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Involvement of Divalent Ions in the Nitric Oxide-Induced Blockade of *N*-Methyl-D-aspartate Receptors in Cerebellar Granule Cells

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SUMMARY

We have previously shown that nitric oxide blocks the *N*-methyl-D-aspartate (NMDA) receptor without affecting the agonist binding site. We now report that in cerebellar granule cells nitric oxide decreases the NMDA channel conductance and open probability, in voltage-dependent and -independent manners, respectively, by acting on an extracellular site different from the redox, glycine, and pH modulatory sites of the receptor-channel complex. This inhibition is not additive with those of Mg^{2+} and Zn^{2+}. Moreover, removal of trace concentrations of metal ions in the external medium by means of metal ion-chelators significantly reduced the inhibitory action of nitric oxide on NMDA currents. These results indicate that divalent ions are required for the blockade of NMDA receptors by NO donors.

Glutamate is a major neurotransmitter of the mammalian central nervous system that stimulates two superfamilies of receptors, i.e., the metabotropic and ionotropic receptors. The glutamate metabotropic receptors are G protein-coupled receptors. The glutamate ionotropic receptors are receptor channels that have been named according to their most specific agonists, AMPA, kainic acid, and NMDA. Stimulation of these ionotropic receptors, and mainly the Ca^{2+}-permeable NMDA receptor subtype, results in activation of a neuronal, Ca^{2+}-dependent, constitutive NO synthase (1). NO has been shown to mediate excitatory amino acid-dependent neurotoxicity (2) and more subtle brain processes, such as synaptic plasticity (3, 4) and gene transcription (5).

We first showed that NO selectively blocks NMDA receptors in cultured cerebellar granule cells and striatal neurons (6), and this has been confirmed in cultured cortical neurons (7). Although we found that NO acted directly on the receptors, the mechanism of this action was not completely elucidated. An oxidative and irreversible effect of NO on the NMDA receptor complex has been proposed in neocortical neurons (7). In this model, NO would nitrosylate putative thiol residues of the redox modulatory site of the NMDA receptor, which then would form disulfide bonds through an oxidative process and stabilize the receptor in an inhibited state. On the other hand, other studies performed in cortical neurons have suggested that NO would not interact with the redox modulatory site of the NMDA receptors but rather would mobilize an unidentified, intracellular, Ca^{2+} homeostatic process (8). Thus, the mechanism of action of NO at the NMDA receptor remains controversial. The aim of the present study was to further examine this issue in cerebellar granule cells. These neurons were chosen because they have been shown to display a particularly elevated NO synthase activity upon NMDA receptor stimulation (9, 10).

In the studies mentioned above, NMDA responses were mainly examined by using intracellular Ca^{2+} monitoring and macroscopic NMDA current recordings. Here, we extended these investigations on NMDA single channels by using the outside-out configuration of the patch-clamp technique. We found that NO blocks NMDA receptors via voltage-dependent and -independent mechanisms, suggesting actions on the pore of the receptor-channel complex and on channel gating processes. Moreover, this NO-induced inhibition involved divalent ions.

Materials and Methods

Granule cell cultures. Primary cultures of granule cells were prepared as described previously (11). Briefly, cerebella were re-

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ABBREVIATIONS: AMPA, (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SNOC, *N*-nitrosocysteine; ISDN, isosorbide dinitrate; SNAP, *S*-nitrosopenicillamine; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; NEM, *N*-ethylmaleimide; SIN-1, 3-morpholinosydnonymine; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; NR1 or -2, *N*-methyl-D-aspartate receptor type 1 or 2.

moved from postnatal 6–7-day-old mice (Iffa-Credo, Lyon, France). The cells were gently dissociated in culture medium with a fire-narrowed Pasteur pipette, and the homogenate was centrifuged at 500 rpm. The pellet of cells was resuspended and plated (1.5×10^6 cells/dish) in 35-mm-diameter culture dishes (Falcon, Lincoln Park, NJ) that had been previously coated with poly-L-ornithine (15 μg/ml; M_r 40,000). Culture medium was composed of a 1:1 mixture of Dulbecco's minimal essential medium and F-12 nutrient, supplemented with 30 mM glucose, 25 mM KCl, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES buffer, decomplemented 5% calf serum and 5% horse serum (both from GIBCO), 100 IU/ml penicillin, and 100 μg/ml streptomycin.

The relatively high KCl concentration reduced glial cell outgrowth and improved neuronal survival. Under these conditions, these cultures have been shown to be highly enriched in neurons, which form many mature and functional synapses (11). Granule cells were identified as 5–10-μm round cells with thin long neurites. This cell type represented 95% of cells in our cultures. Recordings were performed after 7–10 days *in vitro*.

Electrical recordings and analyses. For patch-clamp recording, the culture medium was replaced by a solution containing 140 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 1 μM glycine, and 0.3 μM tetrodotoxin, pH 7.4. The recording pipette solution contained 140 mM CsCl, 0.5 mM $CaCl_2$, 5 mM EGTA, and 10 mM HEPES, pH 7.2, unless specified in the text or figure legends. Whole-cell and unitary currents were recorded using a List EPC-7 amplifier and were stored on VHS videotape after 16-bit digitization at 44 kHz, with a pulse code modulator (Sony PCM-701). Whole-cell currents were then filtered at 200 Hz and digitized at 1 kHz. Unitary currents were filtered at 1 kHz and digitized at 3 kHz. Analyses of these currents were performed using the pClamp 5.5 program (Axon Instruments). Macroscopic NMDA currents were measured at their initial peak amplitude (before desensitization of the response).

For single-channel recordings, the threshold for detection of opening and closing transitions was set at 50% of the open level of each event. Data were thus reduced to values corresponding to open time, close time, and amplitude. These values provided a way of calculating open probabilities (NP_o), open times, and amplitude distributions. For a given level of channel opening, the integrated time during which the channels stayed open was divided by the duration of the NMDA application and this calculation gave the value NP_o, where N is the putative number of recorded channels and P_o is the open probability of each channel. Mean amplitude of unitary currents was calculated using a Gaussian relation fitted to amplitude histograms by the least-squares method. For statistical analyses we used Student's t test, with $p \leq 0.05$.

Drug preparation and perfusion. NO was supplied by means of the NO donors SIN-1, SNOC, ISDN, and SNAP. Drug solutions were prepared in the aforementioned external medium and the pH was adjusted to 7.4. We previously showed that a 1 mM solution of SIN-1 continuously generates NO over a period of at least 8 hr, under physiological conditions (6). The SNOC solution was prepared by mixing $NaNO_2$ and cysteine (both at 100 mM) in acidified (pH 2) deoxygenated distilled water. The solution was then diluted 1000-fold in normal buffered incubation medium. We previously showed that this final solution still contained NO and did not modify the pH (7.4) of the buffered incubation medium (6).

Control and drug solutions (except SNOC, for reasons described below) were applied using a rapid perfusion system. The delay to reach concentration-clamp conditions was <30 msec. This was estimated as follows: a solution containing Cl^- ions was switched to a Cl^--free solution and the recorded direct current shift measured the change in diffusion at the tip of a recording pipette filled with Cl^-. Because of rapid desensitization of NMDA receptors, such a fast perfusion system was necessary to adequately record whole-cell NMDA currents.

The SNOC solution, when used 15 min after preparation, elicited inward currents that could be blocked by selective NMDA receptor antagonists (carboxypiperazin-4-yl-propyl-1-phosphonic acid or MK-801, both at 10 μM). Similar NMDA receptor agonistic effects on NMDA single-channel NP_o values were observed with the SNOC solution. Similar agonistic responses were also observed with pure cysteic acid-containing solution. Therefore, the agonistic effects of the SNOC solution probably resulted from the release of cysteine, which was rapidly oxidized to cysteic acid. Because of its short lifetime, SNOC could be tested only on outside-out patches, as follows. Fresh SNOC solution was prepared and rapidly applied, in the presence of NMDA, by means of a Pipetman. The effect of the NO donor on the NMDA single-channel conductance was then measured.

Peroxinitrite ($ONOO^-$) solution was prepared according to the method previously described by Hughes and Nicklin (12). Briefly, $ONOO^-$ was synthesized by adding H_2O_2 at acidic pH to $NaNO_2$. The reaction was quenched with NaOH and excess H_2O_2 was removed with activated MnO_2. The solution was filtered three times and stored at −80°. The $ONOO^-$ concentration (70 nM) of this stock solution was measured photometrically at 302 nm in 1 N NaOH.

Stable recordings could be obtained in the presence of external EDTA, TPEN, or EGTA for periods of time short enough (≤5 min) to preserve membrane integrity and long enough to measure the effects of these metal-chelators on NMDA currents, in the absence and presence of SIN-1.

NO production measurements. Production of NO by SIN-1 was measured using the method of Ignarro *et al.* (13), as described previously (6). Briefly, the method is based on diazotization of sulfanilic acid by NO, at acidic pH, followed by a coupling reaction with *N*-(1-naphthyl)ethylenediamine. This leads to the formation of a colored compound, the absorbance of which was measured by spectrophotometry.

cGMP measurements. cGMP formation was measured as described previously (6). Briefly, cultured neurons grown in 12-well dishes for 1–2 weeks were washed two times with 1 ml of Krebs bicarbonate buffer (3.5 mM KCl, 124 mM NaCl, 1.25 KH_2PO_4, 26.3 mM $NaHCO_3$, pH 7.4), pre-equilibrated with 95% O_2/5% CO_2, and then incubated for 5 min in this medium. Neurons were stimulated for 2 min with the indicated concentration of drugs in the same medium containing 1 mM 3-isobutyl-1-methylxanthine. The incubation was stopped by replacing the medium with 0.6 ml of 95% ethanol/5% formic acid. The cellular content of cGMP was determined by radioimmunoassay.

Materials. All divalent ions were used as chloride salts. Pharmacological agents used in this study were obtained from the following sources. NMDA receptor agonists and antagonists were from Tocris Neuramin (Bristol, England). SNAP was from TEBU (Le Perray en Yvelines, France), and SIN-1 and SIN-1C were generously donated by Hoechst Laboratories (Paris, France). TCEP was from Interchim (Asnieres, France). All other compounds were of the highest grade from Sigma (Saint-Quentin-Fallavier, France).

Results

Blocking effects of SIN-1 on NMDA currents. Bath application of SIN-1 decreased the amplitude of the whole-cell NMDA current recorded (Fig. 1A). The effect was dose dependent, with inhibitions of 17 ± 4% (mean ± standard error, n = 10), 44 ± 5% (n = 20), and 83 ± 7% (n = 10) with drug concentrations of 100 μM, 1 mM, and 10 mM, respectively, at a membrane potential of −80 mV. As shown in Fig. 1B the effect was present at all membrane potentials but was more pronounced at negative than positive potentials. The action of SIN-1 appeared within <1 sec, was rapidly reversible (<5 sec), was reproducible upon successive applications of the drug, and was antagonized by the potent NO-chelator hemoglobin (10 μM) (data not shown).

In outside-out patches, two effects of SIN-1 were observed,

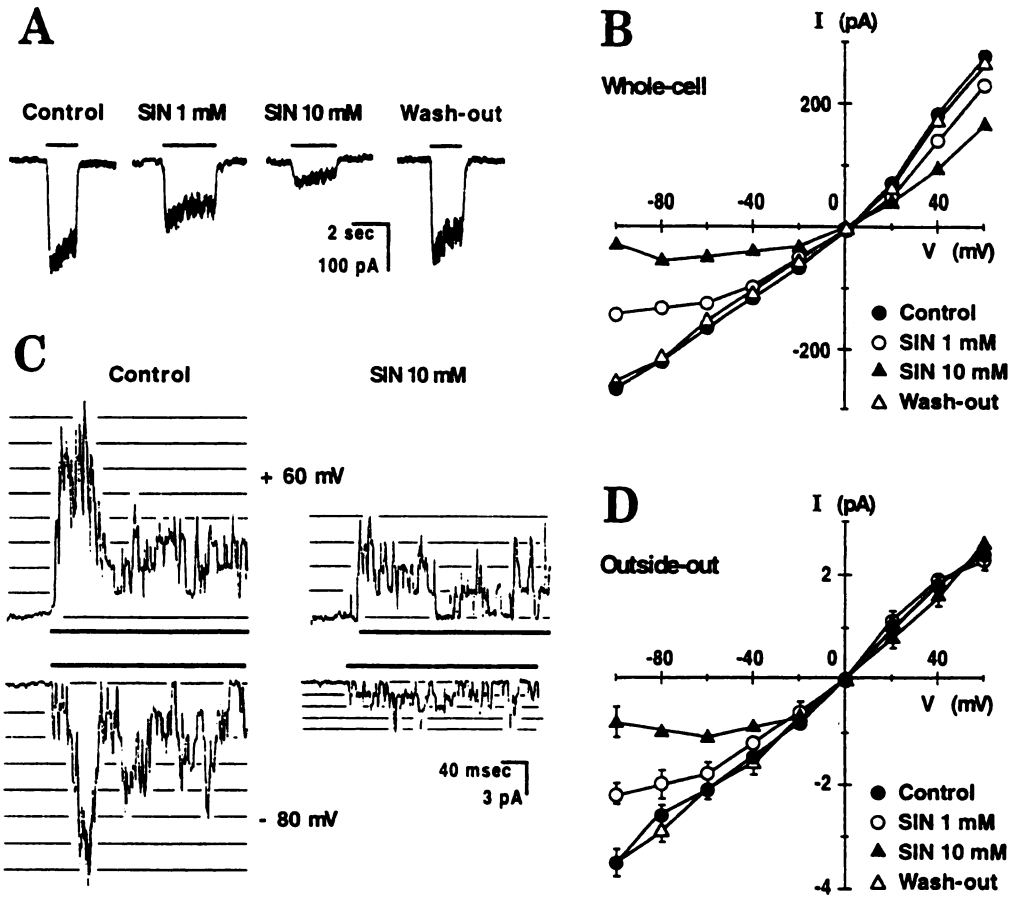

Fig. 1. SIN-1-induced reversible inhibition of NMDA receptors. A, Whole-cell currents elicited by NMDA (100 μM) applications (*horizontal bars*) at −80 mV. B, NMDA current-voltage relations, all obtained in the same cell. C, Outside-out recordings of unitary NMDA currents obtained at +60 mV (*upper traces*) and −80 mV (*lower traces*), under control conditions (*left traces*) and in the presence of 10 mM SIN-1 (*right traces*). *Thick horizontal bars*, NMDA (100 μM) applications. Note the decrease in the number of opening levels (*thin horizontal bars*) at both potentials and in the unitary current amplitude at −60 mV, in the presence of SIN-1. D, Current-voltage relations for NMDA unitary currents, obtained from five outside-out patches (mean ± standard deviation).

i.e., a voltage-independent decrease in the open probability (NP_o) of NMDA channels and a voltage-dependent decrease in their conductance. Thus, 51 ± 10% and 50 ± 13% (n = 11) decreases in NP_o were observed with 1 mM SIN-1 at +40 mV and −80 mV, respectively (Fig. 1C). SIN-1 also induced a reversible and dose-dependent reduction in the amplitude of unitary NMDA currents, but only at negative membrane potentials (Fig. 1C). Thus, decreases in NMDA channel conductance from 39 ± 2 pS (n = 30) to 27 ± 1 pS and 12 ± 2 pS were observed at −80 mV in the presence of 1 mM and 10 mM SIN-1 (n = 10), respectively (Fig. 1D), without any significant change in the mean open time of the channel (Fig. 2, A-D). This effect was reversed by hemoglobin (10 μM) (Fig. 2E).

Neither SIN-1C (up to 10 mM), the breakdown product of SIN-1 (14), nor the NO degradation products nitrite and nitrate (sodium salts, both at 10 mM) affected macroscopic or unitary NMDA currents. In addition to producing NO, SIN-1 has been reported to generate superoxide anions ($O_2^{\cdot -}$), which can react with NO and H^+ to generate $ONOO^-$ anions and hydrogen peroxide (H_2O_2), respectively (15). The $ONOO^-$ anions, at up to 700 μM, did not affect NMDA currents. Superoxide dismutase and catalase (100 units/ml each), which prevent $O_2^{\cdot -}$ and H_2O_2 accumulation, respectively, did not significantly modify the SIN-1 inhibitory effect on macroscopic NMDA currents. Moreover, H_2O_2, at up to 100 μM, did not significantly modify the effect of SIN-1 on these currents (data not shown). Together, these observations suggested that SIN-1 inhibited NMDA receptors through generation of NO.

Blocking effects of other NO donors on NMDA currents. SNAP (0.1 and 1 mM) and ISDN (10 mM) reproduced the effects of SIN-1 on macroscopic NMDA currents (Fig. 3, A and B). SNAP displayed potency similar to that of SIN-1, whereas ISDN was markedly less potent. SNAP and ISDN solutions left for at least 72 hr in open vials at room temperature, as well as penicillamine (1 mM), the breakdown product of SNAP, did not affect these currents. Because of technical difficulties (see Materials and Methods), SNOC could be tested only on unitary NMDA currents. A decrease in the single-channel conductance was observed, but only at negative potentials (Fig. 3C). Neither cystine nor cysteic acid (both at 1 mM), the breakdown products of SNOC (14), affected NMDA single-channel conductance.

Evidence that NO did not alter the redox, glycine, or pH modulatory sites of the NMDA receptor. Irreversible alkylation of the NMDA receptor sulfhydryl residues was performed by means of the following treatment. Neurons were exposed for 5 min to the reducing agent DTT (5 mM). They were then washed with normal solution for 5 min, treated for 3–5 min with NEM (5 mM), and finally washed

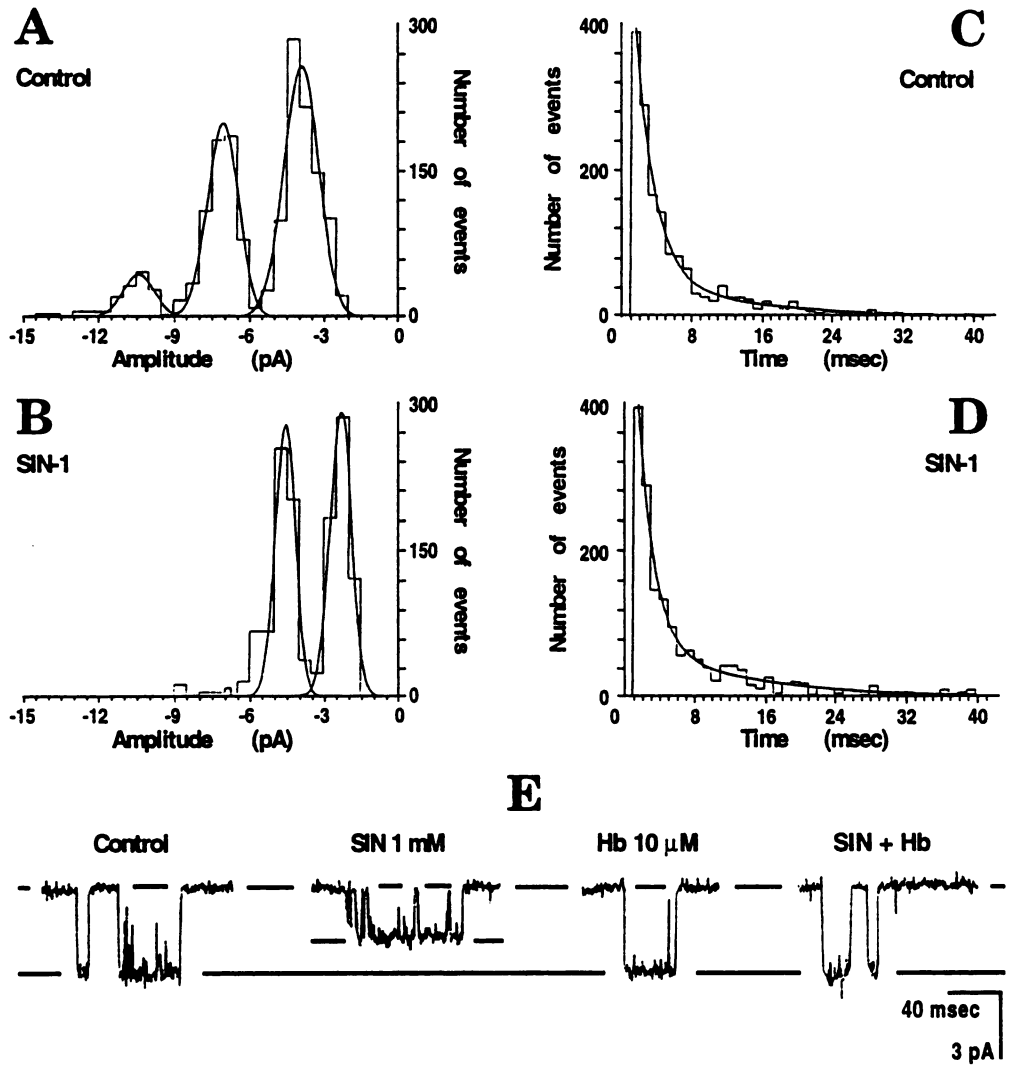

Fig. 2. Effects of SIN-1 on the conductance but not open time of NMDA channels. A–D, Amplitude (A and B) and open time (C and D) histograms for unitary NMDA currents recorded in the same outside-out patch at −100 mV, in the absence and presence of 1 mM SIN-1, as indicated. Peak amplitude values were −3.85 ± 0.7 pA, −7.06 ± 0.6 pA, and −10.39 ± 0.6 pA in A and −2.32 ± 0.4 pA and −4.57 ± 0.4 pA in B. The 0-mV peak amplitude level (closed level) was discarded from these histograms. Note the significant decrease in the amplitude of the first two levels in the presence of SIN-1, indicating a decrease in the conductance of the channels. The disappearance of the third open level in the presence of SIN-1 further indicates a decrease in the NP_o of the channel. Open time histograms were fitted with double-exponential curves of the following time constants: 2.33 msec and 13.86 msec (C) and 1.97 msec and 13.13 msec (D). E, NMDA unitary currents recorded from the same outside-out patch under the conditions indicated. The holding potential was −100 mV. Note the suppression of the SIN-1 inhibitory effect on the current amplitude by hemoglobin (*Hb*). Similar results were obtained in five other outside-out patches.

again with normal solution. Effective reduction of disulfide bonds was confirmed by the significant potentiation (51 ± 11% increase at −80 mV, $n = 5$) of whole-cell NMDA currents induced by this treatment (Fig. 4) (16, 17). This treatment did not significantly alter the inhibitory effect of SIN-1 on NMDA currents at either negative or positive potentials (Fig. 4; Table 1). Similar results were obtained with a TCEP/NEM (0.5 mM/1 mM, 12 min/5 min) treatment (data not shown).

Glycine, at micromolar concentration, interacts at an external allosteric site of NMDA receptors to potentiate NMDA responses in various preparations (18, 19). In our preparation, increasing the glycine concentration from 0.01 to 10 μM potentiated NMDA responses by 207 ± 12% ($n = 6$). Glycine, in the same cells, did not significantly modify the inhibitory effect of 1 mM SIN-1 (Table 1).

Acidic and alkaline solutions have been shown to inhibit and potentiate NMDA currents, respectively (20). In our cultures, switching from pH 7.4 to pH 6.8 or pH 8.0 decreased NMDA currents by 64 ± 9% or increased them by 85 ± 10%, respectively ($n = 5$). In the same cells, the inhibitory effect of SIN-1 on NMDA currents was not significantly modified by such treatments (Table 1).

Role of external divalent cations on NO-mediated inhibition of NMDA receptors. An inhibitory effect of Zn^{2+} on NMDA receptors has been described in cortical (21) and hippocampal (22) neurons. We found that it was also present in cerebellar granule cells. In our preparation, Zn^{2+} decreased whole-cell NMDA currents in voltage-dependent (data not shown) and dose-dependent (Fig. 5A) manners. As shown in Fig. 5, the inhibitory effects of this metal ion and SIN-1 were not additive. Thus, at a membrane potential of −80 mV, both 5 μM Zn^{2+} and 2 mM SIN-1 inhibited 45 ± 5%

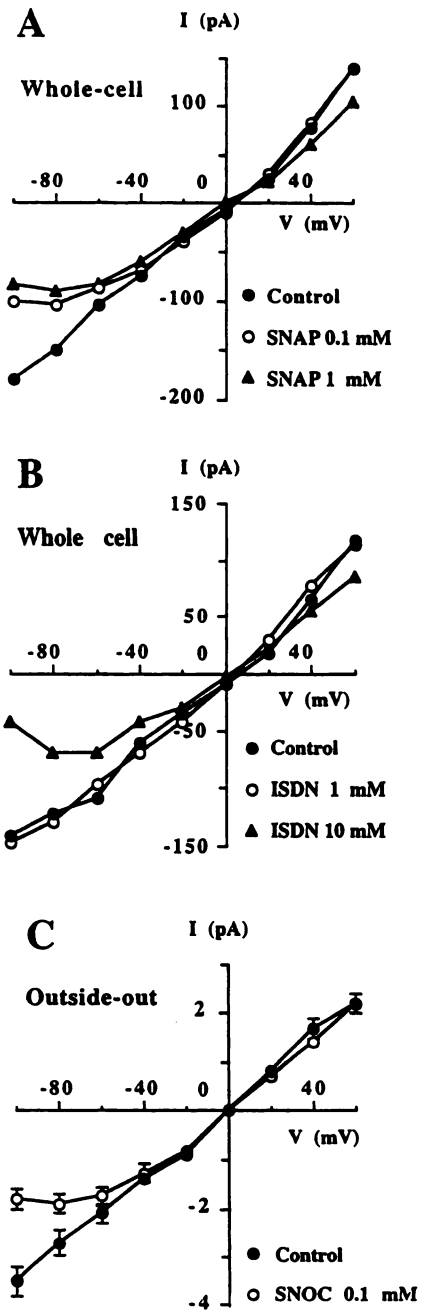

Fig. 3. Inhibition of NMDA receptors induced by various NO donors. Current-voltage relations for NMDA whole-cell (A and B) and unitary (C) currents obtained in the absence (controls) and presence of SNAP (A), ISDN (B), and SNOC (C) are shown. A and B were obtained from two different cells representative of 10 others. In C, each value represents the mean ± standard deviation of five experiments.

of the NMDA current when applied separately, in the same cells (n = 7). They blocked the NMDA current by 57 ± 5% when applied together in these cells. This inhibition was significantly smaller than the theoretical additive inhibition of Zn^{2+} and SIN-1 (90%) (Fig. 5A).

In cerebellar granule cells (present study), as in other preparations (23, 24), external Mg^{2+} decreased whole-cell NMDA currents in voltage-dependent (data not shown) and dose-dependent (Fig. 5B) manners. Similarly to Zn^{2+}, this inhibitory effect of Mg^{2+} was not additive with that of SIN-1 (Fig. 5B). Thus, in seven cells tested, 2 mM SIN-1 and 20 μM

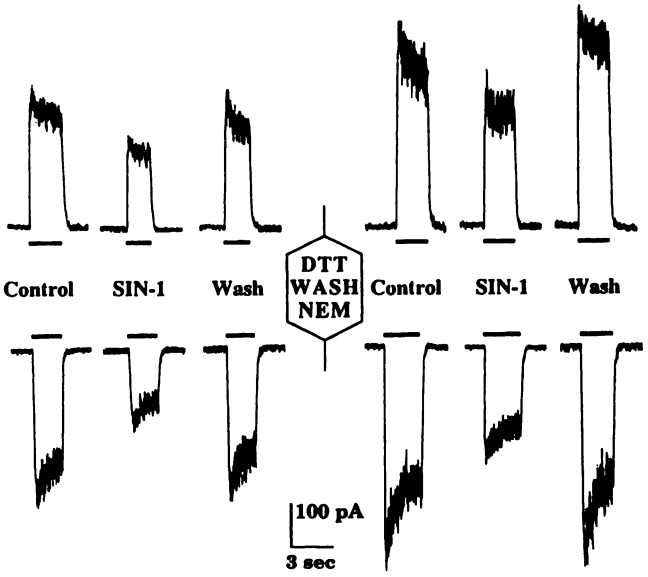

Fig. 4. Absence of interaction between NO and the redox modulatory site of the NMDA receptor. Whole-cell NMDA currents were recorded before (*left traces*) and after (*right traces*) a DTT/NEM treatment (5 mM/5 mM, 5 min/5 min), in the same cell, at +60 mV (*upper traces*) and −80 mV (*lower traces*). Note the slight increase in the control NMDA current after the DTT/NEM treatment, indicating a reduction of the receptor. Note that the inhibitory effects of SIN-1 (1 mM) were identical before and after the redox treatment. *Horizontal bars*, NMDA (100 μM) applications.

Mg^{2+} inhibited 46 ± 4% and 60 ± 3% of the NMDA current, respectively. When applied together in these same cells, these agents inhibited 67 ± 2% of the current, which was significantly different from the arithmetic sum of the inhibitory effects of Mg^{2+} and SIN-1 (100%) (Fig. 5B).

Fe^{2+}-containing solution, prepared with $FeSO_4$ (up to 30 μM), inhibited NMDA currents (up to 17 ± 3%, n = 7), in a dose-dependent manner (Fig. 5C). In the same cells, the inhibitory effect of SIN-1 (1 mM) on these currents was additive with the effect of Fe^{2+} ions (41 ± 3% inhibition with SIN-1 alone and 53 ± 4% inhibition with SIN-1 plus Fe^{2+}) (Fig. 5C). The mechanism of the blocking effect of Fe^{2+} alone on NMDA channels was not studied.

Requirement for external divalent ions for the NO-induced inhibition of NMDA receptors. The divalent ion-chelators EDTA (0.5 mM) (Fig. 6A), TPEN (0.1 mM) (Fig. 6B), and EGTA (0.5 mM) (data not shown) increased macroscopic NMDA currents at negative membrane potentials. These observations suggested the presence of trace concentrations of inhibitory divalent ions in our normal external solution. We compared the actions of SIN-1 on NMDA currents in this external medium (1 mM Ca^{2+} and no added Mg^{2+}) and in solutions (no Ca^{2+} and no Mg^{2+} added) where putative trace concentrations of divalent ions had been chelated by means of EDTA (0.5 mM), TPEN (0.1 mM), or EGTA (0.5 mM). The inhibitory effects of SIN-1 (1 mM) on macroscopic NMDA currents were markedly reduced in the presence of EDTA (Fig. 6A; Table 1) or EGTA (Table 1) and were almost abolished in the presence of TPEN (Fig. 6B; Table 1).

EGTA (data not shown) and TPEN (Fig. 6D) also slightly increased, in a voltage-dependent manner, the amplitude of unitary NMDA currents, without significantly affecting the NP_o values of the channels. We tested the effect of SIN-1 on NMDA channels under the same conditions as for NMDA

TABLE 1

Interactions between various NMDA receptor modulatory sites and SIN-1

Values represent percentage inhibitions of whole-cell NMDA currents induced by 1 mM SIN-1 under control and test conditions, at −80 mV. Control and test conditions are defined as follows. For glycine experiments, controls and tests were performed in the presence of 0.01 and 10 μM glycine, respectively. In redox experiments, control and test values were obtained before and after the DTT/NEM treatment described in the text. For pH experiments, controls were performed at pH 7.4 and tests at pH 6.8 and 8.0, in the same cells. A Ca^{2+}- and Mg^{2+}-free medium was used for EDTA, TPEN, and EGTA experiments. The control solution contained SIN-1 alone, and test solutions contained SIN-1 and either 0.5 mM EDTA, 0.1 mM TPEN, or 0.5 mM EGTA. For each column, control and test values were obtained from the same cells.

	Glycine	Redox	pH 6.8	pH 8.0	EDTA	TPEN	EGTA
Control (% inhibition)	47 ± 7	53 ± 6	46 ± 5	46 ± 5	42 ± 5	42 ± 5	40 ± 3
Test (% inhibition)	45 ± 5	61 ± 7	50 ± 6	41 ± 7	17 ± 3	6 ± 2	21 ± 3
Number of cells	6	5	5	5	6	6	8
Student's *t* test	NSD[a]	NSD	NSD	NSD	SD[b]	SD	SD

[a] NSD, not significantly different.
[b] SD, significantly different (Student's *t* test, $p \leq 0.05$).

macroscopic currents described above. The voltage-dependent inhibitory effects of SIN-1 on the amplitude of NMDA unitary currents were almost abolished in the presence of EDTA (Fig. 6C), EGTA (data not shown but similar to those for EDTA), or TPEN (Fig. 6D). The voltage-independent effect of SIN-1 on the NP_o value of NMDA channels was also significantly reduced in the presence of the ion-chelators. Hence, the SIN-1-induced decrease in NP_o was only 11 ± 4% (n = 5), 19 ± 5% (n = 5), and 3 ± 2% (n = 5) in the presence of EDTA, EGTA, and TPEN, respectively.

The production of NO by 2 mM SIN-1, measured at physiological pH in the absence of divalent cations, was not significantly modified by either EDTA (6 ± 3% decrease), TPEN (5 ± 3% decrease), or EGTA (3 ± 2% decrease, triplicates in three experiments). Consistent with this observation, the activation of guanylate cyclase by 30 μM SIN-1 (formation of 11 ± 1 pmol of cGMP/well under control condition) was not affected by either TPEN (formation of 14.5 ± 1 pmol of cGMP/well), EDTA (formation of 10 ± 1 pmol of cGMP/well), or EGTA (formation of 12.5 ± 1 pmol of cGMP/well, triplicates in two experiments).

Evidence that NO inhibits NMDA receptors by acting preferentially from outside the membrane. Whole-cell recordings were performed with patch pipettes filled with a solution containing 10 mM SIN-1, 0 mM EGTA, and 0 mM Ca^{2+}, to optimize the action of NO (Fig. 7). These cells were abundantly washed with normal external solution to eliminate NO molecules in the immediate extracellular environment. Under such conditions, the NMDA current slowly declined. After 15 min, the current amplitude measured at −80 mV stabilized at 48 ± 6% (n = 6) of the current amplitude recorded 30 sec after rupture of the membrane patch. We tested the effect of an external application of 10 mM SIN-1 in the same cells. SIN-1 inhibited the remaining NMDA current to 25 ± 5% of its original value. This effect appeared within the first 5 sec and was fully reversible upon wash-out of SIN-1.

Discussion

Two reversible actions of NO on NMDA channels of cerebellar granule cells were observed here, i.e., a voltage-dependent decrease in the channel ionic conductance and a voltage-independent blockade of gating processes. These effects of NO did not involve the redox, pH, or glycine modulatory sites of the receptor. On the other hand, our results suggest that NO may act on the receptor in association with divalent ions.

Among the various NO donors that we tested, SIN-1, SNAP, and ISDN appeared to be most suitable for the study of the mode of action of NO at NMDA receptors, because their breakdown products were inactive at these receptors. SIN-1 and SNAP were also the most potent in inhibiting NMDA currents. Hess *et al.* (25) have estimated that, 10 min after preparation, a solution of 1 mM SIN-1 yields a plateau of about 5 μM NO, under physiological conditions. Based on our previous studies, one can assume that such a final NO concentration should be stable for up to 8 hr (6). The NO donor glycerol trinitrate (data not shown) was not suitable for the present study because of a strong inhibitory effect of glycerol itself on the alcohol-sensitive inhibitory site of the NMDA receptor (26). We also observed that, at physiological pH, SNOC solution rapidly decomposes and displays potent NMDA receptor agonistic properties (see Materials and Methods).

Because the inhibitory effects of SIN-1 could be observed in excised patches, it is likely that NO inhibited NMDA currents by acting directly on the NMDA receptor-channel complex. This finding corroborated our previous conclusion (6). Here, we further examined whether the site of action of NO was in an outer or inner domain of the receptor. The slower and less potent effect of internally applied NO, compared with external application, suggested an external location for the site of action of NO on the NMDA receptor.

In cortical neurons, NO has been proposed by Lei *et al.* (7) to irreversibly block NMDA receptors by acting on the so-called redox modulatory site of the receptor. In the same neurons, Hoyt *et al.* (8) reported that ISDN had no direct effect on whole-cell NMDA currents, probably because the concentration of ISDN (1 mM) used in that study was not sufficiently high. However, the same concentration of ISDN was sufficient to block the NMDA-stimulated increase in the intracellular Ca^{2+} concentration. Those authors supported the hypothesis that this effect of NO was independent of the redox modulatory site of the NMDA receptor. We reached the same conclusion, on the basis of the following observations. (i) We found that reduction of disulfide bonds and irreversible alkylation of thiol groups of the redox modulatory site did not affect the inhibitory action of NO on NMDA currents. (ii) In our preparation, alterations of NMDA responses by NO were rapidly reversible, whereas redox treatments are slowly or not reversible (7, 27). (iii) We found that NO decreased the NMDA channel conductance without affecting its mean open time. On the other hand, modification of the redox state of the NMDA receptor changes the mean open time of the channel without affecting its conductance (17). (iv) We did not find

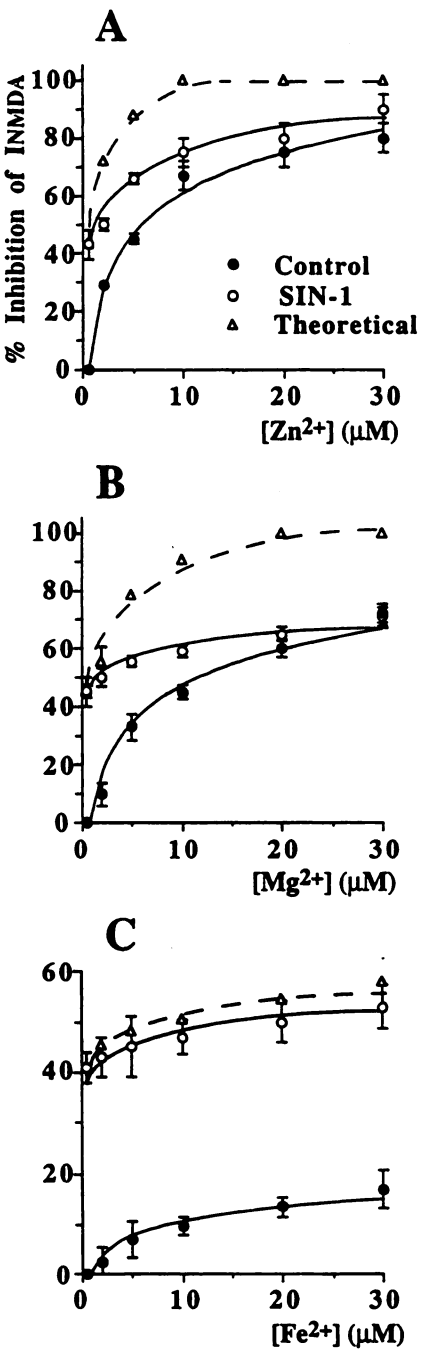

Fig. 5. Nonadditive inhibitory effects of SIN-1 and Zn^{2+}, Mg^{2+}, or Fe^{2+} on NMDA currents. The graphs show percentage inhibitions of whole-cell NMDA currents induced by increasing concentrations of Zn^{2+} (A), Mg^{2+} (B), or Fe^{2+} (C), in the absence (●) and presence (○) of 1 mM SIN-1. Each value represents the mean ± standard deviation of seven experiments. △, Theoretical additive inhibitions induced by SIN-1 and either Zn^{2+} (A), Mg^{2+} (B), or Fe^{2+} (C). Note the difference between the theoretical and experimental (○) curves in A and B but not in C.

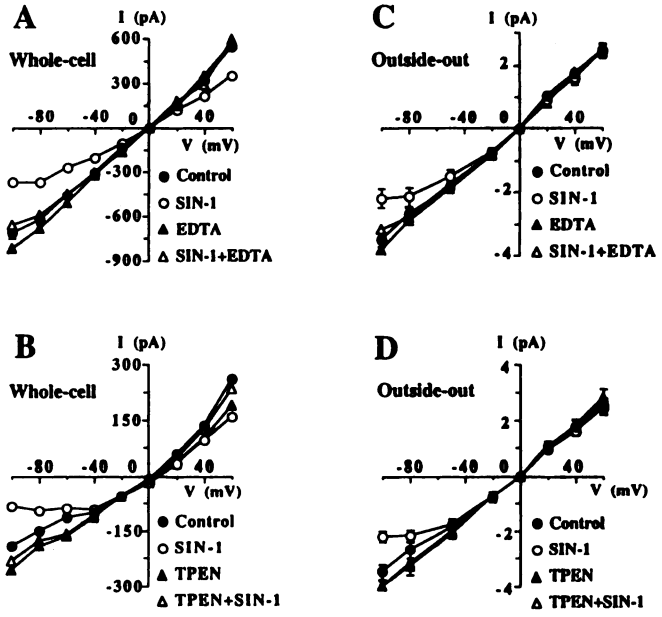

Fig. 6. Antagonism by EDTA (0.5 mM) and TPEN (0.1 mM) of SIN-1 (1 mM) inhibitory effects on macroscopic and unitary NMDA currents. Current-voltage curves for whole-cell (A and B) and unitary (C and D) NMDA currents were recorded under the different conditions indicated. A and B were obtained from two different cells representative of 10 others. In C and D, each value represents the mean ± standard deviation of six or five experiments, respectively.

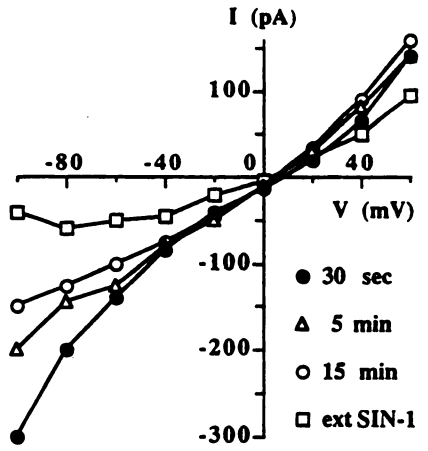

Fig. 7. Evidence for an extracellular site of action of NO on NMDA receptors. The current-voltage relations for whole-cell NMDA currents recorded with a patch pipette containing 10 mM SIN-1 and no EGTA/Ca^{2+} are shown. ●, Curve obtained 30 sec after the whole-cell configuration was established. △ and ○, Curves obtained from the same cell at the indicated times. □, Curve obtained 20 min after a 5–20-sec perfusion with 10 mM external SIN-1, in the same cell. Similar results were obtained in four other cells.

any significant effect of NO on the pH sensitivity (present study) or ligand binding affinity of the NMDA receptors (6), whereas these receptor properties are modified by mutation of the redox site of cloned NMDA receptors (28).

Different NMDA receptors are expressed in the mammalian brain. Each channel appears to be composed of the principal NR1 subunit and at least one of four NR2 subunits (NR2A, -2B, -2C, and -2D). The different NR1/NR2 receptors display distinct properties, regulation, and developmental distribution (29). The reducing agent DTT produces both reversible and irreversible potentiations of NR1/NR2A channels, whereas it elicits only irreversible potentiation of the NR1/NR2B and NR1/NR2C channels (28, 30). In both our preparation and cortical neurons (7, 16), DTT induced only a persistent potentiation of NMDA responses, suggesting expression of NMDA receptors with similar redox sites (NR1/NR2B and/or NR1/NR2C receptor types) in both preparations. What could explain these distinct modes of action of

NO in our preparation and that of Lei *et al.* (7)? One reason could be tissue-specific patterns of post-translational modification of NMDA receptors. Nevertheless, as mentioned above, Hoyt *et al.* (8), who used the same preparation as did Lei and colleagues, also found that NO did not interact with the redox site of NMDA receptors in these cells. A second explanation could be that different NO forms (NO·, NO^+, and NO^-) (31) were present under our experimental conditions and those of Lei and colleagues, due to the use of different NO donors and redox environments. However, like Lei and colleagues we used different types of NO donors. Moreover, the same NO donor, SNOC, was used in both studies. Given the discrepancies between these results, further speculation must await specific studies on mutated NMDA receptors.

We found that neither the glycine nor pH modulatory sites of the NMDA receptor were involved in the receptor blockade by NO. This conclusion is further supported by the fact that, unlike NO (present study), protons do not affect the NMDA channel conductance (22) and do modify the properties of the glycine modulatory site (20) in cerebellar granule cells.

NMDA receptors are sensitive to external divalent ions such as Zn^{2+} (21, 32) and Mg^{2+} (23, 24). According to those authors, Zn^{2+} ions induce both voltage-dependent and -independent inhibitory effects, whereas Mg^{2+} induces only a voltage-dependent block of the channel in various neuronal preparations. We found similar actions of these ions in cerebellar granule cells (data not shown). The voltage-independent block by Zn^{2+} is thought to involve an allosteric site located on the extracellular domain of the NMDA receptor, whereas the voltage-dependent inhibitory effects of both Zn^{2+} and Mg^{2+} may reflect a direct channel block.

Striking similarities exist between the voltage-independent actions of Zn^{2+} and NO. (i) Neither Zn^{2+} nor NO acted at NMDA channels as a classical open-channel blocker, because their effects were not entirely voltage and use dependent. This contrasts with typical NMDA channel blockers such as Mg^{2+} (23, 24) or MK-801 (33). (ii) Although both NO (34) and Zn^{2+} (35) preferentially interact with sulfhydryl and imidazole groups of cysteines and histidines (e.g., "zinc fingers"), neither of these agents seems to block NMDA receptors by interacting with exposed sulfhydryl groups, at least in cerebellar granule cells (Ref. 22 and present results). Although these comparisons *per se* do not at all prove a link between the sites of action of Zn^{2+} and NO, they strongly suggest that these agents block NMDA receptors via common mechanisms. We also found nonadditive inhibitory effects of NO and Mg^{2+} on NMDA responses. As for Zn^{2+}, this result suggests a common step in the action of NO and Mg^{2+}.

The substantial decrease in the inhibitory effect of SIN-1 observed after removal of external divalent ions by EDTA, EGTA, or TPEN indicates that divalent ions were required for this action of NO. TPEN was the most potent of these ion-chelators in inhibiting the action of NO on NMDA currents (Table 1). It is also the one that displays the highest pK_a values for heavy metal ions and the lowest pK_a values for Ca^{2+} and Mg^{2+} (Table 2). Conversely, EGTA was the least potent in antagonizing the action of NO (Table 1) and showed the lowest pK_a values for metal ions and the highest values for Ca^{2+} (Table 2). This suggests that trace concentrations of heavy metal ions were involved in this effect. The absence of additive effects of SIN-1 and Zn^{2+}, compared with the additive effects of SIN-1 and Fe^{2+}, suggested that zinc rather than iron was a likely candidate.

TABLE 2

Apparent pK_a values of metal ion-chelators for various divalent ions

Data are from Refs. 41–43.

	pK_a		
	EDTA	EGTA	TPEN
Mg^{2+}	5.4	5.2	1.7
Ca^{2+}	7.3	11.0	4.4
Mn^{2+}	10.7	12.3	10.3
Fe^{2+}	10.9	11.8	14.6
Zn^{2+}	13.1	12.9	15.6
Cu^{2+}	18.8	17.7	

Aqueous Fe^{2+} ions favor the conversion of NO^+ to NO· and of NO· to NO^-. Our iron-containing solutions did not modify the action of SIN-1, suggesting that the release of NO^+ by NO donors might not be a prerequisite for the inhibition of NMDA receptors.

An open-channel block of NMDA receptors by divalent cations has been shown to correlate with the water substitution rate in the inner coordination sphere of the divalent cation. The dehydration rate for Ca^{2+} (5×10^8 sec^{-1}) is much higher than those for Zn^{2+} (5×10^7 sec^{-1}) and Mg^{2+} (10^5 sec^{-1}) (36). Thus, Ca^{2+} permeates NMDA channels and Mg^{2+} induces a slow channel block, indicated by a flickering activity of the channel. Low concentrations of Zn^{2+} induce a fast channel block, so that unresolved blockages give rise to an apparent reduction in the channel conductance. As with Mg^{2+}, higher concentrations of Zn^{2+} (e.g., 100 μM) produce a flickering activity of NMDA channels (22). We found that these rules also apply to cerebellar granule cells (data not shown). It is worth noting that NO, like low concentrations of the fast open channel blocker Zn^{2+}, decreased NMDA unitary current amplitude in a voltage-dependent manner, without production of flickering activity.

The NO· and NO^- congeners can form metal-NO complexes (31, 37). Therefore, an attractive hypothesis would be that such complexes formed and behaved as open channel blockers on NMDA channels. However, we found additive effects of SIN-1 and Fe^{2+}, indicating that the action of SIN-1 was not potentiated by iron ions. This observation is in contradiction to the hypothesis of NMDA channel block by iron-NO complexes. Nevertheless, this does not exclude the possibility that another metal could be involved. Altogether, our findings support the hypothesis of an allosteric action of NO on the NMDA receptor facilitating both voltage-dependent and -independent receptor blockade by Zn^{2+}.

In conclusion, other studies have shown facilitation of both NMDA currents (38) and glutamate-induced neurotoxicity (39) by EDTA in cultured neurons, suggesting that activation of NMDA receptors induces neuronal release of divalent ions, which in turn block these receptors. Because NO is also released upon NMDA receptor stimulation, our observations suggest that NO blocks the NMDA receptors in combination with released divalent ions.

The voltage-dependent and -independent effects of NO on the NMDA receptor would provide an original negative feedback regulation of receptor activity, which could take place whatever the membrane potential of the neuron. The voltage-independent block of NMDA receptors by NO would be par-

ticularly interesting under conditions where the voltage-dependent block of NMDA receptors by Mg^{2+} is less effective (40). The ionic dependence of NO inhibitory effects on NMDA receptors may have some physiological importance, because both NO and Zn^{2+} can be present at some synapses.

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