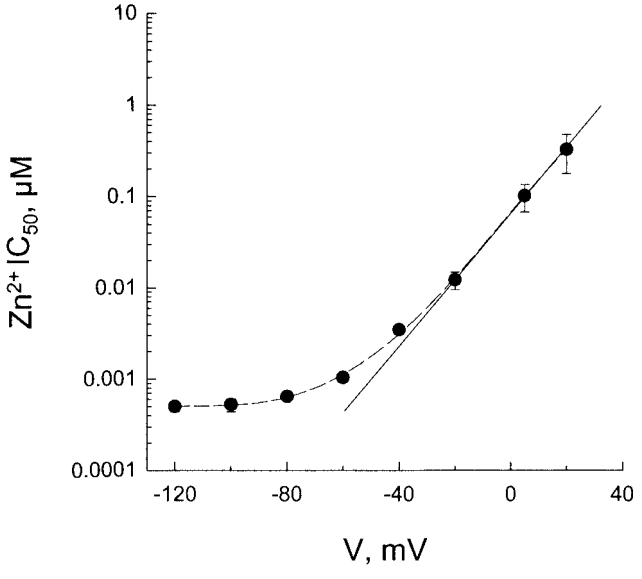

FIGURE 4 The high-affinity Zn inhibition of NR1(NC)/NR2C(N_1C) receptors is voltage dependent. Zn-inhibition dose-response curves were obtained at different steady-state voltages yielding a plot of Zn IC_{50} as a function of voltage. Each point corresponds to the mean and standard deviation of at least two separate experiments. The dashed line is a polynomial fit of the data. The straight line is a linear regression obtained with IC_{50} values measured at V ≥ −20 mV, and its slope is e-fold/14 mV.

Zn binding to and dissociation from the high-affinity inhibitory site of NR1(NC)/NR2C(N_1C) receptors proceeds through open channels only

Fig. 5 *A* shows five recordings obtained sequentially in the same oocyte expressing NR1(NC)/NR2C(N_1C) receptors. The first trace (Fig. 5 *A*, *trace 1*) was obtained under Zn-free conditions (10 μM DTPA). After complete agonist washout, the oocyte was bathed in a solution containing 10 nM free Zn and no agonist. While keeping the Zn concentration constant, two successive agonist applications were performed. The first one started 1.5 min after the beginning of the Zn application and evoked a large initial response, almost as large as the response under Zn-free conditions, which then relaxed to a plateau of much lower amplitude (Fig. 5 *A*, *trace 2*). The second application of agonists induced a response that relaxed to the same amplitude with a very small peak/plateau difference (Fig. 5 *A*, *trace 3*). Traces 4 and 5 in Fig. 5 *A* show recordings obtained in the same oocyte during Zn washout. After complete washout of the agonists applied in trace 3, the oocyte was perfused in a Zn-free solution (10 μM DTPA). The initial amplitude of the response to the first agonist application after a 1.5-min Zn washout (*trace 4*) matched that recorded at the end of the Zn application (see Fig. 5 B_2 for a better resolution). The response then slowly increased until it reached a similar plateau to the Zn-free response. The next application of agonists (*trace 5*) induced a square response of amplitude similar to the plateau in trace 4.

Fig. 5 B_1 shows that the difference between traces 2 and 3 does not result from different Zn preincubation times: after a Zn preincubation of 10 min, the maximum amplitude of the response to the first Zn plus agonist application (*trace 6*) was almost indistinguishable from that measured in trace 2. This result indicates that, despite the continuous presence of Zn in the external solution for several minutes, none (if any) of the channels were blocked at the onset of the first agonist application. A similar observation was made by Huettner and Bean (1988) in a study of the block of NMDA receptors by MK801. We similarly conclude that the channels must first open to enable the blocker, Zn in our study, to reach its blocking site. One may argue that, in such a case, the peak in trace 2 should reach the amplitude of the response in trace 1. However oocyte perfusion systems have a slow time constant (~1–2 s for our setup), and as a

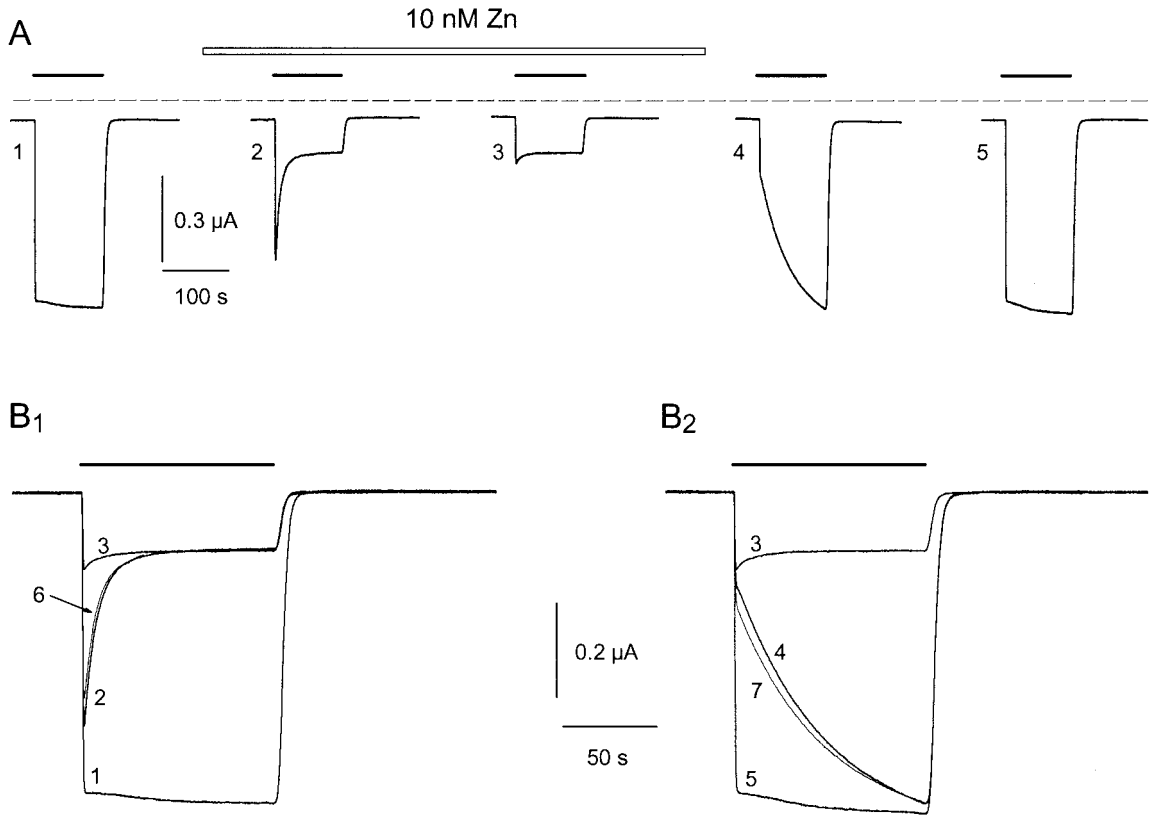

FIGURE 5 Zn binds to and dissociates from open NR1(NC)/NR2C(N_1C) receptors only. (*A*) Responses to five successive agonist applications (*solid bars*) recorded at −60 mV in an oocyte expressing NR1(NC)/NR2(N_1C) receptors. After complete recovery from the first agonist application (*trace 1*) performed under Zn-free conditions, 10 nM Zn was applied for 12 min (*open bar*). Two agonist applications were performed during the Zn application (*traces 2* and *3*) as well as during the subsequent Zn washout (*traces 4* and *5*). The dashed line indicates the zero current level. (B_1) Superimposed traces of the responses obtained before and during Zn application (*1*, *2*, and *3*; *traces* shown in *A*; *6*: first response obtained in the same oocyte after a 10-min preincubation in 10 nM Zn; this response has been corrected for run-down by interpolation of the maximum responses obtained under Zn-free conditions before and after Zn application). (B_2) Superimposed traces of the responses obtained at the end of the Zn application and during Zn washout (*3*, *4*, and *5*: *traces* shown in *A*; *7*: first response obtained in the same oocyte after a 10-min wash; response *7* has been corrected for run-down as indicated above).

consequence, some channels are open and blocked before the activation process reaches equilibrium.

Fig. 5 B_2 shows that the fact that most channels were still blocked at the beginning of the first agonist application during Zn washout (Fig. 5 *A*, *trace 4*) did not result from a short Zn-free preincubation. Increasing the duration of this preincubation to 10 min (Fig. 5 B_2, *trace 7*) did not significantly modify the first washout response. This result suggests that, as in the case of MK801 (Huettner and Bean, 1988), Zn is trapped in the closed channels.

Is there any inter-subunit disulfide bridge formation in the NR1NC/NR2CN_1C receptors?

With a nonreducing heavy metal chelator in the bath, the redox effect of DTE can be separated from its chelating properties. Fig. 6 *A* plots the amplitude of the responses of NR1(NC)/NR2(CN_1C) receptors to a series of agonist applications in the continuous presence of 10 μM DTPA in the bath. This particular oocyte showed very little response run-down. After 20 min under control conditions, 3 mM DTE was added in the bath for a total duration of 20 min. This strong reducing treatment did not affect the response amplitude. Replacement of DTE by 0.5 mM DTNB (a strong oxidizing agent) was similarly without effect.

We considered the possibility that the measured parameter (response amplitude) was not sensitive enough to detect the modification of disulfide bridges and repeated the experiment using as a parameter external Mg block, which has been shown to be strongly affected by modifications at the Q/R/N site. Fig. 6, *B* and *C*, shows the effect of 1 mM Mg on *I*/*V* curves of NR1(NC)/NR2C(N_1C) receptors obtained under Zn-free but different redox conditions with a voltage ramp protocol (see Materials and Methods). The records in Fig. 6 *B* were obtained 10 min after addition of 0.5 mM DTNB in the bath. DTNB was then replaced by DTE for 10 min and the records in Fig. 6 *C* collected. The two redox treatments failed to induce any significant change in the block by Mg. So far we have found no evidence suggesting

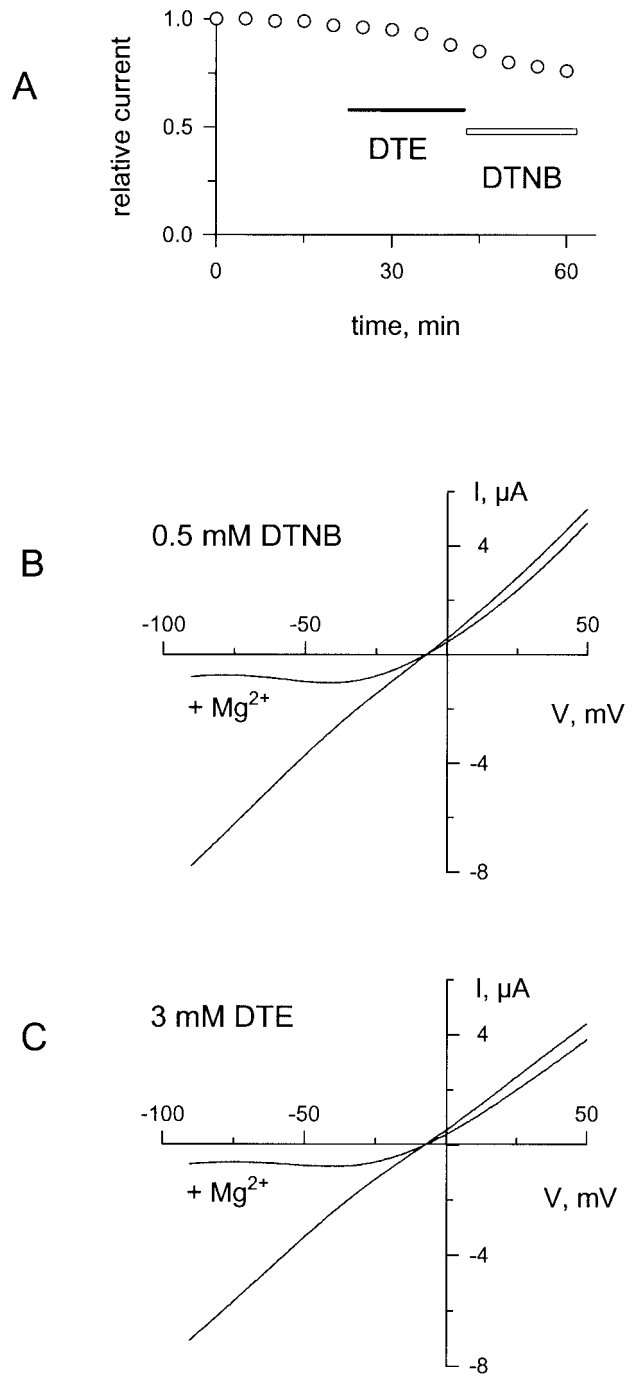

FIGURE 6 Under Zn-free conditions, redox reagents do not affect NR1(NC)/NR2C(N_1C) receptors. (*A*) The response of an oocyte expressing NR1(NC)/NR2C(N_1C) receptors was recorded every 5 min in the presence of 10 μM DTPA (at a steady voltage of -60 mV; response amplitude normalized to the first response). Neither 3 mM DTE nor 0.5 mM DTNB (each applied successively for 20 min) appears to affect significantly the response amplitude. (*B* and *C*) The presence of 10 μM DTPA, DTNB, and DTE has no effect on the voltage-dependent block of mutant receptors by external Mg. The block was measured using a voltage ramp protocol. After a 10-min application of 0.5 mM DTNB, 1 mM Mg induced a 90% inhibition of the current activated by the agonists at -90 mV (*B*). The block was not significantly different in the presence of 3 mM DTE applied subsequently for 10 min (*C*).

that inter-subunit disulfide bridges may form between cysteines at the Q/R/N site of NMDA receptors.

DISCUSSION

In this paper, we show that introducing cysteines at the Q/R/N site in all subunits of a NMDA receptor creates a Zn-inhibitory site of nanomolar affinity. As expected, given the known involvement of the Q/R/N site in the glutamate channel selectivity filter, Zn binding to the engineered inhibitory site is voltage dependent. The results also show that Zn access to and dissociation from this high-affinity site require prior opening of the NMDA channel.

The fact that substituting a cysteine for residues lining the pore of a cationic channel leads to a high-affinity inhibition of cation permeation by heavy metals is not surprising. The nucleophilic sulfur atom of the thiol group likes to coordinate electrophilic soft metals like Zn, Ni, and Cd (Glusker, 1991). In multimeric channel proteins, introduction of cysteines at homologous positions in the pore builds a ring of potential coordinating groups, which, if correctly positioned, can create a high-affinity heavy metal binding site. Long-lasting binding of a heavy metal cation to such a high-affinity site in the pore will block cationic permeation. Heavy metal binding sites in the pore of ionic channels do naturally occur or have already been engineered (Backx et al., 1992; Satin et al., 1992; Chiamvimonvat et al., 1996; Liu et al., 1997; Fahlke et al., 1998; Horenstein and Akabas, 1998). The particularity of the Zn site described in the present study is that it has such a high affinity that it is occupied most of the time if the traces of heavy metals contaminating the experimental solutions are not carefully removed. A similar situation was already described in the case of the voltage-independent Zn inhibition of wild-type NR1/NR2A NMDA receptors (Paoletti et al., 1997).

The possibility of engineering a high-affinity Zn blocking site at the Q/R/N site reveals new structural constraints on the NMDA receptor selectivity filter

Our results show that engineering a high-affinity Zn binding site at the Q/R/N locus of NMDA receptors requires the presence of a cysteine in both types of subunit. This implies that at least two cysteines, one from an NR1 subunit and one from an NR2 subunit are involved. Two structural models can be proposed to account for such a requirement: 1) lateral chains of cysteines from all subunits point toward the center of the pore and participate in a single Zn coordination site, and 2) two cysteines are sufficient to make a high-affinity site that would be located in the pore near a contact between two different subunits. In the latter case a receptor may contain more than one Zn site. The Hill coefficient of the Zn inhibition dose-response curve (see Fig. 3) as well as the

dependence on Zn concentration of the Zn inhibition kinetics (see Fig. 2 *B*) favor a bimolecular interaction of Zn with a single binding site. The Zn affinity for the cysteine-engineered Q/R/N site (~0.1 μM at 0 mV; see Fig. 4) is clearly intermediate between that of most metalloproteins (picomolar range, in which Zn is usually in a tetrahedral coordination; Glusker, 1991) and that observed in cysteine-containing pores of other channels (several tens to hundreds of micromolar; Backx et al., 1992; Satin et al., 1992; Chiamvimonvat et al., 1996; Liu et al., 1997; Fahlke et al., 1998). This is consistent with the fact that more than one cysteine is involved in the Zn binding site of NR1(NC)/NR2C(N_1C) receptors, but does not argue strongly for the simultaneous participation of four thiol groups.

Despite the fact that the (Q/R/N)+1 asparagine of NR2 subunits contribute to the selectivity filter (Kuner et al., 1996; Kupper et al., 1996, 1998; Wollmuth et al., 1996; Wollmuth et al., 1998), there is no high-affinity Zn blocking site in receptors containing NR2 subunits with a cysteine at the (Q/R/N)+1 position and NR1 subunits having a cysteine at position Q/R/N. The cysteine at the (Q/R/N)+1 position in NR2 has been shown to be accessible to 2-aminoethyl methanethiosulfonate (MTSEA) from the internal compartment (Kuner et al., 1996). The fact that this cysteine does not form a high-affinity Zn blocking site with cysteines at the Q/R/N position in NR1 suggests that these cysteines, although they are both accessible to the solvent, never come close enough to coordinate a Zn^{2+} ion together.

Voltage dependence of the high-affinity Zn block in NR1(NC)/NR2(CN$_1$C) receptors

In NR1(NC)/NR2C(N_1C) receptors, the high-affinity Zn block is voltage dependent, but its voltage dependence decreases with hyperpolarization (see Fig. 4). Such a phenomenon, which was also observed with the low-affinity voltage-dependent Zn block of wild-type NMDA receptors (Paoletti et al., 1997), is typical of permeant blockers: with enough hyperpolarization, dissociation from the binding site occurs toward the internal compartment and is accelerated by further hyperpolarization. This effect of voltage on the dissociation rate counteracts that on the blocker binding rate, resulting in a low sensitivity to voltage of the blocker apparent affinity at strongly hyperpolarized potentials.

The voltage dependence of Zn block increases with depolarization up to a limiting value above −40 mV (see Fig. 4). Above that potential, Zn dissociation presumably proceeds mainly toward the external compartment and Zn behaves like an impermeant blocker. The measured limiting voltage dependence is very similar to that of external Mg block (Ascher and Nowak, 1988). This suggests that blocking Zn^{2+} cations bind to a site located very close to the position reached by blocking Mg^{2+} cations, and we observed accordingly that Mg competes against Zn binding. That Mg blocks NMDA channels through binding at the Q/R/N site was suggested but not proved by previous mutagenesis work: the effects of mutations at the Q/R/N locus could have been either direct (the residues at the Q/R/N position participate in the coordination of blocking divalent cations) or indirect (the Q/R/N residues control in some way the shape of the selectivity filter binding sites, which involve other residues as coordinating groups). In NR1(NC)/NR2C(N_1C) receptors, the Zn binding site is made by the side-chain thiol groups of the cysteines introduced at the Q/R/N site. The location of this Zn binding site in the close vicinity of the Mg binding site favors therefore a direct involvement of the Q/R/N residues as coordinating groups in the external Mg blocking site. Our results, however, do not add new information about which part(s) of the Q/R/N asparagines (side chain or backbone) participate(s) in Mg binding.

The localization of the gate in the pore of the NMDA receptor must account for the state-dependent accessibility of the high-affinity Zn binding site

The large peak/plateau relaxation shown by the first response of NR1(NC)/NR2C(N_1C) receptors after a preincubation in the presence of Zn (see Fig. 5, *A* and B_1) indicates that Zn block does not occur in closed channels (or, at least, does not reach the same equilibrium as in open channels). Two possibilities could account for such a phenomenon: 1) the binding reaction is completely prevented by channel closure or 2) the apparent affinity for Zn becomes very weak in closed channels. The fact that a second agonist application in the continuous presence of Zn shows almost no relaxation (given that Zn block has reached equilibrium during the first response) suggests that there is no Zn dissociation during the interval between the two agonist applications and favors the first interpretation. This is confirmed by the Zn washout experiment (see Fig. 5, *A* and B_2), which demonstrates that Zn is actually trapped in the closed channel.

This state dependence of the Zn accessibility to its binding site suggests that a gate shuts the pore at or externally to the Q/R/N locus. Beck et al. (1999) have shown that in each subunit, several amino acid stretches contribute to the external vestibule: a 10-residue pre-TM1 fragment, the C-terminal half of TM3, and the first four residues of TM4. Using the substituted cysteine accessibility method, these authors found that closure of the receptors does not prevent accessibility to any of the external vestibule residues (except NR1-L544). Using the same method, Kuner et al. (1996) had previously concluded that the Q/R/N site is the most external residue of the TM2 pore loop. A first possibility to account for the state dependence of accessibility of the cysteines to Zn at the Q/R/N site is that closure involves the movement of a gate contributed by non-MTS-reactive residues of TM1, TM3 or TM4, which are located deeper in

the pore than the deepest MTS-reactive residues found by Beck et al. (1999). A second possibility is that the external gate is at the Q/R/N site itself. Channel closure would then involve a conformational change at that position, which may create a large energy barrier preventing passage of Zn (and permeant ions).

Given that Zn is a permeant blocker, the trapping result indicates the presence of an additional mechanism, either a second gate on the cytoplasmic side of the Q/R/N site or a conformation change affecting the Q/R/N cysteines upon closure of the channels that confers an extremely high affinity on the Zn binding site such that Zn will have no chance of dissociating toward the cytoplasmic compartment, despite the fact that no gate occludes the passage. Thanks to its very slow kinetic of dissociation from the pore of NR1(NC)/NR2C(N1C) receptors, Zn constitutes a promising probe in studying the gating mechanism of NMDA receptors.

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REFERENCES

Ascher, P., and L. M. Nowak. 1988. The role of divalent cations in the *N*-methyl-D-aspartate responses of mouse central neurones in culture. *J. Physiol.* 399:247–266.

Backx, P. H., D. T. Yue, J. H. Lawrence, E. Marban, and G. F. Tomaselli. 1992. Molecular localization of an ion-binding site within the pore of mammalian sodium channels. *Science.* 257:248–251.

Beck, C., L. P. Wollmuth, P. H. Seeburg, B. Sakmann, and T. Kuner. 1999. NMDAR channel segments forming the extracellular vestibule inferred from the accessibility of substituted cysteines. *Neuron.* 22:559–270.

Bers, D., C. Patton, and R. Nuccitelli. 1994. A practical guide to the preparation of Ca buffers. *Methods Cell Biol.* 40:3–29.

Burnashev, N., R. Schoepfer, H. Monyer, J. P. Ruppersberg, W. Günther, P. H. Seeburg, and B. Sakmann. 1992. Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor. *Science.* 257:1415–1419.

Chen, N., A. Moshaver, and L. A. Raymond. 1997. Differential sensitivity of recombinant *N*-methyl-D-aspartate receptor subtypes to zinc inhibition. *Mol. Pharmacol.* 51:1015–1023.

Chiamvimonvat, N., M. T. Perez-Garcia, R. Ranjan, E. Marban, and G. F. Tomaselli. 1996. Depth asymmetries of the pore-lining segments of the Na^+ channel revealed by cysteine mutagenesis. *Neuron.* 16:1037–1047.

Cornell, N. W., and K. E. Crivaro. 1972. Stability constant for the zinc-dithiothreitol complex. *Anal. Boichem.* 47:203–208.

Dingledine, R., K. Borges, D. Bowie, and S. Traynelis. 1999. The glutamate receptor ion channels. *Pharmacol. Rev.* 51:7–61.

Doyle, D. A., J. Morais Cabral, R. A. Pfuetzner, A. Kuo, J. M. Gulbis, S. L. Cohen, B. T. Chait, and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science.* 280:69–77.

Fahlke, C., T. H. Rhodes, R. R. Desai, and A. L. George, Jr. 1998. Pore stoichiometry of a voltage-gated chloride channel. *Nature.* 394: 687–690.

Glusker, J. P. 1991. Structural aspects of metal liganding to functional groups in proteins. *Adv. Protein Chem.* 42: 1–76.

Hille, B. 1992. Ionic Channels of Excitable Membranes, 2nd ed. Sinauer Associates, Sunderland, MA.

Hollmann, M., and S. Heinemann. 1994. Cloned glutamate receptors. *Annu. Rev. Neurosci.* 17:31–108.

Horenstein, J., and M. H. Akabas. 1998. Location of a high affinity Zn^{2+} binding site in the channel of alpha1beta1 gamma-aminobutyric acid A receptors. *Mol. Pharmacol.* 53:870–877.

Huettner, J. E., and B. P. Bean. 1988. Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. U.S.A.* 85:307–311.

Kuner, T., and R. Schoepfer. 1996. Multiple structural elements determine subunit specificity of Mg^{2+} block in NMDA receptor channels. *J. Neurosci.* 16:3549–3558.

Kuner, T., L. Wollmuth, A. Karlin, P. H. Seeburg, and B. Sakmann. 1996. Structure of the NMDA receptor channel M2 segment inferred from the accessibility of substituted cysteines. *Neuron.* 17:343–352.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. U.S.A.* 82:488–492.

Kupper, J., P. Ascher, and J. Neyton. 1996. Probing the pore region of recombinant *N*-methyl-D-aspartate channels using external and internal magnesium block. *Proc. Natl. Acad. Sci. U.S.A.* 93:8648–8653.

Kupper, J., P. Ascher, and J. Neyton. 1998. Internal Mg^{2+} block of recombinant NMDA channels mutated within the selectivity filter and expressed in *Xenopus* oocytes. *J. Physiol.* 507:1–12.

Liu, Y., M. Holmgren, M. E. Jurman, and G. Yellen. 1997. Gated access to the pore of a voltage-dependent K^+ channel. *Neuron.* 19:175–184.

Martell, A. E., and R. M. Smith. 1989. Critical Stability Constants, Vols. 1–6. Plenum, New York.

Mori, H., H. Masaki, T. Yamakura, and M. Mishina. 1993. Identification by mutagenesis of a Mg^{2+}-block site of the NMDA receptor channel. *Nature.* 358:673–675.

Paoletti, P., P. Ascher, and J. Neyton. 1997. High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *J. Neurosci.* 17:5711–5725.

Paoletti, P., J. Neyton, and P. Ascher. 1995. Glycine-independent and subunit-specific potentiation of NMDA responses by extracellular Mg^{2+}. *Neuron.* 15:1109–1120.

Sakurada, K., M. Masu, and S. Nakanishi. 1993. Alteration of Ca^{2+} permeability and sensitivity to Mg^{2+} and channel blockers by a single amino acid substitution in the *N*-methyl-D-aspartate receptor. *J. Biol. Chem.* 268:410–415.

Satin, J., J. W. Kyle, M. Chen, P. Bell, L. L. Cribbs, H. A. Fozzard, and R. B. Rogart. 1992. A mutant of TTX-resistant cardiac sodium channels with TTX-sensitive properties. *Science.* 256:1202–1205.

Tikhonov, D. B., B. S. Zhorov, and L. G. Magazanik. 1999. Intersegment hydrogen bonds as possible structural determinants of the N/Q/R site in glutamate receptors. *Biophys. J.* 77:1914–1926.

Traynelis, S. F., M. F. Burgess, F. Zheng, P. Lyuboslavsky, and J. L. Powers. 1998. Control of voltage-independent zinc inhibition of NMDA receptors by the NR1 subunit. *J. Neurosci.* 18:6163–6175.

Weber, W. M. 1999. Endogenous ion channels in oocytes of *Xenopus laevis*: recent developments. *J. Membr. Biol.* 170:1–12.

Williams, K. 1996. Separating dual effects of zinc at recombinant *N*-methyl-D-aspartate receptors. *Neurosci. Lett.* 215:9–12.

Wo, Z. G., and R. E. Oswald. 1994. Transmembrane topology of two kainate receptor subunits revealed by N-glycosylation. *Proc. Natl. Acad. Sci. U.S.A.* 91:7154–7158.

Wollmuth, L. P., T. Kuner, and B. Sakmann. 1998. Adjacent asparagines in the NR2-subunit of the NMDA receptor channel control the voltage-dependent block by extracellular Mg^{2+}. *J. Physiol.* 506:13–32.

Wollmuth, L. P., T. Kuner, P. H. Seeburg, and B. Sakmann. 1996. Differential contribution of the NR1- and NR2A-subunits to the selectivity filter of recombinant NMDA receptor channels. *J. Physiol.* 491: 779–797.