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Zinc and Ifenprodil Allosterically Inhibit Two Separate Polyamine-Sensitive Sites at *N*-Methyl-D-Aspartate Receptor Complex[1]

MICHAEL L. BERGER and PATRICK REBERNIK

Institute of Biochemical Pharmacology, University of Vienna, Vienna, Austria

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ABSTRACT

In this study, we investigated the hypothesis that inhibition of the *N*-methyl-D-aspartate (NMDA) receptor complex by zinc involves a polyamine-sensitive regulatory site. We found that the specific binding of the open channel ligand [^{3}H]MK-801 to rat hippocampal membranes 1) was inhibited by low concentrations of Zn^{2+} (IC_{50} = 5.5 μM) by 65%. 2) This high-affinity component of inhibition was reversed by the polyamine spermine to an extent that could be reconciled with competitive interaction between Zn^{2+} and spermine. 3) Partial inhibition by Zn^{2+} was additive with partial inhibition by ifenprodil, an inhibitor of the NMDA receptor complex supposed to act at a polyamine-sensitive regulatory site, and 4) in membranes prepared from several other brain regions, inhibition of [^{3}H]MK-801 binding by Zn^{2+} and by ifenprodil was either less than additive, or superadditive. Our observation that ifenprodil, at concentrations saturating its high-affinity component of inhibition, prevented spermine from reversing the inhibition by Zn^{2+} indicates that spermine did not increase [^{3}H]MK-801 binding by competition with Zn^{2+} but rather via another polyamine regulatory site not sensitive to zinc but sensitive to ifenprodil. We conclude that Zn^{2+} reduces channel opening of the NMDA receptor complex by allosteric inhibition of a polyamine-sensitive regulatory site different from that inhibited by ifenprodil and that these two allosteric sites influence each other in a manner dependent on the brain region investigated. The different proportions of zinc/ifenprodil inhibition in different regions could reflect different percentages of various NMDA receptor subtypes.

Since the first topochemical demonstration of “free” (chelatable) Zn^{2+} in sharply delineated regions of the mammalian hippocampal formation (Maske, 1955), it has been firmly established that zinc is contained in synaptic vesicles of several neuronal pathways. Zinc has anticonvulsant and neuroprotective properties, but a better understanding of the molecular mechanisms of action of zinc appears to be necessary to take selective advantage from this knowledge. Thus, the beneficial effects of zinc in a number of seizure models are contrasted by its possible involvement in processes leading to neurodegeneration (for a review, see Choi and Koh, 1998). Notwithstanding the possible detrimental role of zinc in cerebral ischemia (Koh et al., 1996), it is in a well established animal model of cerebral ischemia in which zinc proved to be beneficial and neuroprotective (Matsushita et al., 1996).

At micromolar concentrations, which might be attained in the synaptic cleft during neuronal activity, Zn^{2+} has pronounced effects on ligand- and voltage-gated ion channels (Harrison and Gibbons, 1994). Particular attention has been devoted to the inhibitory effect of Zn^{2+} at the *N*-methyl-D-aspartate (NMDA) receptor complex mediated by an allosteric regulatory site near the external face of the membrane (Peters et al., 1987; Westbrook and Mayer, 1987). Specific binding of the open NMDA channel blocker [^{3}H]MK-801 to rat neuronal membranes (Wong et al., 1988; Yoneda and Ogita, 1989) is inhibited noncompetitively by micromolar concentrations of Zn^{2+} (Greenberg and Marks, 1988; Reynolds and Miller, 1988). Increasing the concentrations of the coagonists glutamate and glycine has only marginal effects on the inhibition of [^{3}H]MK-801 binding by Zn^{2+} (Reynolds and Miller, 1988), in agreement with the observation that the electrical responses of mouse cultured hippocampal neurons can be blocked by Zn^{2+} independently of the concentrations of NMDA and glycine used to stimulate the cells (Mayer et al., 1989). On the other hand, addition of the polyamine spermidine, which is supposed to increase the opening frequency of the NMDA channel via a separate polyamine-sensitive mechanism (Ransom and Stec, 1988; Williams et al., 1990; Rock and Macdonald, 1991; Benveniste and Mayer, 1993), greatly reduces the inhibitory effect of Zn^{2+} on [^{3}H]MK-801 binding (Enomoto et al., 1992; Reynolds, 1992). The hypothesis that Zn^{2+} interacts as negative modulator

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ABBREVIATIONS: CA, cornu ammonis; NMDA, *N*-methyl-D-aspartate; DG, dentate gyrus; MK-801, dizocilpine; N-12-N, 1,12-dodecanediamine with the same site at the NMDA receptor complex as the positive modulators spermine and spermidine was, however, rejected: the IC_{50} value of Zn^{2+} was not increased to the extent predicted for competitive interaction by increasing the concentration of the agonist spermidine.

Here, we reinvestigate the possibility that Zn^{2+} inhibits the NMDA receptor complex via a polyamine-sensitive regulatory site. We compared Zn^{2+} as an inhibitor of the NMDA receptor complex with three other compounds exhibiting polyamine-sensitive inhibition of the NMDA receptor complex: 1,12-dodecanediamine (N-12-N) (Berger et al., 1992), pentamidine (Reynolds and Aizenman, 1992), and ifenprodil (Carter et al., 1990).

Materials and Methods

Membrane Preparation. Triton-treated membranes were prepared from the hippocampal cornu ammonis 1 and dentate gyrus part (CA1/DG part, the region with the highest density of NMDA receptors) of adult male Wistar rats and stored at −80°C as described previously (Berger et al., 1992). For some experiments, membranes were prepared from the CA3 part of the hippocampus, the piriform cortex and the amygdala, dissected on a cold plate from the unfrozen brain as described previously (Berger et al., 1986), and from the parietal cortex, striatum, bulbus olfactorius, gyrus cinguli, and superior colliculi (also dissected from the unfrozen brain). No EDTA was included into the homogenization medium because in experiments performed in parallel, similar results were obtained with EDTA-treated and untreated membranes.

[^{3}H]MK-801 Binding Assay. Binding assays were performed in polypropylene vials (duplicates or triplicates) in 1.0 ml of 50 mM Tris acetate, pH 7.0, at 24°C. [^{3}H]MK-801 (5 nM, 23.9 Ci/mM; New England Nuclear Research Products, Boston, MA) was incubated for 2 h with glutamic acid (1 μM), glycine (1 μM), and various concentrations of spermine (1, 3, 10, 30, 100, and 300 μM; Serva, Heidelberg, Germany). Incubation times beyond 2 h did not result in any further increase in binding (this will occur only with buffer concentrations lower than 50 mM; unpublished observation). For nonspecific binding, glutamic acid and glycine were replaced by their respective antagonists, D-2-amino-5-phosphonovaleric acid (10 μM) and 5,7-dichlorokynurenic acid (1 μM; both from Tocris Cookson, Northpoint, UK). The incubation was started by adding membranes corresponding to approximately 1 mg fresh tissue and stopped by the addition of 3 ml (room temperature) 20 mM Tris-acetate, pH 7.0, and rapid filtration through Whatman (Hassel, Munich, Germany) GF/C filters presoaked for 1 h in polyethyleneimine (0.3% in H_2O), using a 48-place Brandel (Gaithersburg, MD) harvester. Filters were washed 3 times with 4 ml buffer (room temperature) and transferred into counting vials. After addition of 2.5 ml scintillation standard cocktail (Rotiscint 11; Roth, Karlsruhe, Germany), vials were warmed to 40°C, agitated for 1 h, and counted in a β-scintillation counter. Zinc acetate dihydrate (99.999%) and N-12-N were obtained from Aldrich-Chemie (Steinheim, Germany), pentamidine from Sigma Chemical Co. (St. Louis, MO). Ifenprodil was obtained from Tocris and also as a gift from Synthélabo Recherche (Bagneux, France).

Separation of High- and Low-Affinity Components of Inhibition. Inhibition of [^{3}H]MK-801 binding by Zn^{2+} consisted of two components, with IC_{50} values sufficiently different from each other (3–7 μM and 2.0 mM, see below), to allow nearly complete resolution of the two components. In several experiments, the high-affinity component was masked by 100 μM; in some others, it was masked by 300 μM Zn^{2+}. It can be calculated that under these conditions, only 0.9% to 2.7% of the high-affinity component remained unmasked (0.2–0.6% in presence of 300 μM Zn^{2+}; $n_H = 1.35$), whereas 95% of the low-affinity component was unaffected (87% in presence of 300 μM Zn^{2+}; $n_H = 1.00$). Also, inhibition of [^{3}H]MK-801 binding by ifenprodil consisted of two components, with IC_{50} values sufficiently different from each other (143–231 nM and 362 μM, see below). In several experiments, the high-affinity component was masked by 10 μM; in some others, it was masked by 30 μM ifenprodil. As can be calculated, under these conditions, only 1.7% to 2.7% of the high-affinity component remained unmasked (0.6–1.0% in the presence of 30 μM ifenprodil; $n_H = 0.95$), whereas 97% of the low-affinity component was unaffected (92% in the presence of 30 μM ifenprodil; $n_H = 1.00$).

Data Analysis. Monophasic and biphasic inhibition curves were subjected to computerized curve fitting (Johnson and Faunt, 1992). For the evaluation of biphasic inhibition curves, the Hill coefficient (n_H) for the low-affinity component was fixed to 1.0. In special situations (e.g., in the presence of 30 μM spermine), n_H of the high-affinity component also had to be fixed to 1.0 to avoid inconsistent results. ANOVA was applied to identify significant differences in the components of specific [^{3}H]MK-801 binding in several brain regions (*F* test; posthoc Newman-Keuls test). Computerized curve fitting was used for the determination of EC_{50} values of spermine stimulation of [^{3}H]MK-801 binding, allowing for $n_H \neq 1.0$ (usually $1.1 < n_H < 1.5$). For Schild plot analysis, $IC_{50}/K_i - 1$ was plotted double logarithmically against [spermine]/EC_{50} ($K_i = IC_{50}$ value in the absence of added spermine). To study the influence of ifenprodil on the IC_{50} value of Zn^{2+}, and vice versa, the influence of Zn^{2+} on the IC_{50} value of ifenprodil, the IC_{50} values were determined with and without the influencing agent within the same incubation and filtration procedure. The results of four separate experiments were evaluated by Student's paired *t* test (3 df).

Results

Biphasic Inhibition of [^{3}H]MK-801 Binding by Zn^{2+}. Low concentrations of Zn^{2+} displaced 65 ± 5% of specifically bound [^{3}H]MK-801 from membranes prepared from the CA1/DG part of the rat hippocampus (Fig. 1A, Table 1); the remainder was inhibited by millimolar Zn^{2+}. Increasing the concentrations of glutamic acid and glycine from 1 to 10 μM did not change the IC_{50} value of Zn^{2+} (high-affinity component: 11.9, 6.6 μM with 1 μM; 11.8, 6.5 μM with 10 μM glutamic acid and glycine, $n = 2$). Spermine shifted the high-affinity component to higher IC_{50} values but not the low-affinity component (Fig. 1A and Table 1). In the absence and in the presence of spermine, the inhibition curves were steep (see $n_H > 1$ in Table 1). Spermine (10 μM) (i.e., 2.93 times its EC_{50} for stimulation of [^{3}H]MK-801 binding in these experiments) shifted the high-affinity IC_{50} value by a factor of 5.0 ± 3.1 (range, 2.4–10.4) (i.e., by a factor compatible with competitive interaction between Zn^{2+} and spermine). In Fig. 2, this relationship (in the form of a Schild plot analysis) is compared with results obtained with two compounds inhibiting the NMDA receptor complex via a polyamine-sensitive mechanism: N-12-N (Berger et al., 1992) and pentamidine (Reynolds and Aizenman, 1992). Only the results obtained with Zn^{2+} scatter around a correlation line with unity slope. Linear correlation analysis resulted in the following slopes (±S.D.): 1.05 ± 0.11 (for Zn^{2+}), 0.89 ± 0.03 (for N-12-N), and 0.62 ± 0.04 (for pentamidine), which are significantly different from each other ($p < .001$, ANOVA). All IC_{50}, EC_{50}, and K_i values have been obtained by computer analysis of several independent experiments. In the case of Zn^{2+}, computer analysis had to operate on a greater number of parameters than in the case of N-12-N and pentamidine due to the existence of a high- and a low-affinity component; this might explain the relatively high extent of scattering in the Zn^{2+} data.

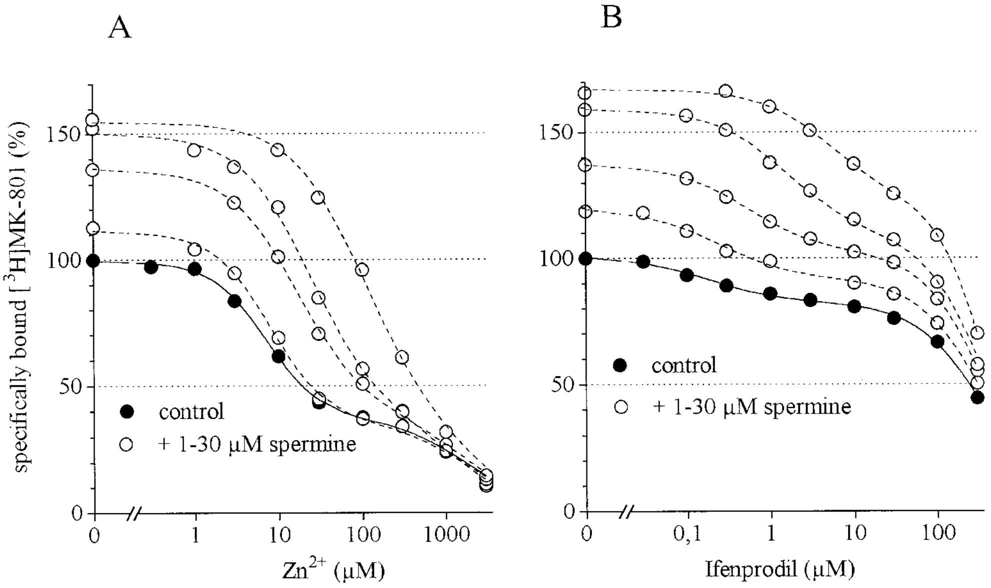

Fig. 1. Inhibition of specific [^{3}H]MK-801 binding to membranes prepared from the CA1/DG part of rat hippocampus (1 μM glutamic acid and glycine) by Zn^{2+} (A) and by ifenprodil (B). Mean values of normalized data were pooled from six independent experiments; 100% is 40.8 ± 2.6 fmol/mg tissue in A and 38.7 ± 4.8 fmol/mg tissue in B (mean ± S.D.). Influence is shown of increasing concentrations of spermine (1, 3, 10, and 30 μM, ○).

TABLE 1

Inhibition of [^{3}H]MK-801 binding to rat hippocampal membranes (CA1/DG) by Zn^{2+} and by ifenprodil

Values are mean ± S.D., with the number of experiments in parentheses. The mean EC_{50} value for spermine stimulation of [^{3}H]MK-801 binding has been 3.41 ± 0.90 μM (4) for experiments with Zn^{2+} and 2.76 ± 0.90 μM (7) for experiments with ifenprodil.

	Extent of Inhibition		IC_{50}		n_H	
	Control	10 μM Spermine	Control	10 μM Spermine	Control	10 μM Spermine
	fmol/mg tissue		μM			
Inhibition by Zn^{2+}						
High-affinity component	26.4 ± 2.4 (8)	40.6 ± 3.2 (8)[b]	5.5 ± 1.6 (8)	24.4 ± 8.6 (8)[b]	1.35 ± 0.22 (8)	1.29 ± 0.14 (4)
Low-affinity component	14.4 ± 3.4 (8)	17.2 ± 4.2 (8)[a]	1970 ± 910 (6)	1560 ± 1010 (6)	N.D.	N.D.
Inhibition by ifenprodil						
High-affinity component	7.6 ± 2.3 (11)[d]	17.4 ± 4.1 (11)[b,d]	0.18 ± 0.08 (11)	1.09 ± 0.52 (11)[b]	0.95 ± 0.21 (8)[c]	1.06 ± 0.34 (10)
Low-affinity component	29.5 ± 5.1 (11)[d]	40.2 ± 6.4 (11)[b,d]	362 ± 81 (9)	330 ± 93 (9)	N.D.	N.D.

[a] $P < .01$, [b] $P < .001$, significantly different from control (paired Student's t test). [c] $P < .01$, [d] $P < .001$, significantly different from corresponding value obtained with Zn^{2+} (ANOVA).

N.D., not determined (for computerized curve fitting, the Hill coefficient n_H was set equal to 1.0).

Biphasic Inhibition of [^{3}H]MK-801 Binding by Ifenprodil. Ifenprodil displaced only 20.7 ± 7.0% of specifically bound [^{3}H]MK-801 (CA1/DG membranes) with high affinity (Fig. 1B, Table 1); the remainder was inhibited by high micromolar concentrations. The addition of spermine shifted the high-affinity component to higher IC_{50} values but not the low-affinity component (Fig. 1B, Table 1). The Hill coefficients n_H did not deviate significantly from unity, neither without nor with 10 μM spermine (Table 1). The shift of the high-affinity IC_{50} value by spermine was compatible with competitive interaction: 10 μM spermine (i.e., 3.62 times its EC_{50} value for stimulation of [^{3}H]MK-801 binding in these experiments) shifted the IC_{50} value by a factor 6.2 ± 2.3 (range, 4.1–11.2). Schild plot analysis of the dependence of the IC_{50} value on a more extended range of spermine concentrations yielded data with an even higher degree of scattering than observed with zinc (not shown). Obviously, it is more difficult to obtain accurate data on a relatively small high-affinity component (as in the case of inhibition by ifenprodil) than on a high-affinity component representing the main effect of the inhibitor (as in the case of zinc).

Additivity of Inhibition by Zn^{2+} and by Ifenprodil. Figure 3 illustrates that in the CA1/DG part of the hippocampus, inhibitions of [^{3}H]MK-801 by Zn^{2+} and by ifenprodil were additive. Low concentrations of ifenprodil (up to 10 μM) inhibited the same fraction, in the absence (Fig. 3A, shaded) and in the presence (Fig. 3B, shaded area) of 100 μM Zn^{2+}. Similarly, the fraction inhibited by low Zn^{2+} concentrations (up to 100 μM) did not change after the addition of 10 μM ifenprodil (arrows in Fig. 3).

Inhibition of [^{3}H]MK-801 Binding by Zn^{2+} and by Ifenprodil in Other Brain Regions. In all brain regions analyzed, inhibition of [^{3}H]MK-801 binding by Zn^{2+} and by ifenprodil was biphasic. The IC_{50} value for the high-affinity components did not vary between the regions by more than a factor of 2 (for neither inhibition by Zn^{2+} nor inhibition by ifenprodil; Table 2). Also, the corresponding n_H values were similar in all regions. However, the regions differed from each other in the extent to which [^{3}H]MK-801 binding was sensitive to low concentrations of either Zn^{2+} or ifenprodil. For example, from piriform cortex membranes, only 40.1% of specifically bound [^{3}H]MK-801 was displaced by 100 μM

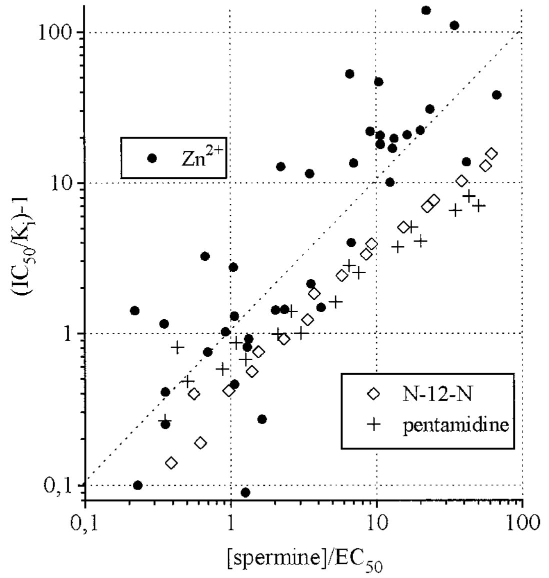

Fig. 2. Spermine-stimulated [^{3}H]MK-801 binding showing Schild plot analysis of inhibition by Zn^{2+} (●, high-affinity component only), by 1,12-dodecanediamine (N-12-N, ◇), and by pentamidine (crosses). The relationship indicated by the dotted diagonal is predicted for simple competitive interaction between the inhibitor and spermine.

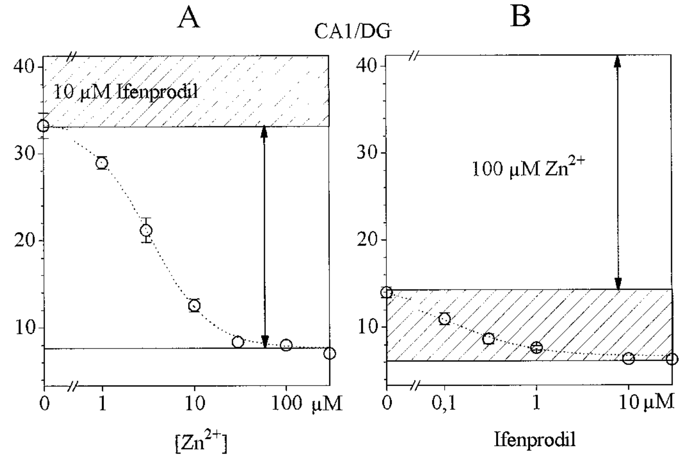

Fig. 3. Additivity of the inhibition of [^{3}H]MK-801 binding by Zn^{2+} and by ifenprodil in membranes from hippocampal CA1/DG part. Data are representative for two independent experiments performed in triplicate (fmol/mg tissue); bars indicate S.D. Displacement curves by Zn^{2+} yielded the same high-affinity component under control conditions (arrow in B) as in the presence of 10 μM ifenprodil (arrow in A). In separate experiments, displacement curves obtained with various concentrations of ifenprodil revealed the same high-affinity component under control conditions (shaded in A) as in the presence of 100 μM Zn^{2+} (shaded in B).

Zn^{2+} (Table 3, column B), but 74.1% was displaced from gyrus cinguli membranes. Also, the sensitivity to 10 μM ifenprodil varied among the regions, from 20.6% (CA3) to 39.7% (bulbus olfactorius; Table 3, column E).

Pronounced interregional variability was also observed for a fraction of [^{3}H]MK-801 binding exhibiting neither high sensitivity to Zn^{2+} nor high sensitivity to ifenprodil. It can be calculated that 100 μM Zn^{2+} (IC_{50} = 5.5 μM, Table 1) should displace 98% of the component sensitive to Zn^{2+} from sites on CA1/DG membranes and that 10 μM ifenprodil also should displace 98% of the component with high sensitivity to ifenprodil (IC_{50} = 183 nM, Table 1). Nevertheless, computer analysis of biphasic inhibition by ifenprodil in the presence of 100 μM Zn^{2+} and of biphasic inhibition by Zn^{2+} in the presence of 10 μM ifenprodil yielded low-affinity components of around 10% (columns D and G in Table 3). With membranes prepared from the piriform cortex, computer analysis revealed that, as a mean of three experiments (±S.D.), 22.5 ± 2.5% of specific [^{3}H]MK-801 binding was not displaced by Zn^{2+} with high affinity in the presence of 10 μM ifenprodil and that 24.0 ± 2.5% of specific [^{3}H]MK-801 binding was not displaced by ifenprodil with high affinity in the presence of 100 μM Zn^{2+}. As can be calculated, under these circumstances, only 2.8% to 4.6% of specifically bound [^{3}H]MK-801 should have remain bound if zinc- and ifenprodil-sensitive components add up to 100%.

Apparent Deviation from Additivity in Many Brain Regions. In contrast to the results obtained with membranes prepared from the CA1/DG part of the hippocampus, inhibition by Zn^{2+} and by ifenprodil was apparently nonadditive in several other brain regions. For example, in the piriform cortex, 40.1% of specific [^{3}H]MK-801 binding was sensitive to 100 μM Zn^{2+} [i.e., 11.9 fmol of 29.7 fmol specifically bound (mean value of three experiments, Table 3, column B; Fig. 4B, arrow)]. However, in the presence of 10 μM ifenprodil, Zn^{2+} displaced with high affinity 16.9 fmol specifically bound [^{3}H]MK-801 (56.8%, Table 3, column F; Fig. 4A, arrow); this is significantly more than 11.9 fmol ($P < .001$). Micromolar concentrations of Zn^{2+} had a similar effect on the ifenprodil sensitivity of [^{3}H]MK-801 binding to piriform cortex membranes. Without Zn^{2+}, only 20.7% (i.e., 6.15 fmol) could be inhibited by 10 μM ifenprodil (Table 3, column E; Fig. 4A, shaded), but in the presence of Zn^{2+}, this fraction amounted to 35.9% (column C; i.e., 10.7 fmol; Fig. 4B, shaded), significantly more than 6.15 fmol ($P <$ 001). Thus, in the piriform cortex, ifenprodil increased the fraction of bound [^{3}H]MK-801 sensitive to low Zn^{2+}, and Zn^{2+} increased the fraction of bound [^{3}H]MK-801 sensitive to low ifenprodil. In membranes prepared from the amygdala (no significance) and from the hippocampal CA3 part (weak significance), a tendency into the same direction could be observed (Table 3), but membranes prepared from several other regions demonstrated opposite relationships. In the gyrus cinguli, one of the most extreme examples (one of three experiments is illustrated in Fig. 4, C and D), the fraction of [^{3}H]MK-801 binding sensitive to low Zn^{2+} was significantly reduced by ifenprodil, from 74.1% (i.e., 13.9 fmol, arrow in Fig. 4D) to 58.6% (i.e., 11.0 fmol, $P < .001$; Table 3; Fig. 4C, arrow), and the fraction sensitive to low ifenprodil was reduced by Zn^{2+} from 35.3% (i.e., 6.64 fmol; shaded in Fig. 4A) to 18.0% (i.e., 3.38 fmol, $P < .001$, Table 3; Fig. 4D, shaded).

Mutual Elimination of Spermine Sensitivity. In four experiments, the sensitivity of the inhibition of [^{3}H]MK-801 binding by Zn^{2+} to spermine (i.e., the factor, by which the IC_{50} value of the high-affinity component was increased by the addition of 10 μM spermine) was determined simultaneously in the absence and in the presence of 30 μM ifenprodil. In the absence of ifenprodil, the addition of 10 μM spermine resulted in a 4-fold shift of the high-affinity IC_{50} value of Zn^{2+} (in agreement with data given in Table 1). Ifenprodil (30 μM) not only reduced the stimulatory effect of spermine but also almost eliminated the spermine-induced shift in the IC_{50} value of Zn^{2+} (to 1.49-fold; Table 4 and Fig. 5A). In four other experiments, the sensitivity of the inhibition of [^{3}H]MK-801 binding by ifenprodil to spermine was

TABLE 2

Inhibition of [^{3}H]MK-801 binding by Zn^{2+} (high-affinity component) in presence of 10 μM ifenprodil and by ifenprodil (high-affinity component) in presence of 100 μM Zn^{2+} in nine different rat brain regions

IC_{50} values and Hill coefficients (n_H) are given as mean ± S.D. (number of experiments in parentheses).

Region	Inhibition by Zn^{2+} in Presence of 10 μM Ifenprodil		Inhibition by Ifenprodil in Presence of 100 μM Zn^{2+}	
	IC_{50}	n_H	IC_{50}	n_H
	μM		*nM*	
Piriform cortex	6.69 ± 0.73	1.12 ± 0.09	231 ± 49	0.94 ± 0.05 (3)
Amygdala	7.47 ± 1.29[a]	1.13 ± 0.14	155 ± 9	0.92 ± 0.20 (4)
CA3	6.20 ± 0.87	1.34 ± 0.06	187 ± 56	0.78 ± 0.14 (3)
Parietal cortex	4.72 ± 0.72[b]	1.26 ± 0.08	160 ± 17	0.87 ± 0.15 (3)
CA1/DG	3.84, 3.30	1.37, 1.34	157, 255	0.83, 0.75 (2)
Striatum	5.27 ± 0.49	1.42 ± 0.06	168 ± 29	0.86 ± 0.10 (3)
Bulbus olfactorius	4.82 ± 1.40[b]	1.15 ± 0.06	147 ± 25	0.96 ± 0.17 (3)
Gyrus cinguli	4.34 ± 0.69[b]	1.29 ± 0.03	143 ± 113	0.94 (2) (3)
Superior colliculus	3.12, 3.94	1.37, 1.35	83, 202	0.98, N.D. (2)

[a] Significantly higher than values designated with [b] ($P < .05$, ANOVA).
N.D., not determined (for computer analysis, n_H was set equal to 1.0).

TABLE 3

Components of specific [^{3}H]MK-801 binding sensitive to low concentrations of Zn^{2+} (1–100 μM, columns B and F), to low concentrations of ifenprodil (0.03–10 μM, columns C and E), and to neither of these (columns D and G) in nine different rat brain regions

Values are mean ± S.D. (n). Values in columns B–G represent percentage of corresponding value in column A. Values in columns B–D and in columns E–G sum up to 99% to 101%.

Region	Specific Binding	Percentage Inhibition by 100 μM Zn^{2+}	Percentage Inhibition by Ifenprodil in Presence of 100 μM Zn^{2+}		Percentage Inhibition by 10 μM Ifenprodil	Percentage Inhibition by Zn^{2+} in Presence of 10 μM Ifenprodil	
			High-Affinity Component	Low-Affinity Component		High-Affinity Component	Low-Affinity Component
	A	B	C	D	E	F	G
	fmol/mg tissue						
Piriform cortex	29.7 ± 2.3 (3)	40.1 ± 3.8	35.9 ± 1.6[b]	24.0 ± 2.5	20.7 ± 2.2	56.8 ± 2.0[d]	22.5 ± 2.5
Amygdala	21.4 ± 0.9 (4)	53.9 ± 6.2	32.3 ± 2.8	13.7 ± 3.8	29.4 ± 5.2	58.9 ± 3.4	11.6 ± 3.1
CA3	31.0 ± 1.0 (3)	65.1 ± 0.5	23.6 ± 1.2[a]	11.3 ± 0.7	20.6 ± 1.9	68.0 ± 1.0[c]	11.3 ± 1.1
Parietal cortex	27.6 ± 1.8 (3)	64.0 ± 2.7	22.4 ± 2.5	13.6 ± 0.4	23.4 ± 1.3	64.0 ± 2.6	12.4 ± 0.9
CA1/DG	35.9 ± 1.8 (4)	66.7 (2)	23.2 (2)	9.9 (2)	22.6 (2)	68.2 (2)	10.5 (2)
Striatum	20.1 ± 0.6 (3)	66.6 ± 2.8	24.5 ± 2.8[b]	8.9 ± 1.0	34.9 ± 0.4	55.0 ± 1.4[d]	10.1 ± 1.6
Bulbus olfactorius	13.9 ± 0.3 (3)	68.5 ± 4.5	24.8 ± 2.3[b]	6.6 ± 2.3	39.7 ± 0.9	54.6 ± 2.6[d]	5.7 ± 2.5
Gyrus cinguli	18.8 ± 1.2 (3)	74.1 ± 1.5	18.0 ± 0.9[b]	7.7 ± 0.8	35.3 ± 2.8	58.6 ± 2.5[d]	6.0 ± 0.5
Superior colliculi	7.1 (2)	79.1 (2)	17.2 (2)	3.8 (2)	38.6 (2)	55.0 (2)	6.1 (2)

[a] $P < .05$, [b] $P < .001$, significantly different from corresponding value in absence of Zn^{2+} (column E) (ANOVA).
[c] $P < .05$, [d] $P < .001$, significantly different from corresponding value in absence of ifenprodil (column B) (ANOVA).

determined simultaneously in the absence and in the presence of 300 μM Zn^{2+}. In the absence of Zn^{2+}, the addition of 10 μM spermine resulted in a 5-fold shift of the high-affinity IC_{50} value (again in agreement with data given in Table 1). This shift was eliminated (to 0.96-fold; Table 4 and Fig. 5B) by 300 μM Zn^{2+}.

Discussion

The main results of this study are that 1) the dose-response curves for the inhibition of [^{3}H]MK-801 binding by Zn^{2+} and ifenprodil consisted of a low-affinity and a high-affinity component, respectively; 2) the respective high-affinity components were roughly additive and were shifted to the right by the addition of spermine; and 3) the spermine reversal of Zn^{2+} inhibition was prevented by ifenprodil and, vice versa, the spermine reversal of ifenprodil inhibition was prevented by Zn^{2+}.

Possible Complex Formation between Zn^{2+} and Spermine. The interpretation of results obtained with metal ions like Zn^{2+} and Cu^{2+} is complicated by the propensity of these ions to form tight complexes with several organic molecules, including glutamic acid, glycine, and spermine (Smith and Martell, 1975; Prince, 1987). The free concentration of Cu^{2+}, which forms stronger complexes than Zn^{2+}, is sensitive to the presence of amino acids (Vlachová et al., 1996). The effects of Zn^{2+} at the NMDA receptor complex, however, seem to be largely independent of the concentration of amino acids (Westbrook and Mayer, 1987; Mayer et al., 1989; Vlachová et al., 1996; and our own observations). Furthermore, 30 μM ifenprodil abolished the influence of spermine on the inhibitory potency of Zn^{2+}, making it unlikely that our results may be explained simply by spermine forming an inactive complex with Zn^{2+} (although the formation of this complex cannot be excluded, see 933 ff in Prince, 1987, and 101 ff in Smith and Martell, 1975).

Biphasic Inhibition. Several reports have described monophasic inhibition of [^{3}H]MK-801 binding by Zn^{2+} (Greenberg and Marks, 1988; Reynolds and Miller, 1988; Reynolds, 1992), in contrast to our results. A reason for the discrepancy may be that under the conditions of low ionic strength and slightly alkaline pH (as used in these studies), the two components of Zn^{2+} inhibition are practically indistinguishable (M. L. Berger and P. Rebernik, unpublished observation). Electrophysiological experiments leave no doubt that the inhibition of the NMDA receptor complex by

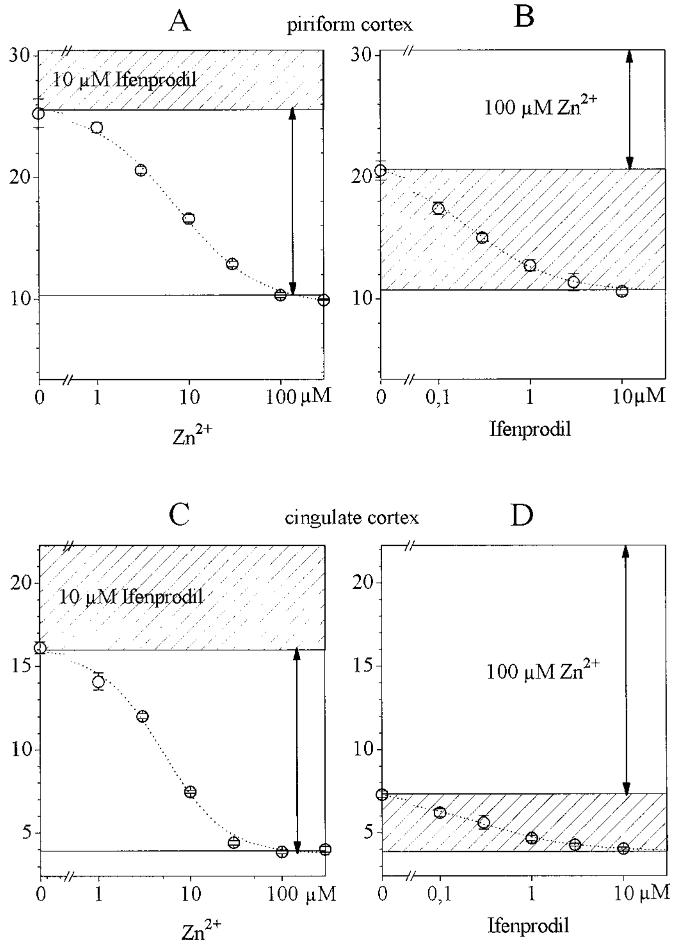

Fig. 4. Inhibition of [^{3}H]MK-801 binding by Zn^{2+} (A and C) and by ifenprodil (B and D). Absolute values (fmol [^{3}H]MK-801 totally bound/mg tissue) are shown; the bottom x-axis is at the level of the nonspecific binding, and the top x-axis at the control level, representative of three independent experiments (mean results are given in Table 3). Positive interaction between the two inhibitors in rat piriform cortex membranes (A and B). In the presence of 10 μM ifenprodil, a greater amount of radioligand is displaced by Zn^{2+} with high affinity (arrow in A) than in the absence of ifenprodil (arrow in B); vice versa, in the presence of 100 μM Zn^{2+}, a greater amount of radioligand is displaced by ifenprodil with high affinity (shaded in B) than in the absence of Zn^{2+} (shaded in A). No interaction could be observed in membranes prepared from the CA1/DG part of the hippocampus (see Fig. 3), whereas negative interaction was seen in cingulate cortex membranes (C and D).

Zn^{2+} involves at least two separate mechanisms: low micromolar (Christine and Choi, 1990; Legendre and Westbrook, 1990), or even lower (Chen et al., 1997; Paoletti et al., 1997), concentrations of Zn^{2+} act at the outer surface of the membrane; a second mechanism mediates direct inhibition of the channel at higher concentrations. Thus, our detection of two components of inhibition by Zn^{2+} also with biochemical techniques is not unexpected.

For the inhibition of [^{3}H]MK-801 binding by ifenprodil, more than one component has been described by several authors (Reynolds and Miller, 1989; Ogita et al., 1992). In electrophysiological experiments, a high-affinity component independent of voltage and of glycine has been described (Legendre and Westbrook, 1991). Ifenprodil acts with high affinity only at NMDA receptors containing the NR2B subunit (Williams, 1993). The binding of [^{3}H]1-(1-(2-thienyl)cyclohexyl(piperidine) (another ligand for the NMDA receptor associated ion channel) can be stimulated by spermine and spermidine, and both stimulations can be eliminated by low concentrations of ifenprodil (Carter et al., 1990).

Additivity of Independent Components? In membranes prepared from the CA1/DG part of the rat hippocampus, the inhibition of [^{3}H]MK-801 binding by Zn^{2+} and by ifenprodil was additive (i.e., each of the two substances inhibited its own fraction of bound [^{3}H]MK-801, apparently independent of the presence of the other substance). In both cases, the inhibition was reversed by the addition of spermine to the extent predicted for competitive interaction; therefore, we adopted the hypothesis that both substances inhibited the NMDA receptor complex via independent polyamine regulatory sites, with the first site sensitive to stimulation by polyamines like spermine and spermidine and at the same time sensitive to inhibition by Zn^{2+}, and the second site also sensitive to stimulation by polyamines and not sensitive to inhibition by Zn^{2+} but sensitive to inhibition by ifenprodil. However, more detailed investigations revealed that this additivity was limited to certain brain regions such as the hippocampal CA1/dentate gyrus and the parietal cortex. In membranes prepared from several other brain regions, Zn^{2+} and ifenprodil mutually influenced the extent to which they inhibited the NMDA receptor complex with high affinity; for example, specific [^{3}H]MK-801 binding to membranes prepared from the gyrus cinguli could be reduced (see Table 3) by 74.1% by 100 μM Zn^{2+} and by 35.3% by 10 μM ifenprodil. Nevertheless, 6.0% to 7.7% proved insensitive to either. These fractions sum up to 100% if mutual influences are taken into consideration: in the gyrus cinguli, Zn^{2+} inhibited only 58.6% of specifically bound [^{3}H]MK-801 under the influence of ifenprodil (instead of 74.1% in its absence). In contrast, pronounced positive interaction was seen in the piriform cortex. Taking together the results from nine brain regions, negative interaction appears to correlate with relatively pronounced high-affinity components of inhibition by zinc and by ifenprodil, whereas small high-affinity components for both inhibitors seem to favor positive interaction. This regional variability does not correlate with the regional distribution of any of the known NMDA receptor subunits. An exception might be the striatum and the olfactory bulb, where a higher level of NR2B expression has been found than in many other brain regions (Portera-Cailliau et al., 1996; Wenzel et al., 1997) and where a relatively high fraction of [^{3}H]MK-801 binding was sensitive to inhibition by ifenprodil.

Competitive or Allosteric Interaction? Reversal of Zn^{2+} inhibition of [^{3}H]MK-801 binding by the polyamine spermidine was first demonstrated by Reynolds (1992), who, while considering a direct competitive interaction between Zn^{2+} and spermidine unlikely, did not take into account components of high- and low-affinity Zn^{2+} inhibition. Direct competitive interaction between stimulatory polyamines and inhibitory ifenprodil has also been questioned (Reynolds and Miller, 1989; Ogita et al., 1992), but here, too, the investigations did not distinguish between high- and low-affinity components in the inhibitory action of ifenprodil. Our data characterizing the interaction between zinc (high-affinity component) and spermine, although compatible with a competitive mechanism, exhibited a high degree of scattering (Fig. 2) and represent no direct proof for such a mechanism; analogous data for ifenprodil (not shown) exhibited an even higher degree of variability. One reason for the difficulty in

TABLE 4

Inhibition of [^{3}H]MK-801 binding to rat hippocampal membranes (CA1/DG) by Zn^{2+} and by ifenprodil (high-affinity components only) showing the influence of ifenprodil and Zn^{2+} on sensitivity of inhibition to spermine

Values are given as mean ± S.D., with the number of experiments in parentheses.

	Without Spermine		10 μM Spermine		Sensitivity to Spermine
	IC_{50}	n_H	IC_{50}	n_H	
					change in IC_{50}
Inhibition by Zn^{2+}					
Control (4)	5.48 ± 0.63 μM	1.36 ± 0.20	22.0 ± 6.3 μM	1.08 ± 0.27	×3.97 ± 0.71
30 μM ifenprodil (4)	3.62 ± 0.78 μM^b	1.24 ± 0.10	5.42 ± 1.42 μM^b	1.13 ± 0.22	×1.49 ± 0.09^b
Inhibition by ifenprodil					
Control (4)	141 ± 75 nM	0.80 ± 0.15	710 ± 322 nM	1.02 ± 0.35	×5.27 ± 1.20
300 μM Zn^{2+} (4)	230 ± 49 nM	0.75 ± 0.06	220 ± 58 nM^a	0.89 ± 0.10 (3)	×0.96 ± 0.18^b

[a] $P < .05$, [b] $P < .01$, significantly different from respective control value (paired Student's *t* test).

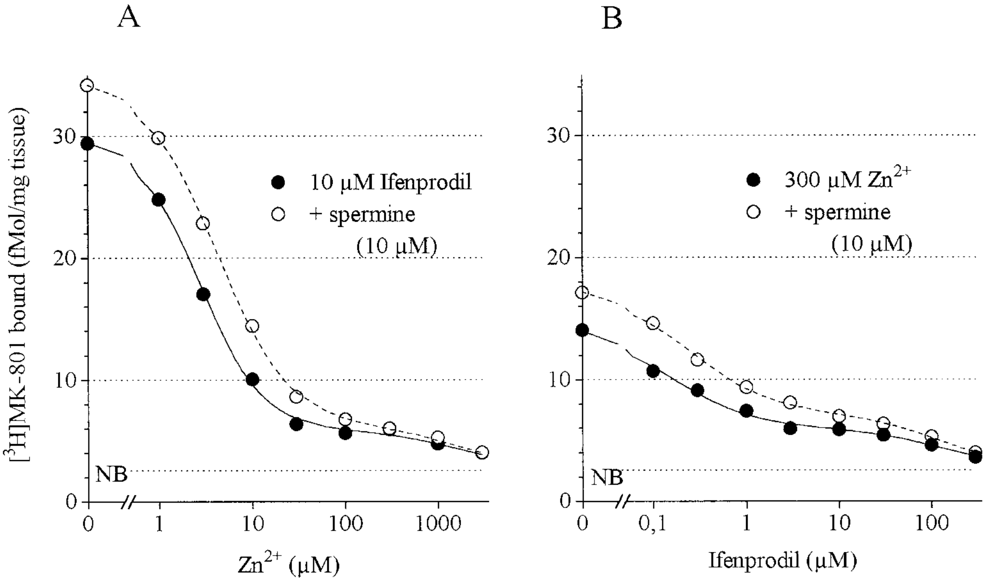

Fig. 5. Inhibition of [^{3}H]MK-801 binding (membranes from hippocampal CA1/DG part) by Zn^{2+} in the presence of 30 μM ifenprodil (A) and by ifenprodil in the presence of 300 μM Zn^{2+} (B). Results are representative for four independent experiments. Note that the addition of 10 μM spermine did not result in a shift of the inhibition curves (in contrast to Fig. 1).

obtaining accurate data on the reversibility of the high-affinity inhibitions produced by zinc and by ifenprodil over a more extended concentration range could be the direct channel blockade at spermine concentrations slightly above stimulating concentrations (Rock and Macdonald, 1991; Benveniste and Mayer, 1993; and our own observations). On the other hand, our observation that spermine reversibility of Zn^{2+} inhibition was lost in the presence of ifenprodil (and vice versa, that spermine reversibility of ifenprodil inhibition was lost in the presence of Zn^{2+}) strongly argues against a competitive and in favor of an allosteric mechanism of action. This hypothesis is in agreement with molecular biology studies, indicating that polyamine stimulation is primarily mediated by NR1 receptor subunits (Williams et al., 1995; Kashiwagi et al., 1996), whereas high-affinity inhibition by zinc depends on the presence of the NR2A (Chen et al., 1997; Paoletti et al., 1997) and high-affinity inhibition by ifenprodil on the presence of the NR2B subunit (Williams, 1993; Gallagher et al., 1996). Furthermore, it has been demonstrated with site-directed mutagenesis that other amino acid residues of the NR2B subunit are involved in polyamine stimulation than those responsible for inhibition by ifenprodil (Gallagher et al., 1996).

Comparison with Other Polyamine-Sensitive Inhibitors. Other compounds postulated to inhibit the NMDA receptor complex via a polyamine regulatory site, such as N-12-N and pentamidine, exhibit monophasic inhibition of [^{3}H]MK-801 binding; the concentrations of polyamines needed to overcome the inhibitions are higher than those needed to overcome high-affinity inhibition by zinc or by ifenprodil. In contrast to Zn^{2+} and ifenprodil, these compounds have two positive charges separated from each other by some distance essential for their potency (Romano et al., 1992). It may be speculated that these compounds interact with the NMDA receptor complex via two separate sites, with the first site corresponding to the interaction of Zn^{2+} and the second corresponding to the interaction of ifenprodil with the NMDA receptor complex. In this case, the zinc site could only be occupied with concomitant occupation of the ifenprodil site, and vice versa, the ifenprodil site could only be occupied with concomitant occupation of the zinc site. Because occupation of one of these sites compromises the spermine reversibility of inhibition via the other site (as shown in this report; see above), concomitant occupation of both sites by "bidentate" compounds would provide an explanation for the reduced spermine sensitivity of this type of inhibition in comparison to inhibition by Zn^{2+} or by ifenprodil alone (with only one of the two sites inhibited).

In conclusion, the results of this study suggest that Zn^{2+} and ifenprodil interact with separate (although not independent) sites at the NMDA receptor complex. Both sites seem to regulate allosterically polyamine stimulation of the NMDA receptor complex. Our results may aid the search for drugs with a new pharmacological profile, interacting selectively with the high-affinity zinc site at the NMDA receptor complex.

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References

Benveniste M and Mayer ML (1993) Multiple effects of spermine on NMDA receptor responses of rat cultured hippocampal neurones. *J Physiol* **464:**131–163.

Berger ML, Charton G and Ben-Ari Y (1986) Effects of seizures induced by intra-amygdaloid kainic acid on kainic acid binding sites in rat hippocampus and amygdala. *J Neurochem* **47:**720–727.

Berger ML, Seifriz I, Letschnig M, Schödl C and Noe CR (1992) Interaction of long chain n-alkyl diamines with the NMDA receptor complex. *Neurosci Lett* **142:**85–88.

Carter CJ, Lloyd KG, Zivkovic B and Scatton B (1990) Ifenprodil and SL 82.0715 as cerebral anti-ischemic agents. III. Evidence for antagonistic effects at the polyamine modulatory site within the NMDA receptor complex. *J Pharmacol Exp Ther* **253:**475–482.

Chen N, Moshaver A and Raymond LA (1997) Differential sensitivity of recombinant NMDA receptor subtypes to zinc inhibition. *Mol Pharmacol* **51:**1015–1023.

Choi DW and Koh JY (1998) Zinc and brain injury. *Annu Rev Neurosci* **21:**347–375.

Christine CW and Choi DW (1990) Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. *J Neurosci* **10:**108–116.

Enomoto R, Ogita K, Han D and Yoneda Y (1992) Differential modulation by divalent cations of [^{3}H]MK-801 binding by brain synaptic membranes. *J Neurochem* **59:** 473–481.

Gallagher MJ, Huang H, Pritchett DB and Lynch DR (1996) Interactions between ifenprodil and the NR2B subunit of the NMDA receptor. *J Biol Chem* **271:**9603–9611.

Greenberg DA and Marks SS (1988) Cation interactions with putative NMDA receptor-gated channels labeled by [^{3}H]MK-801 in rat cerebral cortex. *Neurosci Lett* **95:**236–240.

Harrison NL and Gibbons SJ (1994) Zn^{2+}: An endogenous modulator of ligand- and voltage-gated ion channels. *Neuropharmacology* **33:**935–952.

Johnson ML and Faunt LM (1992) Parameter estimation by least-squares methods. *Methods Enzymol* **210:**1–37.

Kashiwagi K, Fukuchi J-I, Chao J, Igarashi K and Williams K (1996) An aspartate residue in the extracellular loop of the NMDA receptor controls sensitivity to spermine and protons. *Mol Pharmacol* **49:**1131–1141.

Koh J-Y, Suh SW, Gwag BJ, He YY, Hsu CY and Choi DW (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science (Wash DC)* **272:**1013–1016.

Legendre P and Westbrook GL (1990) The inhibition of single NMDA-activated channels by zinc ions on cultured rat neurones. *J Physiol* **429:**429–449.

Legendre P and Westbrook GL (1991) Ifenprodil blocks NMDA receptors by a two-component mechanism. *Mol Pharmacol* **40:**289–298.

Maske H (1955) Über den topochemischen Nachweis von Zink im Ammonshorn verschiedener Säugetiere. *Naturwiss* **42:**424.

Matsushita K, Kitagawa K, Matsuyama T, Uhtsuki T, Taguchi A, Mandai K, Mabuchi T, Yagita Y, Yanagihara T and Matsumoto M (1996) Effect of systemic zinc administration on delayed death in the gerbil hippocampus. *Brain Res* **743:**362–365.

Mayer ML, Vyklický L Jr and Westbrook GL (1989) Modulation of excitatory amino acid receptors by group IIB metal cations in cultured mouse hippocampal neurons. *J Physiol* **415:**329–350.

Ogita K, Ohkawara A, Suzuki T, Ohgaki T, Uchida S, Meguri H and Yoneda Y (1992) Effects of ifenprodil on the NMDA receptor ionophore complex in rat brain. *Neurochem Int* **21:**135–147.

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

Peters S, Koh J and Choi DW (1987) Zinc selectively blocks the action of NMDA on cortical neurons. *Science (Wash DC)* **236:**589–593.

Portera-Cailliau C, Price DL and Martin LJ (1996) NMDA receptor proteins NR2A and NR2B are differentially distributed in the developing rat central nervous system as revealed by subunit-specific antibodies. *J Neurochem* **66:**692–700.

Prince RH (1987) *Comprehensive Coordination Chemistry Chapter 56.1*. Oxford.

Ransom RW and Stec NL (1988) Cooperative modulation of [^{3}H]MK-801 binding to the NMDA receptor ion channel complex by L-glutamate, glycine, and polyamines. *J Neurochem* **51:**830–836.

Reynolds IJ and Miller RJ (1988) [^{3}H]MK-801 binding to the NMDA receptor/ionophore complex is regulated by divalent cations: Evidence for multiple regulatory sites. *Eur J Pharmacol* **151:**103–112.

Reynolds IJ and Miller RJ (1989) Ifenprodil is a novel type of NMDA receptor antagonist: Interaction with polyamines. *Mol Pharmacol* **36:**758–765.

Reynolds IJ (1992) Interactions between zinc and spermidine on the NMDA receptor complex: Clues to the mechanism of action of 1,10-bis(guanidino)decane and pentamidine. *J Pharmacol Exp Ther* **263:**632–638.

Reynolds IJ and Aizenman E (1992) Pentamidine is an NMDA receptor antagonist and is neuroprotective in vitro. *J Neurosci* **12:**970–975.

Rock DM and Macdonald RL (1991) The polyamine spermine has multiple actions on NMDA receptor single-channel currents in cultured cortical neurons. *Mol Pharmacol* **41:**83–88.

Romano C, Williams K, DePriest S, Seshadri R, Marshall GR, Israel M and Molinoff PB (1992) Effects of mono-, di-, and triamines on the NMDA receptor complex: A model of the polyamine recognition site. *Mol Pharmacol* **41:**785–792.

Smith RM and Martell AE (1975) *Critical Stability Constants. Volume 2: Amines*. Plenum Press, New York.

Vlachová V, Zemková H and Vyklický L Jr (1996) Copper modulation of NMDA responses in mouse and rat cultured hippocampal neurons. *Eur J Neurosci* **8:**2257–2264.

Wenzel A, Fritschy JM, Möhler H and Benke D (1997) NMDA receptor heterogeneity during postnatal development of the rat brain: Differential expression of NR2A, NR2B, and NR2C subunit proteins. *J Neurochem* **68:**469–478.

Westbrook GL and Mayer ML (1987) Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons. *Nature (London)* **328:**640–643.

Williams K (1993) Ifenprodil discriminates subtypes of the NMDA receptor: Selectivity and mechanisms at recombinant heteromeric receptors. *Mol Pharmacol* **44:**851–859.

Williams K, Dawson VL, Romano C, Dichter MA and Molinoff PB (1990) Characterization of polyamines having agonist, antagonist, and inverse agonist effects at the polyamine recognition site of the NMDA receptor. *Neuron* **5:**199–208.

Williams K, Kashiwagi K, Fukuchi J-I and Igarashi K (1995) An acidic amino acid in the NMDA receptor that is important for spermine stimulation. *Mol Pharmacol* **48:**1087–1098.

Wong EHF, Knight AR and Woodruff GN (1988) [^{3}H]MK-801 labels a site on the NMDA receptor complex in rat brain membranes. *J Neurochem* **50:**274–281.

Yoneda Y and Ogita K (1989) Labeling of NMDA receptor channels by [^{3}H]MK-801 in brain synaptic membranes treated with Triton X-100. *Brain Res* **499:**305–314.

Send reprint requests to: Dr. Michael Berger, Institute of Biochemical Pharmacology, Borschkegasse 8a, A-1090 Vienna, Austria. E-mail: michael.berger@univie.ac.at