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ACCELERATED COMMUNICATION

Evidence that Zinc Inhibits *N*-Methyl-D-aspartate Receptor-Gated Ion Channel Activation by Noncompetitive Antagonism of Glycine Binding

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SUMMARY

Zinc noncompetitively antagonizes *N*-methyl-D-aspartate (NMDA) receptor-mediated responses in cultured neurons. We investigated the mechanism of this inhibition by examining the effect of zinc on ligand binding to three distinct sites on the NMDA receptor in rat hippocampal membranes. Zinc dose-dependently inhibited both the association and dissociation of the NMDA channel blocker [^{3}H]*N*-(1-[thienyl]cyclohexyl)piperidine ([^{3}H]TCP) but had no effect on steady state levels of [^{3}H]TCP binding. This suggests that zinc inhibits the receptor-gated access of [^{3}H]TCP to its site in the ion channel but has no effect on the binding site itself. Zinc inhibition of [^{3}H]TCP association was not mediated by an action at the NMDA recognition site, because zinc had no effect on NMDA-displaceable L-[^{3}H]glutamate binding. On the other hand, zinc dose-dependently inhibited [^{3}H]glycine binding by a noncompetitive interaction. Stoichiometric analysis of equilibrium binding data indicated the presence of two [^{3}H]glycine binding sites/[^{3}H]TCP binding site. Comparison of the potencies of zinc in inhibiting glycine-dependent [^{3}H]TCP association and [^{3}H]glycine binding suggests that blockade of only one of the two glycine sites is sufficient to prevent [^{3}H]TCP association. We hypothesize that synaptically released zinc inhibits NMDA receptor-mediated responses by binding to a site on the receptor/channel complex, reducing glycine binding, and thereby decreasing what would otherwise be a tonically present action of endogenous extracellular glycine.

Zinc, a divalent cation, is an important trace element in a variety of metabolic processes (1). Within the hippocampus, zinc is found in high concentrations in synaptic vesicles in the terminals of excitatory mossy fibers; this zinc is released upon depolarizing stimuli (2–6). *In vitro* studies have documented the Ca^{2+} dependence of depolarization-induced zinc release (2).

Zinc selectively blocks NMDA receptor-mediated currents, neurotoxic responses, and excitatory synaptic transmission (7–12). In patches of cultured neurons, low micromolar concentrations of zinc block NMDA-evoked currents by a voltage-insensitive inhibition of channel opening frequency; higher concentrations inhibit currents by a voltage-dependent magnesium-like action (13). Zinc-mediated inhibition is noncompetitive with respect to NMDA (10). Biochemical studies of ligand binding to the NMDA receptor have found that zinc has relatively little effect on [^{3}H]glutamate binding to the NMDA receptor (14) but, nevertheless, decreases the NMDA-dependent dissociation of [^{3}H]MK-801 (15, 16). Because MK-801 is an NMDA channel blocker (17–19) whose binding (and unbinding) is dependent upon glycine and NMDA activation of the receptor-gated ion channel (20–22), this suggests that zinc noncompetitively reduces activation of the NMDA receptor-gated ion channel in membrane preparations.

To further elucidate the mechanism by which zinc interacts with the NMDA receptor, we examined the effects of zinc on the binding of ligands to three distinct sites on the NMDA receptor/channel complex. [^{3}H]Glycine was used to examine the effect of zinc on the strychnine-insensitive glycine recognition site of the NMDA receptor (23, 24). [^{3}H]Glutamate was used to examine the effect of zinc on ligand binding to the NMDA recognition site (25). [^{3}H]TCP, an uncompetitive NMDA antagonist whose binding is dependent upon NMDA and glycine activation of the NMDA receptor-gated ion channel (26–31), was used to examine the effect of zinc on activation of the NMDA receptor-gated ion channel and on ligand binding to the phencyclidine binding site.[1]

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[1] [^{3}H]TCP, rather than [^{3}H]MK-801, was used in these studies because the association rate for [^{3}H]TCP is significantly faster than that of [^{3}H]MK-801, thereby allowing shorter duration incubations in equilibrium studies (unpublished observations). The use of [^{3}H]TCP resulted in no loss of selectivity because in this preparation, [^{3}H]TCP binding is completely dependent upon agonist binding to the NMDA and glycine recognition sites (26, 27).

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; TCP, *N*-(1-[thienyl]cyclohexyl)piperidine.

Materials and Methods

Membrane Preparation

Adult (300–350 g) male Sprague-Dawley rats were killed by decapitation. Hippocampi were removed, homogenized (Polytron setting 6, 20 sec) in 10 ml of ice-cold Tris-acetate buffer (50 mM with 10 mM EDTA, pH 7.4), and centrifuged (30,000 × *g*, 20 min). The membranes were then washed by eight additional cycles of homogenization, centrifugation, and resuspension in fresh buffer. Before the third and fourth centrifugations, the membranes were frozen in a methanol/dry ice bath. Before the fifth centrifugation, the membranes were incubated at 37° for 15 min. All membrane preparation steps after the third centrifugation were conducted on the day of the binding experiment.

Ligand Binding Assays

[^{3}H]Glycine binding assay. Membranes were incubated with [^{3}H]glycine for 40 min in 1 ml of 5 mM Tris-acetate buffer (pH 7.4, 25°, no added EDTA). The reactions were terminated by centrifugation at 10,000 × *g* for 15 min. The pellets were rinsed twice with 2 ml of cold buffer and digested in 200 μl of Protosol (New England Nuclear). Specific binding was defined as that binding displaced by 1 mM nonradioactive glycine. Specific binding was linear with protein concentration (0.05 to 0.3 mg/ml) and ranged from 60% of total binding ([^{3}H]glycine concentration of 10 nM) to 15% of total binding ([^{3}H]glycine concentration of 1000 nM). Binding isotherms were generated with 12 concentrations of [^{3}H]glycine, ranging from 10 to 1000 nM.

[^{3}H]Glutamate binding assay. Membranes were incubated with 10 nM [^{3}H]glutamate in 1 ml of Tris-acetate buffer (50 mM, pH 7.4, 4°) for 30 min. Reactions were terminated by vacuum filtration using a Skatron cell harvester and Skatron filtermats (catalog no. 7034). After filtration, the filters were rinsed for 7 sec with ice-cold buffer. NMDA-sensitive [^{3}H]glutamate binding was defined as that binding displaced by 100 μM NMDA. Specific NMDA-sensitive [^{3}H]glutamate binding was linear with protein concentration (0.05 to 0.3 mg/ml) and accounted for 65–75% of total binding.

[^{3}H]TCP binding assay. [^{3}H]TCP binding was measured in 1 ml of 5 mM Tris-acetate buffer, pH 7.4, at 25°. Reactions were terminated by vacuum filtration using a Skatron cell harvester and Skatron filters. [^{3}H]TCP binding to the filters was reduced by pretreatment with 5 ml of 0.075% polyethylenimine (32). After filtration the filters were rinsed with ice-cold buffer for 10 sec. [^{3}H]TCP binding was performed in the presence of 30 μM NMDA and 3 μM glycine, unless otherwise specified. Specific binding was defined as that binding displaced by 1.25 μM nonradioactive TCP. Under steady state conditions, [^{3}H]TCP binding was linear with protein concentration (0.05 to 0.3 mg/ml) and accounted for greater than 90% of total binding.

[^{3}H]TCP binding in hippocampal membranes is affected by three factors, the affinity of the [^{3}H]TCP binding site, the number of [^{3}H]TCP binding sites, and the accessibility of [^{3}H]TCP to its binding site (presumably located within the channel itself). The last factor is controlled by agonist occupancy of the NMDA- and strychnine-insensitive glycine binding sites (26–28). All three factors (affinity, number, and access) influence [^{3}H]TCP binding under nonequilibrium conditions; under equilibrium conditions, only affinity and number regulate [^{3}H]TCP binding. To determine whether zinc modifies the [^{3}H]TCP binding site itself (affinity or number of sites) or modifies the channel-gated access of [^{3}H]TCP to its site (without modifying the interaction of the ligand with its recognition site *per se*), [^{3}H]TCP binding was measured under both equilibrium and nonequilibrium conditions.

[^{3}H]TCP binding isotherms, generated under equilibrium conditions, yielded K_d and B_{max} values for [^{3}H]TCP binding. For these experiments, [^{3}H]TCP was incubated with membranes, NMDA, and glycine for 400 min, because previous experiments documented that steady state is reached under these conditions (26, 27). To determine the effect of zinc on steady state [^{3}H]TCP binding, the membranes were first incubated with [^{3}H]TCP, NMDA, and glycine for 400 min, whereupon zinc (0–100 μM final concentration) was added; the membranes were filtered 30 min after the addition of zinc. The rationale for establishing steady state [^{3}H]TCP binding before the addition of zinc was based on preliminary findings indicating that zinc greatly slowed the rate of [^{3}H]TCP equilibration.

Dose-response curves, measuring drug effects (glycine or zinc) on [^{3}H]TCP (2.5 nM) binding under nonequilibrium conditions (amount bound in a 30-min incubation), yielded EC_{50} and R_{max} (maximum responses) values for stimulation of [^{3}H]TCP association. It should be noted that, because these R_{max} values were calculated using a 30-min incubation, they are underestimates of initial association rates. As a consequence, EC_{50} values calculated in this manner will slightly overestimate the potency of ligands that stimulate [^{3}H]TCP binding (e.g., glycine and NMDA) and slightly underestimate the potency of ligands that inhibit [^{3}H]TCP association (e.g., zinc).

[^{3}H]TCP association curves were generated by incubation of membranes with 2.5 nM [^{3}H]TCP for varying periods of time before filtration. [^{3}H]TCP dissociation curves were generated by addition of nonradioactive TCP (final concentration of 1.25 μM) after incubation of the membranes with 2.5 nM [^{3}H]TCP for 400 min.

Data Analysis

To determine EC_{50} and IC_{50} values from dose-response curves (NMDA or glycine stimulation of [^{3}H]TCP binding or zinc inhibition of [^{3}H]TCP binding), the curve-fitting programs in ENZFITTER (33) were used. To calculate the K_d and B_{max} values for ligand binding (under equilibrium conditions), the nonlinear least squares regression program LIGAND was used (34). Values reported are means and standard errors. Statistical analysis was conducted using either *t* tests or analysis of variance with *post hoc* tests.

Results

Effects of zinc on [^{3}H]TCP binding. The effect of zinc on [^{3}H]TCP binding was examined under both nonequilibrium and equilibrium conditions. Under nonequilibrium conditions, zinc inhibited [^{3}H]TCP binding in a dose-dependent manner, with a calculated IC_{50} of 6 ± 1 μM (Fig. 1). By contrast, once [^{3}H]TCP binding was equilibrated, addition of zinc (0 to 100 μM) had no effect on [^{3}H]TCP binding (Fig. 1). The lack of effect of zinc on steady state [^{3}H]TCP binding was further demonstrated by generation of equilibrium binding isotherms following addition of zinc. Neither 10 nor 100 μM zinc modified the calculated K_d or B_{max} for [^{3}H]TCP binding (Table 1).

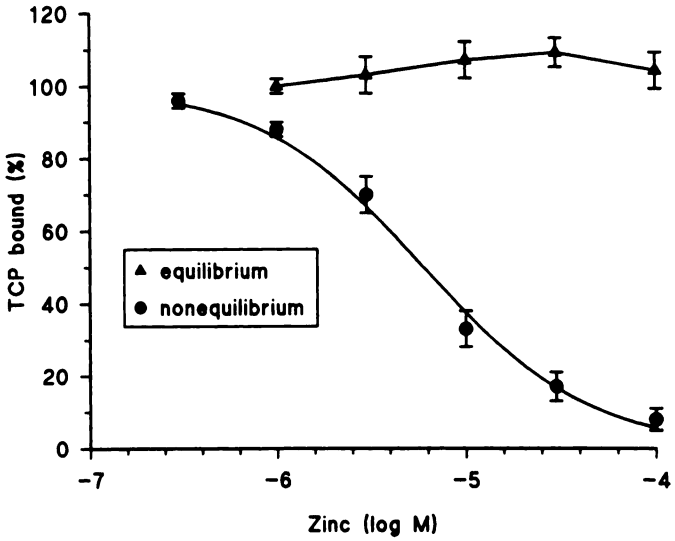

Fig. 1. Effects of zinc on [^{3}H]TCP association (2.5 nM) and on previously equilibrated [^{3}H]TCP binding (2.5 nM). In both cases membranes were exposed to zinc for 30 min (see Results). Values are the mean ± standard error of at least three experiments. [^{3}H]TCP binding in the absence of added zinc in the nonequilibrium and equilibrium studies was 0.23 and 0.41 pmol/mg of protein, respectively.

TABLE 1

Effect of zinc on [^{3}H]TCP binding

Values are the mean ± standard error of at least three separate experiments. K_d and B_{max} values were determined 30 min after the addition of zinc (430 min after the addition of [^{3}H]TCP, NMDA, and glycine). Because only 30 min were allowed for equilibration of [^{3}H]TCP binding after addition of zinc, it is possible that these values do not reflect equilibrium conditions. However, in these incubations and in incubations (with 100 μM zinc) carried out for an additional 170 min, there was no detectable effect of zinc on steady state [^{3}H]TCP binding. Values for [^{3}H]TCP association and dissociation are rates (not rate constants) and are based on 30-min incubations.

	0 μM zinc	10 μM zinc	100 μM zinc
K_d (nM)	8.8 ± 0.6	9.1 ± 0.2	9.1 ± 0.2
B_{max} (pmol/mg)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
Association rate (fmol mg^{-1} min^{-1})	4.3 ± 0.1	2.5 ± 0.3	1.3 ± 0.2
Dissociation rate (fmol mg^{-1} min^{-1})	3.4 ± 0.1	2.6 ± 0.2	1.1 ± 0.2

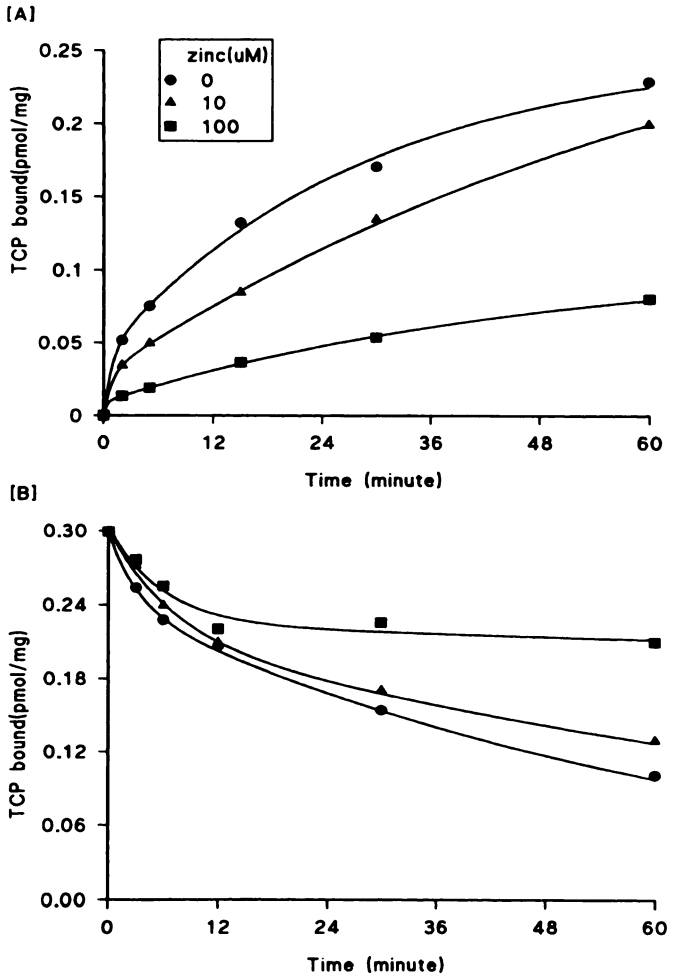

Fig. 2. Effects of zinc on the association and dissociation of [^{3}H]TCP (2.5 nM) binding. A, Association curves generated in the presence of different concentrations of zinc; B, dissociation curves generated in the presence of different concentrations of zinc. Similar results have been obtained in three separate experiments.

The absence of effects of zinc on previously equilibrated steady state [^{3}H]TCP binding suggested that the inhibitory effect of zinc on [^{3}H]TCP binding under nonequilibrium conditions was not due to a reduction in binding site affinity. Rather, these findings suggested that zinc reduced nonequilibrium [^{3}H]TCP binding by limiting the access of [^{3}H]TCP to its binding site. To further test this idea, the effects of zinc on the

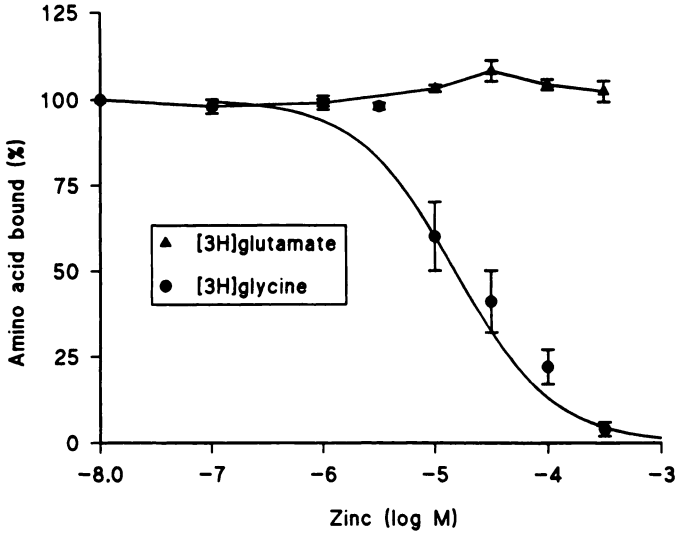

Fig. 3. Effect of zinc on [^{3}H]glutamate and [^{3}H]glycine binding. Values are mean ± standard error (three experiments for [^{3}H]glutamate and five experiments for [^{3}H]glycine). Binding in the absence of added zinc was 0.56 and 0.48 pmol/mg of protein for [^{3}H]glycine and [^{3}H]glutamate, respectively.

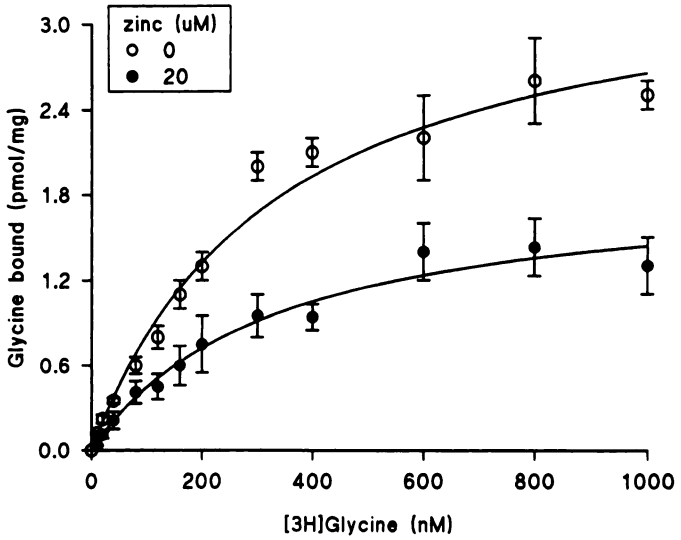

Fig. 4. Effect of zinc on the [^{3}H]glycine binding isotherm. Values are mean ± standard error (five experiments for control and three experiments for zinc).

association and dissociation of [^{3}H]TCP binding were examined (Fig. 2). Zinc (10 and 100 μM) reduced both the association and dissociation rates of [^{3}H]TCP binding at concentrations that did not alter steady state [^{3}H]TCP binding (Table 1).

Effects of zinc on [^{3}H]glycine and [^{3}H]glutamate binding. To determine whether the effects of zinc on [^{3}H]TCP binding were mediated by an effect at the glycine or NMDA binding sites, the effects of zinc on the binding of ligands to these sites were examined. Zinc dose-dependently inhibited [^{3}H]glycine binding, with an IC_{50} of 15 ± 4 μM (Fig. 3). Zinc produced this effect by decreasing the apparent number of [^{3}H]glycine binding sites without modifying the affinity. Zinc (20 μM) decreased the B_{max} for [^{3}H]glycine binding from a control value of 3.5 ± 0.2 pmol/mg to 1.9 ± 0.2 pmol/mg ($p < 0.05$). The K_d for glycine binding in the absence and presence of 20 μM zinc was 334 ± 44 and 328 ± 65 nM (not significantly

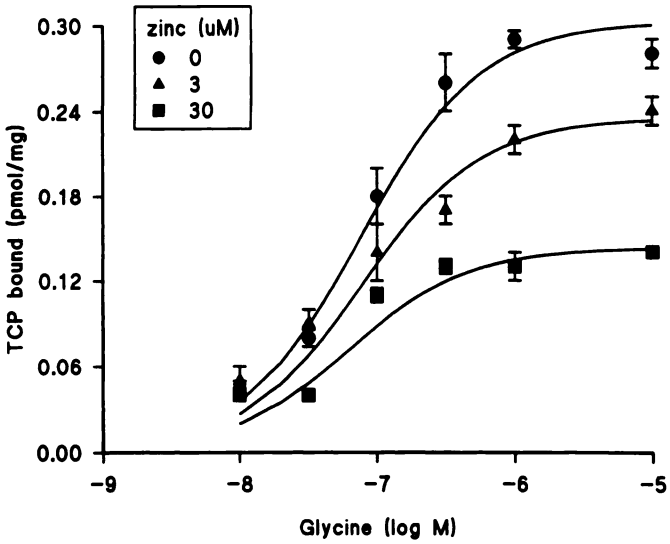

Fig. 5. Effect of zinc on glycine-stimulated [^{3}H]TCP binding. Membranes were incubated with 30 μM NMDA, 2.5 nM [^{3}H]TCP, and various concentrations of glycine for 30 min in the presence of 0, 3, or 30 μM zinc. Values are from six experiments with no added zinc, four experiments with 3 μM zinc, and two experiments with 30 μM zinc. *Error bars*, standard errors (standard deviation for experiments with 30 nM zinc). Missing *error bars* are obscured by the *symbol* denoting the mean value.

different), respectively (Fig. 4). By contrast, zinc did not modify NMDA-sensitive [^{3}H]glutamate binding (Fig. 3).

Effects of zinc on glycine stimulation of [^{3}H]TCP binding. The noncompetitive inhibitory effect of zinc on [^{3}H]glycine binding predicted that zinc would inhibit glycine stimulation of [^{3}H]TCP association in a noncompetitive fashion. Zinc dose-dependently decreased the R_{max} of glycine-stimulated [^{3}H]TCP binding while having no effect on the EC_{50}. Zinc (30 μM) decreased the R_{max} from 0.28 ± 0.02 pmol mg^{-1} 30 min^{-1} to 0.12 ± 0.02 pmol mg^{-1} 30 min^{-1} ($p < 0.01$) (Fig. 5). Zinc similarly decreased the R_{max} (with no effect on EC_{50}) of D- and L-serine and D- and L-alanine-stimulated [^{3}H]TCP binding (data not shown).

Discussion

The principal findings of this report are that 1) zinc inhibited [^{3}H]TCP association and dissociation but had no effect on previously equilibrated [^{3}H]TCP binding; 2) zinc inhibited glycine binding and glycine-stimulated [^{3}H]TCP binding by a noncompetitive mechanism; and 3) zinc had no effect on glutamate binding to the NMDA recognition site.

Electrophysiologic studies of cultured neurons demonstrate that noncompetitive NMDA antagonists such as MK801, TCP, and ketamine block NMDA-evoked currents; both the formation and the removal of the block are use and voltage dependent (35–37). This led to the suggestion that these antagonists bind to sites in the ion channel and that their binding and unbinding are dependent upon activation of the receptor (activation is defined here as a conformational change in the receptor/channel complex associated with increased ion permeability). Biochemical studies of isolated hippocampal membranes demonstrate that the binding of [^{3}H]TCP is regulated in a manner similar to that inferred in electrophysiologic studies. That is, both the association and dissociation of the ligand are dependent upon NMDA and glycine agonists. This suggests that the conformational change that allows noncompetitive antagonists to bind (and unbind) in electrophysiologic studies of intact cells may be identical to that which allows [^{3}H]TCP access to and from its binding site in biochemical studies of isolated membranes. Thus, the rate of [^{3}H]TCP association and dissociation likely reflects the degree to which the population of NMDA receptors in a membrane preparation is being activated. Specifically, the rate of [^{3}H]TCP association (or dissociation) is proposed to be proportional to the product of the frequency of channel activation and the duration of individual "openings."

Based on this model, the finding that zinc decreased the association and dissociation of [^{3}H]TCP binding suggests that zinc decreased NMDA- and glycine-dependent activation of the NMDA receptor in hippocampal membranes. This decreased receptor activation may be an *in vitro* reflection of the inhibitory effect of zinc on channel opening frequency detected in intact cell membranes. Thus, the effects of zinc on [^{3}H]TCP binding may be an *in vitro* measure of the voltage-independent inhibitory action of zinc on NMDA-evoked currents (10, 13).

If zinc inhibits [^{3}H]TCP association by preventing transition of the ion channel into an activated (presumably open) conformation, this could be mediated by blocking agonist binding to the NMDA receptor. Because both NMDA and glycine agonists are absolute requirements for sustained activation of the ion channel (38), zinc could act as either an NMDA or a glycine antagonist. The lack of effect of zinc on glutamate binding argues against the former possibility. However, the dose-dependent inhibition of glycine binding by zinc strengthens the possibility that zinc acts as a glycine antagonist.

If zinc inhibits [^{3}H]TCP association by inhibiting glycine binding to the NMDA receptor, then it would be expected that the potency of zinc in inhibiting [^{3}H]TCP association and inhibiting [^{3}H]glycine binding should be equivalent. Interestingly, the IC_{50} for zinc inhibition of glycine binding (15 ± 4 μM) was slightly greater than that for inhibition of glycine-stimulated [^{3}H]TCP binding (6 ± 1 μM). One explanation for this apparent discrepancy is the 2:1 ratio of [^{3}H]glycine to [^{3}H]TCP binding sites found here (B_{max} for [^{3}H]glycine and [^{3}H]TCP of 3.5 ± 0.2 and 1.7 ± 0.1 pmol/mg, respectively) and elsewhere (39, 40).

Previous investigations of glycine and NMDA stimulation of [^{3}H]TCP binding suggest that the rate of [^{3}H]TCP association is proportional to the product of the fractional occupancy of all the agonist sites on the NMDA receptor/channel complex (26, 27). If this idea is correct and if there are two zinc-sensitive glycine sites/channel binding site, then 50% glycine receptor occupancy (at each glycine binding site) should produce a rate of [^{3}H]TCP association equivalent to only 25% of that found with 100% glycine receptor occupancy.[2] Thus, a concentration of zinc (15 μM) sufficient to inhibit 50% of the [^{3}H]glycine binding (with no effect on NMDA receptor occupancy) should yield a [^{3}H]TCP association rate 25% of R_{max}, because on average only 25% of the receptor/channel complexes would have both glycine sites occupied. This prediction is close to the

[2] This hypothetical model holds that the rate of [^{3}H]TCP association is proportional to $f[N] \times f[G_1] \times f[G_2]$, where $f[N]$ refers to the fractional occupancy of the NMDA receptor, $f[G_1]$ refers to the fractional occupancy of one glycine site on the receptor/channel complex, and $f[G_2]$ refers to the fractional occupancy of a second (pharmacologically equivalent) glycine site on the receptor/channel complex. The model does not preclude the presence of multiple NMDA binding sites and is undoubtedly incomplete, inasmuch as it disregards the potential for allosteric interactions between NMDA and glycine receptor ligands (13, 41, 42).

experimentally determined value of 28% R_{max} [based on a 6 μM IC_{50} for zinc inhibition of [^{3}H]TCP association (Fig. 1)]. Moreover, the data more closely fit a two-glycine site model (predicted rate of 25% R_{max}) than either a one-site model (predicted rate of 50% R_{max}) or a three-site model (predicted rate of 12.5% R_{max}). Thus, based on the observed stoichiometry of two [^{3}H] glycine sites/[^{3}H]TCP binding site, these data are consistent with the hypothesis that zinc inhibits opening of the NMDA channel by blocking glycine binding.

The experiments examining zinc regulation of [^{3}H]glycine binding and glycine stimulation of [^{3}H]TCP binding shed light on the mechanism by which zinc inhibits glycine binding. Zinc is not merely chelating glycine, because the action of zinc was not surmountable with increasing concentrations of glycine and because zinc similarly inhibited the actions of D- and L-serine and D- and L-alanine. Moreover, zinc is not likely directly competing for glycine binding, because zinc inhibition of [^{3}H] glycine binding was not surmountable with increasing glycine concentrations. The most parsimonious explanation is that zinc binds to a site distinct from the glycine binding sites to induce conformational changes in the receptor/channel complex, thereby preventing glycine binding and, consequently, glycine-dependent activation of the NMDA channel.

These results suggest that inhibition of glycine binding mediates the voltage-independent inhibitory effect of zinc on channel opening frequency. These results do not address the question of how zinc mediates its voltage-dependent magnesium-like action. The voltage-dependent inhibitory action of zinc may be mediated by a separate binding site located within the ion channel (10, 13). The use of [^{3}H]TCP to study the interaction of zinc with an intrachannel binding site will be problematic, because the inhibitoty effects of zinc on channel activation will greatly reduce the interaction of zinc, [^{3}H]TCP, and other channel-binding ligands. It should be noted that, whereas [^{3}H]TCP binding to its "guarded receptor" (43, 44) is predictive of manipulations that alter channel opening frequency (i.e., changes in NMDA, glycine, and now zinc receptor occupancy), it has not been validated as a tool for predicting the effects of voltage-dependent channel-binding ligands on NMDA receptor-mediated currents.

These findings, suggesting that zinc mediates its voltage-independent inhibition of channel opening frequency by inhibiting glycine binding, raise intriguing possibilities regarding the physiologic interactions of zinc and glycine in controlling NMDA receptor-mediated neurotransmission. The glycine concentration in the extracellular space has been estimated to be at least 1 μM (45–47); this concentration is sufficient to almost saturate the glycine receptor [as measured in radioligand binding studies (13, 24, 26)] and to produce nearly maximal facilitation of NMDA-evoked currents (38, 48). These findings suggest that the glycine binding site is occupied by endogenous glycine to a considerable extent at all times. This has raised questions as to whether and how the glycine recognition site regulates NMDA receptor-mediated neurotransmission. However, in contrast to glycine, zinc is released in a calcium- and stimulus-dependent manner (2, 3, 6). We propose that synaptically released zinc removes the tonic excitatory influence of glycine. The noncompetitive action of zinc circumvents the persistently high concentrations of glycine. In this way, zinc release may be a physiologic mechanism for limiting excitatory neurotransmission and, consequently, cell damage. The extent to which a failure of zinc inhibition contributes to neurotoxicity and other pathologic processes remains to be determined.

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