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ACCELERATED COMMUNICATION

Multiple Sites for the Regulation of the *N*-Methyl-D-Aspartate Receptor

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SUMMARY

The *N*-methyl-D-aspartate (NMDA) receptor consists of a recognition site for NMDA, a cation-selective ion channel, and binding sites for glycine, Zn^{2+}, and phencyclidine-like compounds. In addition, the channel can be blocked by Mg^{2+}. We have studied the NMDA receptor using the potent and specific phencyclidine-like compound [^{3}H]MK-801. Drugs that bind to the NMDA, glycine, Zn^{2+}, and Mg^{2+} recognition sites profoundly affect both the association and the dissociation rate of [^{3}H]MK-801. NMDA-like agonists, glycine, and Mg^{2+} all increase the rates of association and dissociation of [^{3}H]MK-801, whereas the NMDA antagonists AP5 and Zn^{2+} decrease these rates. These data allow the construction of a model of drug interaction at the NMDA receptor that is based on the binding of MK-801 within the NMDA-operated channel. Using this model it is possible to clearly distinguish between drug action at any of the five binding sites proposed.

The NMDA subtype of the glutamate receptor is an important modulator of synaptic activity in the central nervous system. In this receptor complex, binding of NMDA to its recognition site is believed to activate an ionophore that is permeable to both Ca^{2+} and monovalent cations (1–4). NMDA receptors can be blocked by competitive antagonists such as AP5 and CPP^+ (5, 6) and by a range of noncompetitive antagonists, including phencyclidine, Mg^{2+}, and Zn^{2+} (7–12). The novel phencyclidine-like antagonist MK-801 has recently been used as a probe for the NMDA receptor (13–17). Binding of [^{3}H]MK-801 to rat brain membranes is dependent on the presence of NMDA agonists and can be regulated by competitive and noncompetitive NMDA antagonists (14, 15). In addition glycine, which enhances the activity of NMDA electrophysiologically, also increases [^{3}H]MK-801 binding (15, 18–21). Thus, it appears that [^{3}H]MK-801 can "sense" some agonist-induced alteration in the receptor associated with channel opening. In this study we have examined the kinetics of [^{3}H]MK-801 binding to the NMDA receptor and have found specific effects of a number of important agents that modulate channel opening. These purely biochemical data allow us to construct a model of the receptor that is entirely consistent with current biophysical concepts.

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Materials and Methods

Drugs and chemicals. Labeled (±)-[^{3}H]MK-801 and unlabeled (+)-MK-801 were obtained from Dr. Geoffrey Woodruff, Merck, Sharp and Dohme, Terlings Park, U.K. All other chemicals were obtained from commercial sources.

Radioreceptor binding assays. Well washed rat brain membrane preparations were prepared as previously described (15). Association rate constants were determined as follows. Binding assays typically contained 0.5 mg of membrane protein, ligand, and drugs where appropriate in a volume of 0.5 ml of HEPES, pH 7.4, at room temperature (20–24°). Four ligand concentrations were used between 0.5 and 30 nM. Association curves were generated by measuring binding at up to 10 time points between 1 and 120 min. Values for the association rate constant were derived by linear regression of the apparent association rate constant (k_{obs}) plotted against ligand concentration as previously described (22). Curves thus obtained did not appear to deviate from linearity and had regression coefficients of >0.94. Dissociation rate constants were obtained from the analysis of association data (22) or from direct experiments that were performed as follows. Membranes, at a protein concentration of 3 mg/ml in 20 mM HEPES, pH 7.4, were incubated with 1–2 nM [^{3}H]MK-801 for at least 90 min. Aliquots (50 μl) of the tissue/ligand mixture were added to 4 ml of buffer containing drugs, cations, and glutamate (100 μM) and glycine (30 μM) as appropriate and vortexed to terminate the association reaction. After an appropriate period of time, between 1 and 120 min, the dissociation reaction was terminated by filtration as previously described (15). The dissociation rate constant was determined by linear regression of the curve derived by plotting $\ln(B_t/B_o)$ against time (22) where B_o is the

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; CPP, 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid; AP5, 2-amino-5-phosphonopentanoate.

fraction bound at the termination of association, and B_t is the amount bound at time t.

Results and Discussion

We and others have previously demonstrated that [^{3}H]MK-801 binds to well washed rat brain membranes and that binding can be completely blocked by NMDA antagonists such as CPP$^+$ and AP5 (14, 15, 23). In contrast, the binding of [^{3}H]MK-801 can be further enhanced by the addition of exogenous glutamate and glycine, which increase drug affinity (15, 19–21). The addition of a combination of saturating concentrations of glutamate (100 μM) and glycine (30 μM) increased both the association and dissociation rates of [^{3}H]MK-801 binding to brain membranes (Fig. 1; Tables 1 and 2). Glutamate or glycine alone produced similar but smaller effects (data not shown). Addition of the competitive NMDA antagonist DL-AP5 slowed both the association and dissociation rates of [^{3}H]MK-801 (Fig. 1; Tables 1 and 2), an effect that was more pronounced when these had been increased by the addition of exogenous glutamate and glycine. Much higher concentrations of DL-AP5 were required to produce these actions in the presence of glutamate, suggesting a competitive interaction (Tables 1 and 2). In contrast, the addition of excess unlabeled MK-801 did not increase the dissociation rate of [^{3}H]MK-801 (Table 2).

We have also previously demonstrated that several cations can block [^{3}H]MK-801 binding to rat brain membranes (16, 23, 24). We found that there were two classes of inhibitory cations. The first class, typified by Mg^{2+}, and including Co^{2+}, Mn^{2+}, and Ni^{2+}, increased in potency in the presence of exogenously added agonist. The second group, which included Cd^{2+} and Zn^{2+}, became less potent in the presence of exogenous glutamate and glycine. An evaluation of the effects of these respective classes of cation on the kinetics of [^{3}H]MK-801 binding revealed striking differences in the mechanism of action of ions from the two classes. Mg^{2+} produced a marked increase in the association and dissociation rates of [^{3}H]MK-801 (Fig. 1; Tables 1 and 2). Other cations in this class also increased the rate of [^{3}H]MK-801 dissociation (Fig. 2; Table 2). The divalent cation Zn^{2+} is the most potent cation inhibitor of [^{3}H]MK-801 binding (24) and of the electrophysiological and neurotoxic effects of NMDA (11, 12). In complete contrast to the actions of Mg^{2+} and related cations, Zn^{2+} decreased the association and dissociation rates of [^{3}H]MK-801 binding (Tables 1 and 2). This is qualitatively similar to the effects of DL-AP5. However, Zn^{2+} is clearly not a competitive antagonist of NMDA or glycine as we have previously shown that the addition of an excess of glutamate and glycine only slightly decreases the potency of Zn^{2+} (24). We also observed that Cd^{2+} decreased the dissociation rate of [^{3}H]MK-801 in a similar fashion to Zn^{2+} (Fig. 2; Table 2).

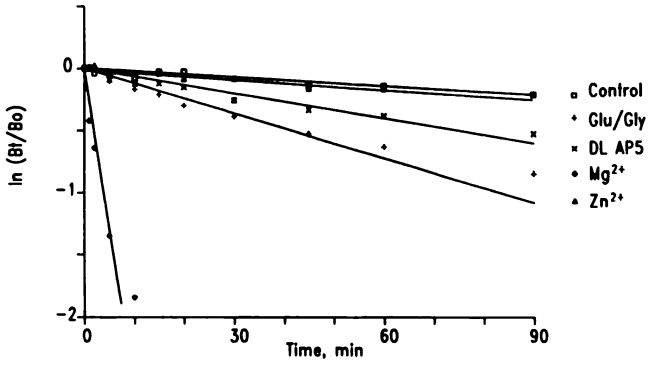

Fig. 1. Effects of Mg^{2+}, Zn^{2+}, and DL-AP5 on the dissociation on [^{3}H]MK-801 from well washed rat brain membranes. Dissociation experiments were performed as described in Materials and Methods. Tissue/ligand mixtures were added to buffer containing 100 μM glutamate and 30 μM glycine except in the control curve. In addition, 0.33 mM Mg^{2+}, 0.015 mM Zn^{2+}, or 1.0 mM DL-AP5 were added as shown. The data shown represent a typical experiment that was repeated three to seven times with essentially similar results.

TABLE 1

Effects of drugs and divalent cations on the association and dissociation rates of [^{3}H]MK-801 binding to rat brain membranes

Association assays were performed as described in Materials and Methods. Drugs and cations were added to the [^{3}H]MK-801 association reaction at concentrations that half maximally inhibit equilibrium binding (24). Glutamate and glycine when used were added at concentrations of 100 μM and 30 μM, respectively, which saturate the NMDA and glycine receptors (15). The data shown are the mean of three to five separate experiments. Statistical values were calculated using ANOVA.

	[Inhibitor]	Association rate		Dissociation rate	
		Mean	SE	Mean	SE
	mM	min^{-1} M^{-1} × 10^6		min^{-1} × 10^{-3}	
No added agonists					
Control		1.75	0.42	17.9	2.1
DL-AP5	0.002	0.81	0.25	19.5	2.8
Mg^{2+}	10.0	10.52[a]	2.88	115[b]	8.0
Zn^{2+}	0.007	1.01	0.29	12.7	2.1
+ Glutamate and glycine					
Control		6.57[a]	2.33	28.5[b]	2.8
DL-AP5	1.00	1.75[c]	0.53	23.3	1.9
Mg^{2+}	0.33	18.15[c]	3.37	224[d]	56
Zn^{2+}	0.015	1.29[c]	0.55	15.8[d]	0.5

[a] Significantly different from the control association rate in the absence of glutamate and glycine, $p < 0.05$.
[b] Significantly different from the control dissociation rate in the absence of glutamate and glycine, $p < 0.05$.
[c] Significantly different from the control association rate in the presence of glutamate and glycine, $p < 0.05$.
[d] Significantly different from the control dissociation rate in the presence of glutamate and glycine, $p < 0.05$.

TABLE 2

Effects of drugs and divalent cations on the dissociation rate of [^{3}H]MK-801 bound to rat brain membranes.

Binding assays were performed as described in Materials and Methods. Drugs and cations were added to the [^{3}H]MK-801 dissociation reaction, after equilibration of [^{3}H]MK-801, at concentrations that half maximally blocked equilibrium binding (24). Glutamate and glycine when used were added at concentrations of 100 μM and 30 μM, respectively, which saturate the NMDA and glycine binding sites (15), after equilibration with ligand. MK-801 did not further increase the dissociation rate produced by diluting the reaction mixture 80-fold, indicating that the dilution was effectively infinite. The results shown are the mean of three to seven experiments. Statistical values were calculated using ANOVA.

Condition	Control			+ Glutamate and glycine		
	[Inhibitor]	Dissociation rate		[Inhibitor]	Dissociation rate	
		Mean	SE		Mean	SE
	mM	min^{-1} × 10^{-3}		mM	min^{-1} × 10^{-3}	
Control		1.8	0.2		11.5[a]	0.8
DL-AP5	0.002	2.0	0.4	1.00	7.3[b]	0.8
Mg^{2+}	10.00	14.9[a]	8.7	0.33	81.7[b]	14.1
MK-801	0.0001	1.7	0.2	0.0001	15.0	1.9
Zn^{2+}	0.007	1.9	0.8	0.015	3.6[b]	1.3
Cd^{2+}				0.015	3.5[b]	0.3
Co^{2+}				0.10	32.3[b]	1.2
Mn^{2+}				0.08	81.2[b]	17.9
Ni^{2+}				0.115	21.2[b]	1.5

[a] Significantly different from the control dissociation rate in the absence of glutamate and glycine, $p < 0.01$.
[b] Significantly different from the control dissociation rate in the presence of glutamate and glycine, $p < 0.01$.

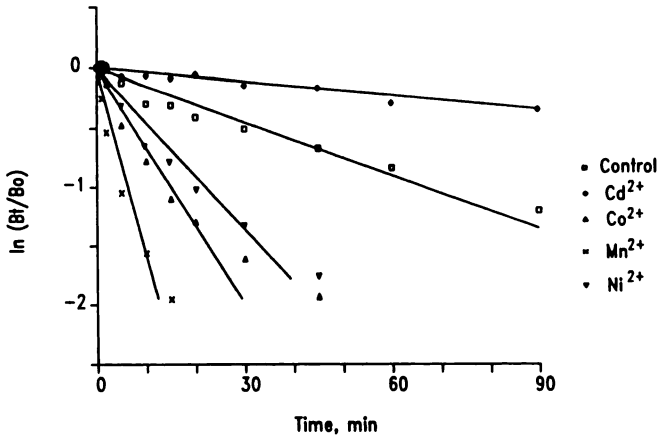

Fig. 2. Effects of divalent cations on the dissociation of [^{3}H]MK-801 from well washed rat brain membranes. Mg^{2+}-like cations, including Co^{2+} (0.10 mM), Mn^{2+} (0.08 mM), and Ni^{2+} (0.115 mM) at concentrations sufficient to half maximally decrease equilibrium binding of [^{3}H]MK-801 (24) increase its dissociation rate in the presence of glutamate and glycine (*Control*). Conversely, the Zn^{2+}-like cation Cd^{2+} (0.015 mM) decreases the dissociation rate. See also Table 2. The data represent a typical experiment performed in duplicate and was repeated twice with essentially similar results.

Assuming a simple bimolecular interaction, one can calculate values for the affinity of [^{3}H]MK-801 from the kinetic values shown in Table 1. These calculations give values of 10.2, 24.1, 11.0, and 12.6 nM for binding in the absence of added agonists and in the presence of DL-AP5, Mg^{2+}, and Zn^{2+}, respectively. In the presence of glutamate and glycine these values become 4.33, 13.3, 13.3, and 12.2 nM. Thus, in the presence of DL-AP5, Mg^{2+}, and Zn^{2+} the affinity of [^{3}H]MK-801 decreases, consistent with the ability of these agents to inhibit equilibrium [^{3}H]MK-801 binding (24). It should be noted that there is some discrepancy in the dissociation rate constants measured using association and dissociation experiments (Tables 1 and 2, respectively). Thus although the same trends exist, in that glutamate, glycine, and Mg^{2+} increase whereas DL-AP5 and Zn^{2+} decrease the dissociation rate of [^{3}H]MK-801, the values measured directly indicate rates significantly slower than those shown in Table 1. The basis of this discrepancy is not clear but it may imply a mechanism of MK-801 binding that is more complicated than the simple bimolecular model used here. The slower dissociation rate is unlikely to reflect rebinding of [^{3}H]MK-801, as the addition of unlabeled MK-801 does not increase the dissociation rate of [^{3}H]MK-801. Inspection of the dissociation curves shown in Figs. 1 and 2 suggests that they may be biphasic, particularly when the rates were accelerated by the addition of glutamate and glycine or certain divalent cations. Although this may reflect some complexity in the kinetics of [^{3}H]MK-801 binding not apparent from our present analysis, we are cautious in making such an interpretation as the ligand used in this study was racemic.

It has been shown that the inhibition of NMDA-induced currents by MK-801 and ketamine is use dependent (13, 17, 25). Thus, channel block occurs only in the presence of agonist. Similarly, recovery from block is also dependent on the presence of agonist. Indeed, under certain conditions MK-801 and ketamine can apparently become trapped within the ionophore (17, 25). Our data provide a biochemical correlate of these findings. The increase in the rate of association and dissociation of [^{3}H]MK-801 produced by agonists can be viewed as reflecting an increase in access of the drug to its binding site within the ionophore. The agonist-induced increase in the potency of Mg^{2+} and related cations may also be a reflection of an increased ability to enter the channel (24). The binding sites for Mg^{2+} and MK-801-like drugs are both apparently located inside the channel, as suggested from the strong voltage dependence of the block they produce (3, 7, 8). However, the present study clearly demonstrates that the interaction of Mg^{2+} with the [^{3}H]MK-801 binding site is noncompetitive, as Mg^{2+} increases the dissociation rate of [^{3}H]MK-801. Thus, it is likely that the sites are allosterically coupled.

Our findings also demonstrate the presence of a third site for the regulation of channel activity, namely, that occupied by Zn^{2+}. This is in agreement with recent electrophysiological studies (11). Zn^{2+} appears to act by blocking the ability of glutamate to open the ionophore. Several observations are consistent with this idea. It has been shown that Zn^{2+} will inhibit the binding of agonists to the NMDA recognition site whereas Mg^{2+} does not (26). Furthermore, in contrast to the

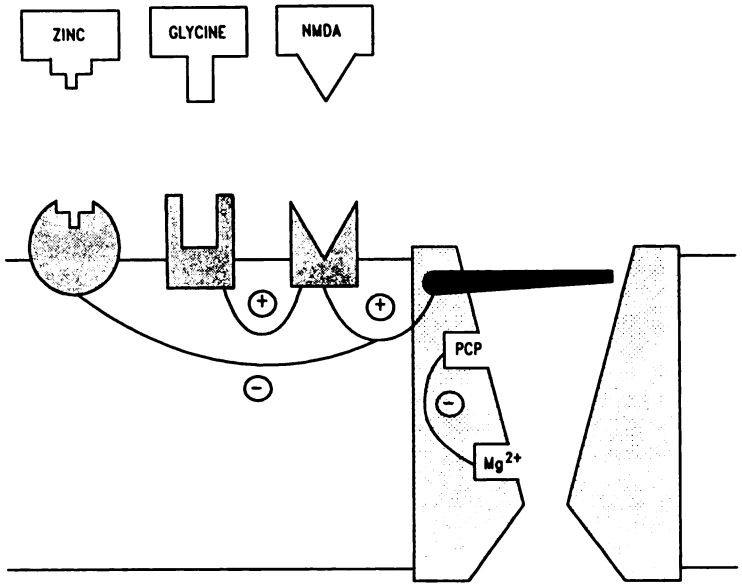

Fig. 3. Schematic model of drug and cation interaction with the NMDA receptor. The details of this model are described in the text.

actions of Mg^{2+}, the blocking effects of Zn^{2+} are not markedly voltage dependent, suggesting that Zn^{2+} acts at a superficial site that is not within the channel pore (11). Our results also explain the failure of drugs acting at the phencyclidine binding site to inhibit ligand binding to the NMDA recognition site (27). Thus, the phencyclidine binding site is not allosterically coupled to the NMDA recognition site. Instead, NMDA controls the access of phencyclidine to its binding site within the channel.

On the basis of our binding studies together with the appropriate biophysical observations we propose the model illustrated in Fig. 3. According to this model, the phencyclidine binding site, to which [^{3}H]MK-801 also binds, is located inside the channel. This location is based on the voltage dependency of the blocking action of phencyclidine-like drugs (25). Access to this site is controlled by drugs that act at the NMDA receptor and modify the opening of the channel. This is also true for Mg^{2+} and other cations that become more potent in the presence of agonist (24). Mg^{2+}-like cations act at a site that is allosterically coupled to the phencyclidine binding site. In turn, the action of NMDA is modulated by agents acting at the binding sites for glycine and Zn^{2+}.

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