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Ifenprodil is a Novel Type of *N*-Methyl-D-aspartate Receptor Antagonist: Interaction with Polyamines

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SUMMARY

We have investigated the interactions of polyamines and the *N*-methyl-D-aspartate (NMDA) receptor antagonist ifenprodil with the binding of [^{3}H]MK801 to the NMDA receptor. Spermine and spermidine but not putrescine substantially increase [^{3}H]MK801 binding to well washed rat brain membranes in the absence or presence of saturating concentrations of glutamate and glycine. Spermine also increased the association and dissociation of [^{3}H]MK801 from its binding site, suggesting that polyamines activate the NMDA receptor in a similar manner to glycine. Ifenprodil inhibited the binding of [^{3}H]MK801 in a biphasic fashion. The high affinity phase of binding (K_i of approximately 15 nM) accounted for 50–60% of total [^{3}H]MK801 binding in the nominal absence of glutamate, glycine, and polyamines or in the presence of 100 μM glutamate. This fraction was reduced to 20% by the addition of 30 μM glycine and could be abolished by the addition of 50 μM spermine. However, ifenprodil apparently did not act by binding to the polyamine recognition site. The low affinity phase (K_i of 20–40 μM) was insensitive to the presence of positive modulators and may represent binding to the Zn^{2+} regulatory site. Ifenprodil decreased NMDA and glycine-induced Ca^{2+} influx into cultured rat brain neurons. The potency of ifenprodil suggests that spermine may activate NMDA receptors *in vivo*. These data indicate that ifenprodil may bind to the NMDA receptor in a state-dependent fashion and preferentially stabilize an inactivated form of the channel.

Many compounds normally found in the central nervous system can profoundly influence the activity of the NMDA-preferring subtype of glutamate receptor. Such compounds include Mg^{2+} (1, 2) and Zn^{2+} (3, 4), which decrease the current carried by the NMDA-gated ion channel, and glycine, which enhances the effects of NMDA and glutamate (5–8). A recent addition to the list of modulators has been the polyamines spermine and spermidine. These compounds, which are found in virtually all cells, are normally associated with the control of cell division. However, a recent study found that spermine and spermidine could profoundly enhance [^{3}H]MK801 binding to the NMDA receptor (9). By analogy with the observed effects of glycine and glutamate in similar circumstances (10–12), these data suggest that polyamines may enhance activation of the NMDA receptor or decrease desensitization (13).

There are a number of neuropathological conditions that are believed to be associated with the "overstimulation" of NMDA receptors. These include ischemia, including stroke (14), epilepsy (15), and possibly some neurodegenerative disorders such as Huntingdon's disease (16). Effective antagonists of the NMDA receptor might have therapeutic utility in these disorders. However, the antagonists that have been tested so far, which include competitive NMDA antagonists such as AP7 and CPP and channel blockers such as MK801, also have significant behavioral effects in animal models that may limit their use (17). An alternative approach would involve blockade of the effects of one of the positive modulators of receptor activity. To this end, competitive glycine antagonists have recently been described (18–20). Reducing the positive modulation by endogenous spermine may also have therapeutic potential.

In this study, we have explored the effects of polyamines on [^{3}H]MK801 binding by monitoring the actions of spermine on nonequilibrium binding and binding kinetics. We have also investigated the effects of the NMDA receptor antagonist ifenprodil. Ifenprodil is a neuroprotective agent that has been reported to act as an NMDA antagonist, although its precise mechanism of action remains unclear (21, 22). In addition, ifenprodil also alters cerebral blood flow (23) by interaction with α-adrenoceptors and possibly voltage-sensitive Ca^{2+} channels (24, 25). We have employed the [^{3}H]MK801 binding assay to determine the mechanism of action of ifenprodil, because this assay can differentiate drug action at five or more binding sites on the NMDA receptor, including the sites that recognize

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AP7, aminophosphoneheptanoic acid; CPP, 3-(2-carboxypiperazin-4 yl)propylphosphonic acid; MK801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate; HEPES, 4-(2-hydroxyethyl)-1-piperezineethanesulfonic acid.

glutamate, glycine, Mg^{2+}, and Zn^{2+} as well as MK801 and phencyclidine-like drugs (10). In comparison with ifenprodil, we have examined the interactions between spermine and three NMDA antagonists that share a similar site of action but have widely differing potencies, namely phencyclidine, ketamine, and milacemide. These drugs are all competitive inhibitors of [^{3}H]MK801 binding. We have also measured NMDA and glycine-induced intracellular Ca^{2+} changes in cultured neurons to assay physiological effects of the antagonists under study. We have found that, although the actions of ifenprodil are significantly altered by the addition of spermine, this compound apparently does not simply compete with the polyamine. Furthermore, ifenprodil appears to block the NMDA receptor in a novel manner that is not shared by any previously described antagonist.

Materials and Methods

Radioreceptor binding assays. Nonequilibrium [^{3}H]MK801 binding assays to well washed rat brain membranes were performed as previously described (10, 20). Ligand (0.3–0.5 nM), tissue, and drugs as appropriate were incubated in a volume of 1 ml of HEPES/NaOH (pH 7.4) for 2 hr at room temperature (20–24°). Previous studies have demonstrated that, in the absence of agonists, ligand binding to the phencyclidine site does not reach equilibrium within 2 hr. However, when glutamate or glycine is added, equilibrium is reached more quickly, because agonists increase access of the ligand to the binding site (10–12). However, the affinity of the site is not altered (12). By using conditions where equilibrium will not be reached without agonist being present, the [^{3}H]MK801 assays can be used to detect agonist effects on the NMDA receptor.

Association rates were measured using 18–22 time points up to 240 min, using a single concentration of [^{3}H]MK801 (2–3 nM). Nonspecific binding was determined using 30 μM unlabeled MK801 at 1 min and 240 min and was usually similar. Values of nonspecific binding at 24 min were used in all cases. Dissociation curves were performed as previously described (10), using 10 time points between 1 and 180 min. In order to determine drug effects on the dissociation of [^{3}H]MK801, a modification of a previously employed technique was used (10). We wanted to determine the dose dependence of drug effects on the dissociation rate of [^{3}H]MK801. Thus, we determined the effect of a range of drug concentrations on [^{3}H]MK801 binding at a single time point on the dissociation curve, 60 min. In this paradigm, drugs that enhance binding are expected to increase the dissociation rate and, therefore, decrease the amount of [^{3}H]MK801 bound at any given time point. Conversely, inhibitors such as Zn^{2+}, which act by closing the channel, will trap the ligand inside the channel and increase the bound [^{3}H]MK801 remaining at any given time. To assay inhibitor action in this paradigm, we also added glutamate, glycine, and Mg^{2+} to the diluting buffer, as previously described (26).

Data from inhibitor competition curves were analyzed using nonlinear least squares curve-fitting (EBDA and LIGAND, purchased from Elsevier Biosoft, New York). Biphasic inhibition curves employed 20–22 drug concentrations in duplicate. When fitting data to two sites, the affinity of [^{3}H]MK801 for each site was assumed to be identical, because there is no evidence for a multiplicity of [^{3}H]MK801 binding sites. As described above, effective measurement of the interaction between agonists and antagonists is aided by employing nonequilibrium conditions. The term K_{app} is used to describe the apparent inhibition constants found in this study. It is unlikely that these values represent absolute parameters for the interaction of inhibitors with the NMDA receptor. However, a comparison of these values is appropriate, because experiments were performed using the same time of incubation. Monophasic curves were estimated using 10 or 11 drug concentrations, also in duplicate. Differing values for the apparent affinity of [^{3}H]MK801 were used in curve fitting as appropriate for the assay conditions. Parameters for binding to two sites are given where this provided a significantly better fit of the data ($p < 0.05$, partial F test). Data from association and dissociation curves were fitted using the KINETIC program (Elsevier Biosoft). Estimates for EC_{50} values, as well as basal and maximum levels of binding for compounds that increased [^{3}H]MK801 binding, were made using a Simplex routine provided by Dr. Lane Hirning (Natural Product Sciences Inc., Salt Lake City, Utah), using the equation:

$$E = E_{max} \cdot \frac{D}{(D + EC_{50})} + B$$

where E is the increase in binding for a given drug concentration, D, with a maximal response E_{max} and basal (unstimulated) binding of B.

Intracellular Ca^{2+} measurements. NMDA and glycine-induced changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured in individual rat brain neurons in primary culture, as previously described (6, 20). Control responses were obtained in the presence of 10 μM NMDA and 3 μM glycine in the absence of extracellular Mg^{2+}. The agonists were then washed out. When the $[Ca^{2+}]_i$ level returned to baseline (usually 4–5 min), inhibitors were added as appropriate. After a further incubation of 4–5 min, a second dose of NMDA and glycine was added. Control responses obtained without inhibitor were 94 ± 7% of the first response. Second responses were corrected for this small discrepancy before statistical evaluation.

Materials. (+)-[^{3}H]MK801 (15 Ci/mmol) was obtained from Du Pont/New England Nuclear. Unlabeled (+)-MK801 was obtained from Merck Sharp and Dohme (Westpoint, PA). Ifenprodil was provided by Synthelabo (Paris, France). Milacemide was obtained from G. D. Searle (St. Louis, MO). Polyamines were purchased from Sigma (St. Louis, MO). Other chemicals were from commercial sources.

Results

We initially evaluated the effects of the polyamines spermine, spermidine, and putrescine on [^{3}H]MK801 binding to well washed rat brain membranes. As previously reported (9), spermine and spermidine substantially increased [^{3}H]MK801 binding, whereas putrescine was without effect (Fig. 1). Spermine was somewhat more potent than spermidine (EC_{50} of 8.89 ± 3.66 and 13.93 ± 0.91 μM, mean ± SE; six and three experiments for spermine and spermidine, respectively). Both were similarly efficacious. Polyamines did not enhance binding in the presence of the competitive NMDA antagonist CPP (data not shown). Putrescine (500 μM) did not prevent the enhancement of binding produced by spermine (data not shown). Glutamate (100 μM) and glycine (30 μM) in combination increased [^{3}H]MK801 binding by 3–5 fold. Spermine and spermidine produced a smaller increase in binding in the presence of a saturating concentration of glutamate and glycine (Fig. 1B), although the maximum level of binding was similar. In the presence of glutamate and glycine, EC_{50} values were 2.60 ± 0.57 and 5.20 ± 0.78 μM (mean ± SE; three experiments) for spermine and spermidine, respectively. Under both conditions, higher concentrations of polyamines reduced the maximum enhancement seen. This was most pronounced for spermine in the presence of glutamate and glycine (Fig. 1B). We also tested lysyllysine and poly-L-lysine (mean molecular weight, 3800). Polylysine increased binding of [^{3}H]MK801 between 2 and 20 μM, whereas lysyllysine was substantially less potent (data not shown).

We next examined the effects of ifenprodil on [^{3}H]MK801 binding. Ifenprodil inhibited binding over the concentration range 1 nM to 0.5 mM in the nominal absence of glutamate, glycine, and polyamines (Fig. 2A). Analysis of the inhibition

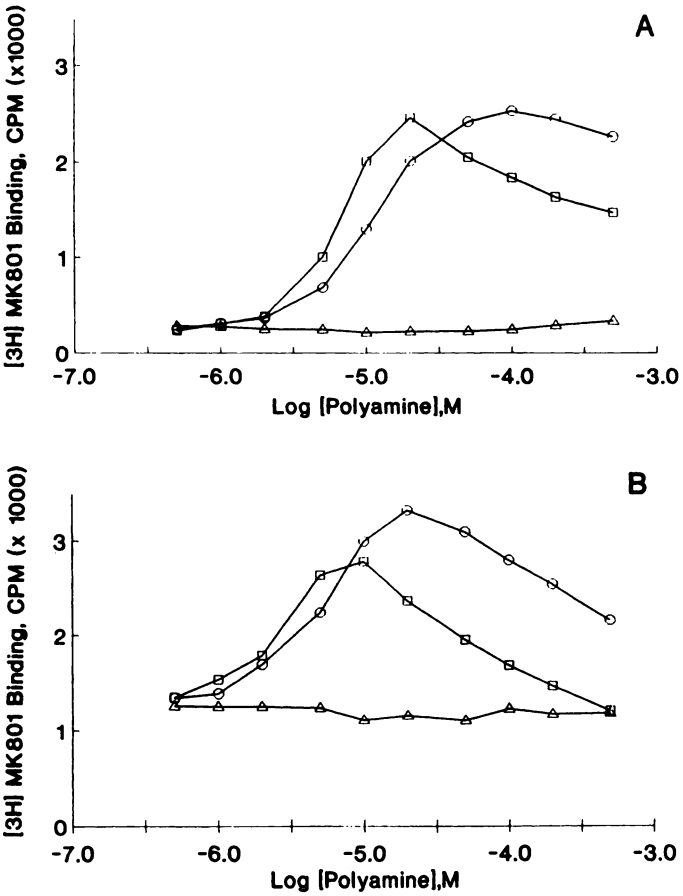

Fig. 1. Effects of polyamines on [^{3}H]MK801 binding to well washed rat brain membranes. Spermine (□), spermidine (○), and putrescine (△) were added to membranes in the nominal absence (A) or presence (B) of glutamate (100 μM) and glycine (30 μM). Results represent a typical experiment performed in duplicate that was repeated two further times with similar results.

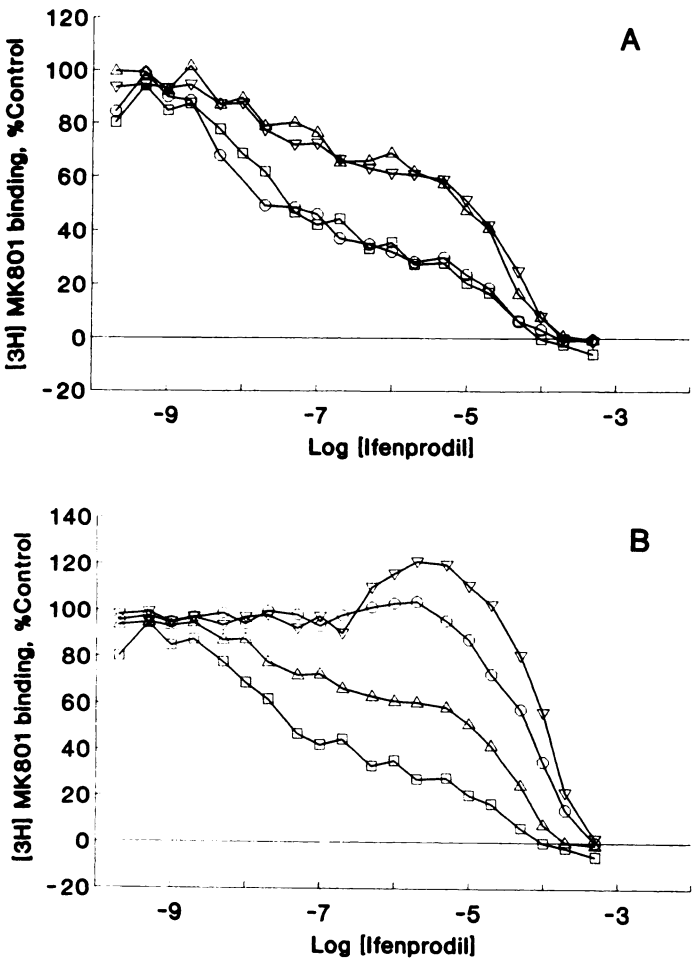

Fig. 2. Effects of glutamate, glycine, and spermine on the inhibition of [^{3}H]MK801 binding by ifenprodil. Ifenprodil dose-response curves shown in A were obtained in the nominal absence of glutamate and glycine (□) or in the presence of 100 μM glutamate (○), 30 μM glycine (△), or glutamate and glycine (▽). Curves shown in B were obtained in the nominal absence of glutamate and glycine (□) or in the presence of 50 μM spermine (○), 100 μM glutamate and 30 μM glycine (△) or glutamate, glycine, and spermine (▽). The results shown are from a typical experiment performed in duplicate, which was repeated two or three further times with similar results (see Table 1) and are expressed as a percentage of the appropriate control condition with agonists added but no inhibitor. Thus, control binding was lowest in the absence of agonists and was highest in the presence of spermine (see Fig. 1). As indicated, ifenprodil at high concentrations reduced [^{3}H]MK801 binding below the nonspecific binding level defined by 10 μM unlabeled MK801.

curve indicated that there were two phases of inhibition. The high affinity portion was observed between 1 and 100 nM, whereas a second, lower affinity, phase was seen between 1 and 100 μM. Binding parameters for ifenprodil are shown in Table 1. We have previously shown that manipulation of the concentrations of the various positive modulators of [^{3}H]MK801 binding can distinguish between drug action at glutamate and glycine binding sites. Addition of 100 μM glutamate, although increasing levels of control binding, did not alter the interaction of ifenprodil with [^{3}H]MK801 binding. The inclusion of 30 μM glycine decreased the fraction of binding sensitive to low concentrations of ifenprodil (Fig. 2A; Table 1) from approximately 50% to 20%. However, the affinity of ifenprodil for this site was unchanged by the addition of glycine. The combination of glutamate and glycine increased levels of control binding still further but produced qualitative effects similar to those produced by glycine alone (Fig. 2A; Table 1). The affinity of ifenprodil for the low affinity site was unchanged by these manipulations of the binding conditions.

A further determination of the nature of the interaction of ifenprodil with [^{3}H]MK801 binding was provided by the data in Table 2 and Fig. 3. The effects of increasing concentrations of ifenprodil on dose-response curves to glutamate and glycine were monitored. The results shown in Table 2 indicate that ifenprodil, over a wide concentration range, did not alter the affinity of either glutamate or glycine for their respective sites on the NMDA receptor. However, it is clear that ifenprodil decreased the maximum response to both glutamate and glycine. This clearly indicates that the interaction between ifenprodil and either glutamate or glycine is not competitive in nature. Moreover, the response to glycine was affected less by ifenprodil than glutamate, which is consistent with data shown in Fig. 1 and Table 1.

An examination of the effects of spermine on ifenprodil dose-response curves is shown in Fig. 2B and Table 1. A supramaximal concentration of spermine (50 μM) completely abolished high affinity effects of ifenprodil, while having relatively minor effects on the low affinity binding parameters. In contrast, 0.2 mM putrescine had no effects on the ifenprodil dose-response curve (data not shown). Interestingly, a combination of 100 μM

TABLE 1

Effects of glutamate and glycine on ifenprodil inhibition of [^{3}H] MK801 binding

Binding assays were performed and analyzed as described in Materials and Methods. Results are the mean ± standard error of three experiments performed in duplicate. Twenty to twenty-two drug concentrations were used for each curve. Binding parameters for two sites are given when a two-site model provided a significantly better fit of the data (partial F test; $p < 0.05$).

Condition	$K_{app HIGH}$	$K_{app LOW}$	Fraction high
	nM	μM	
No additions	34.9 ± 12.4	12.6 ± 8.6	0.53
+100 μM Glutamate	13.2 ± 3.3	22.1 ± 7.2	0.59
+30 μM Glycine	17.7 ± 15.1	18.8 ± 0.9	0.21
+Glutamate and glycine	8.22 ± 1.4	15.3 ± 1.9	0.27
+50 μM Spermine		31.0 ± 3.4	0.0
+Glutamate, glycine, and spermine		51.6 ± 11.1	0.0

TABLE 2

Effects of ifenprodil on enhancement of [^{3}H]MK801 binding by glutamate and glycine

Binding assays were performed and analyzed as described in Materials and Methods. Results are the mean ± standard error of four or five experiments performed in duplicate. Fold increase in binding represents the ratio of maximum specific binding to the basal (i.e., low glutamate or glycine) binding and is expressed as a percentage of the fold increase in the absence of added ifenprodil. The changes in maximum and basal binding are also expressed as a percentage of the appropriate values in the absence of added ifenprodil.

[Ifenprodil]	EC_{50}	Maximum	Basal
nM	μM	% control	% control
Glutamate			
Control	0.29 ± 0.09	100	100
1	0.23 ± 0.04	88 ± 15	86 ± 11
10	0.23 ± 0.05	58 ± 9	69 ± 9
100	0.31 ± 0.19	38 ± 9	66 ± 12
1000	0.29 ± 0.16	24 ± 3	53 ± 11
Glycine			
Control	0.36 ± 0.16	100	100
1	0.37 ± 0.06	90 ± 10	98 ± 12
10	0.25 ± 0.06	88 ± 12	72 ± 6
100	0.36 ± 0.07	78 ± 10	57 ± 6
1000	0.40 ± 0.07	78 ± 10	47 ± 7

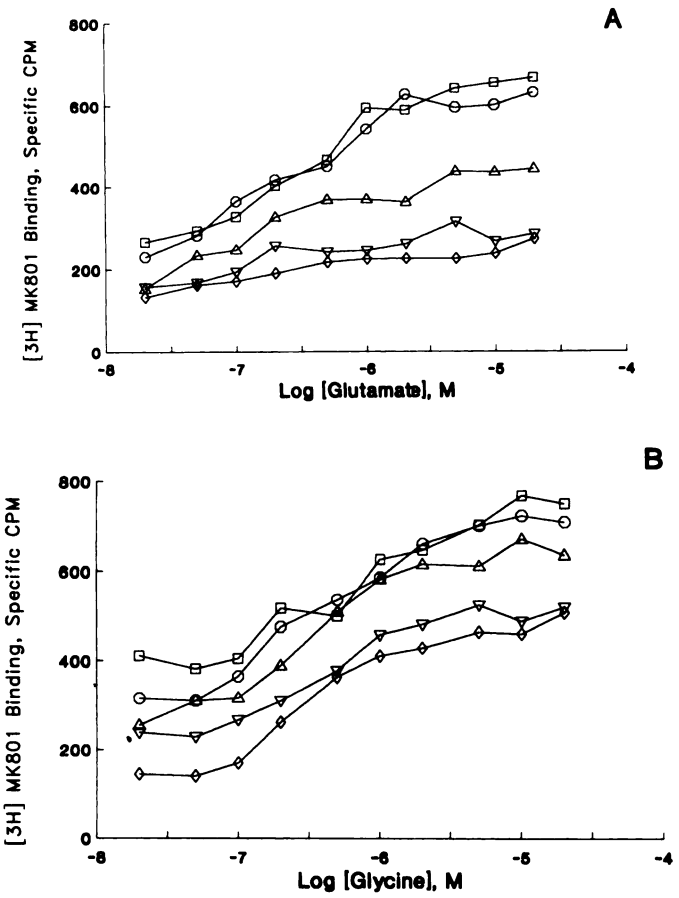

Fig. 3. Effects of ifenprodil on the enhancement of [^{3}H]MK801 binding by glutamate (A) and glycine (B). Dose-response curves were performed in the absence (□) or presence of 0.001 (○), 0.01 (△), 0.1 (▽), or 1.0 (◇) μM ifenprodil. Dose-response curves to glycine included 0.1 μM glutamate whereas dose-response curves to glutamate included 0.03 μM glycine. The curves are from a single experiment performed in duplicate that was repeated three or four times with similar results.

glutamate, 30 μM glycine, and 50 μM spermine revealed a stimulatory effect of ifenprodil in the concentration range 0.1 to 10 μM (Fig. 2B). A 20–40% increase in binding was seen in three of three experiments employing these conditions. The pharmacological basis of this effect is not clear. However, the bell-shaped nature of the response to spermine is more pronounced in the presence of glutamate and glycine (Fig 1B). Thus, if ifenprodil were to block the inhibitory effect of spermine, an enhancement of binding might be observed.

In order to elucidate the possible mechanism of action of ifenprodil, we compared the effects of spermine on the binding parameters of ifenprodil with the effects of the polyamine on the binding of phencyclidine, ketamine, and milacemide. These drugs are believed to be competitive antagonists of [^{3}H]MK801 binding. The increase in [^{3}H]MK801 binding produced by spermine suggests an increase in the apparent affinity of [^{3}H] MK801, as previously described (9). In contrast, the apparent affinity of phencyclidine, ketamine, and milacemide was decreased by spermine, although the effects were rather small (2-, 3-, and 6-fold, respectively; Table 3). Thus, spermine has a much greater effect on the inhibition of [^{3}H]MK801 binding by ifenprodil (Fig. 2; Table 1) than on the inhibition of binding by phencyclidine, ketamine, and milacemide. These data suggest that the mechanism underlying the inhibition of [^{3}H]MK801 binding by ifenprodil is different from simple competitive antagonism.

The effects of spermine on the inhibition of [^{3}H]MK801 binding produced by ifenprodil, acting at its high affinity site, suggested that this inhibitor might actually be competitive with respect to spermine. To test this hypothesis, we performed dose-response curves to spermine in the presence of increasing concentrations of ifenprodil, in an attempt to determine whether a parallel or nonparallel shift in the spermine curve would be observed. The results of this analysis are shown in Fig. 4. Concentrations of ifenprodil that were sufficient to depress basal levels of binding had no effect on either the EC_{50} for spermine or the maximal increase in binding produced by spermine. This indicated that spermine overcame the effects of ifenprodil in a noncompetitive fashion. Concentrations of ifenprodil corresponding to the low affinity phase of inhibition decreased the peak response and shifted the EC_{50} slightly to the right. We compared these results with those obtained with the channel blocker milacemide (Fig. 4B). Inhibitory concentrations of milacemide depressed the peak response to spermine and decreased its apparent potency. The decrease in peak response to spermine is consistent with a noncompetitive interaction between milacemide and spermine and serves to further accentuate the qualitative differences between the inhibi-

TABLE 3

Effects of spermine on the inhibition of [^{3}H]MK801 by milacemide, phencyclidine, and ketamine

Binding assays were performed and analyzed as described in Materials and Methods. Assays were performed with no additions, with 50 μM spermine alone, with 100 μM glutamate and 30 μM glycine, or with all three combined. Results represent the mean ± standard error of three to six experiments performed in duplicate.

Inhibitor	K_{app}			
	No addition	Spermine	Glutamate and Glycine	Combined
Phencyclidine (μM)	0.024 ± 0.003	0.037 ± 0.004	0.019 ± 0.006	0.048 ± 0.019
Ketamine (μM)	0.18 ± 0.03	0.45 ± 0.26	0.24 ± 0.10	1.08 ± 0.46
Milacemide (mM)	0.17 ± 0.06	0.96 ± 0.27	0.23 ± 0.06	1.50 ± 0.26

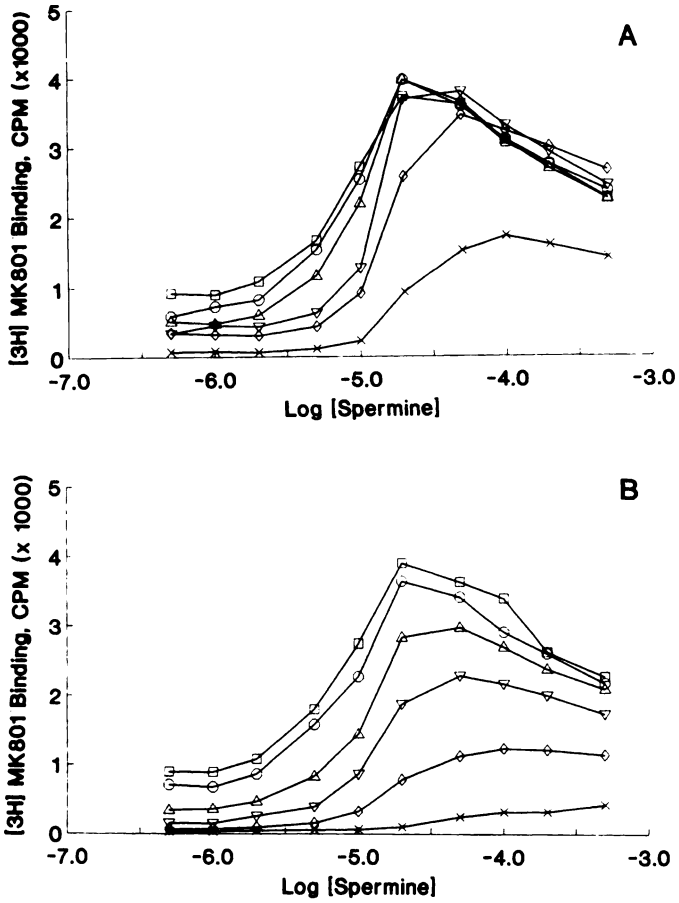

Fig. 4. Effects of ifenprodil and milacemide on the enhancement of [^{3}H] MK801 binding by spermine. A, Dose-response curves to spermine were performed in the absence (□) or presence of 0.01 (○), 0.1 (△), 1.0 (▽), 10 (◇) or 100 μM (×) ifenprodil in the absence of glutamate and glycine. B, Dose-response curves to spermine were performed in the absence (□) or presence of 0.1 (○), 0.3 (△), 1.0 (▽), 3.0 (◇), or 10 mM (×) milacemide in the absence of glutamate and glycine. Results shown are typical experiments performed in duplicate that were repeated two further times with similar results.

tion of [^{3}H]MK801 binding produced by ifenprodil and the phencyclidine-like compounds.

In order to further understand the action of the polyamines in this system, we explored the effects of spermine on the association and dissociation of [^{3}H]MK801 (Fig. 5). In the presence of low levels of glutamate and glycine, [^{3}H]MK801 associated very slowly. The addition of saturating amounts of these amino acids increased the association rate dramatically. Similarly, 50 μM spermine also increased the association rate (Fig. 5A). In two of three experiments, the association curves were better described by a biphasic fit of the data. Both glutamate and glycine in combination and spermine alone increased the dissociation rate of [^{3}H]MK801 (Fig. 5B). However, whereas the dissociation curve in the absence or presence of glutamate and glycine was monophasic, the curve in the presence of spermine was clearly biphasic. The combination of monophasic and biphasic curves in these experiments highlights the complexity of the binding process for [^{3}H]MK801. We have previously shown that it is possible to distinguish between modulators acting at various sites on the NMDA receptor complex by monitoring drug effects on the dissociation rate of [^{3}H]MK801. Fig. 5C shows the dose dependence of ifenprodil actions on the amount of ligand dissociated in 60 min in the presence of Mg^{2+}. Lower concentrations of ifenprodil had rather modest effects on the dissociation of [^{3}H]MK801. Higher concentrations, corresponding to the low affinity phase of inhibition, slowed the dissociation. The effects of Zn^{2+} and milacemide are shown for comparison. Zn^{2+} also decreased the amount of [^{3}H]MK801 dissociated from the receptor at concentrations that inhibit equilibrium [^{3}H]MK801 binding. Milacemide clearly had no effect on the dissociation of [^{3}H]MK801 at any concentration, again consistent with its proposed competitive action at the [^{3}H]MK801 binding site.

Finally, we examined the actions of ifenprodil and milacemide on $[Ca^{2+}]_i$ changes produced by NMDA and glycine in cultured neurons from rat cortex (Fig. 6). Consistent inhibition of NMDA and glycine-induced calcium changes was only seen when ifenprodil concentrations above 300 nM were used. Thus, although significant inhibition was seen at 3 and 10 nM, the amount of inhibition was small (<20%), and significant inhibition was not observed between 30 and 300 nM. The potency of the inhibition produced by ifenprodil (half-maximal effects at approximately 10 μM) was similar to that observed for its low affinity binding site. Milacemide also prevented NMDA- and glycine-induced $[Ca^{2+}]_i$ changes. Milacemide produced half-maximal effects between 1 and 3 mM, corresponding to binding parameters observed when spermine was included in [^{3}H]MK801 binding assays.

Discussion

In this study we have investigated the interaction of polyamines with the NMDA receptor complex. We have also studied the effects of ifenprodil, an NMDA receptor antagonist whose mechanism of action has not previously been elucidated. Ifenprodil appears to be a novel type of NMDA antagonist, because its profile of inhibition of [^{3}H]MK801 binding is unique. Thus, low concentrations of ifenprodil do not compete with glutamate, glycine, or spermine for activation of the NMDA receptor, nor do its effects mimic those of Zn^{2+} or phencyclidine-like NMDA receptor antagonists. The analysis of the effect of ifenprodil on the dissociation of [^{3}H]MK801 suggests that, in common with

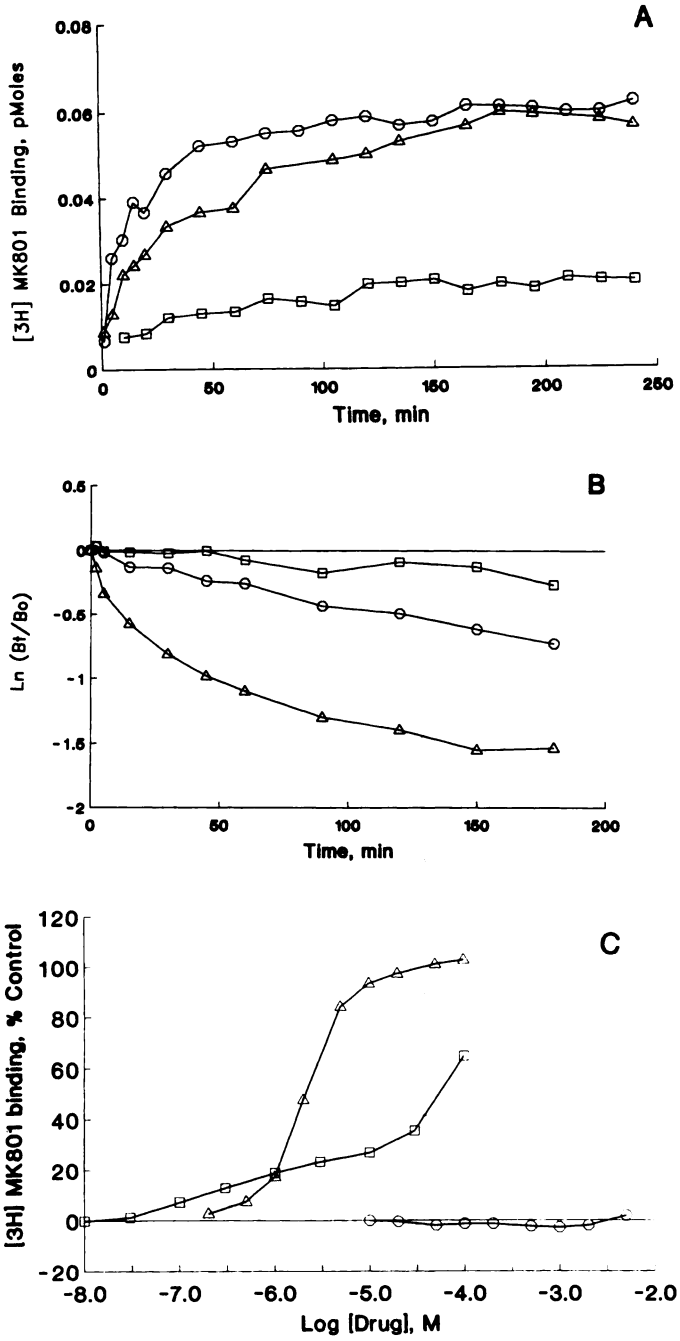

Fig. 5. Effects of polyamines and inhibitors on the kinetics of [^{3}H]MK801 binding. A, Association curves with [^{3}H]MK801 were performed as described in Materials and Methods. Curves were generated using 0.1 μM glutamate and 0.03 μM glycine (□), 100 μM glutamate and 30 μM glycine (△), or 50 μM spermine with 0.1 /M glutamate and 0.03 μM glycine (○). The results represent a single experiment ([[^{3}H]MK801] = 2.19 nM) performed in duplicate that was repeated two further times with similar results. The values of k_{obs} in this experiment were 0.0358 min^{-1} in the absence of glutamate and glycine, 1.42 and 0.0217 min^{-1} with glutamate and glycine (18.0% of binding to the fast phase), and 0.242 and 0.0249 min^{-1} with spermine (46.7% of binding to the fast phase). B, Dissociation curves with [^{3}H]MK801 were performed as described in Materials and Methods. Curves were generated using 0.1 μM glutamate and 0.03 μM glycine (□), 100 μM glutamate and 30 μM glycine (△), or 50 μM spermine with 0.1 μM glutamate and 0.03 μM glycine (○). The values of K_{-1} obtained in this experiment were 0.00147 min^{-1} in the absence of glutamate and glycine, 0.00384 min^{-1} with glutamate and glycine, and 0.0838 and 0.00540 min^{-1} with spermine (48.7% of binding to the fast phase). The results represent a single experiment performed in duplicate that was repeated two further times with similar results. C, Ifenprodil (□), milacemide (○), and Zn^{2+} (△) were added to the diluting buffer at the concentration shown, along with 100 μM glutamate, 30 μM glycine, and 300 μM Mg^{2+}. Control binding represents binding remaining 60 min after 120-fold dilution with the above mixture and 200 μM Zn^{2+}. This presumably represents the slowest dissociation rate pharmacologically obtainable and represents a fully closed state of the channel. The results shown represent a typical experiment performed in duplicate that was repeated two further times with similar results.

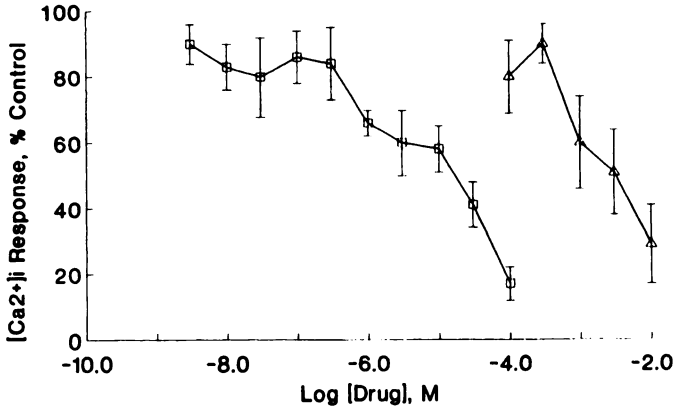

Fig. 6. Effects of ifenprodil and milacemide on changes in [Ca^{2+}] produced by NMDA and glycine. [Ca^{2+}]$_i$ measurements were as described under in Materials and Methods. The results shown are the response to NMDA (10 μM) and glycine (3 μM) in the presence of ifenprodil (□) or milacemide at the concentration shown, expressed as a percentage of the first (control) response. These values have been corrected to allow for the slight decay in the response seen without the addition of inhibitor. The results represent the mean ± standard error of 8–10 cells for ifenprodil and 6–8 cells for milacemide.

several other compounds (26), the low affinity phase of its action may be mediated by the Zn^{2+} site. However, the pharmacological basis of the high affinity portion of the actin of ifenprodil is more complicated.

The only compound previously shown to inhibit [^{3}H]MK801 binding in a biphasic or incomplete manner is HA-966 (20). HA-966 is a selective antagonist at the glycine site (18, 20). However, ifenprodil does not bind to the glycine site under the conditions employed in this study, inasmuch as glycine does not affect its potency. Interestingly, glycine alters the fraction of [^{3}H]MK801 binding that is sensitive to low concentrations of ifenprodil (Table 1), which suggests a noncompetitive interaction between ifenprodil and glycine. Similarly, it is unlikely that ifenprodil is binding to the NMDA/glutamate recognition site, inasmuch as glutamate does not alter any of its binding parameters. The concentrations of glutamate and glycine used increase [^{3}H]MK801 binding to a similar extent. However, the proportion of [^{3}H]MK801 binding sensitive to low concentrations of ifenprodil is different between glutamate- and glycine-stimulated binding (Table 1). Thus, the fraction of high affinity ifenprodil binding sites does not simply correlate with the level of [^{3}H]MK801 binding. Because spermine profoundly altered the ability of low concentrations of ifenprodil to inhibit [^{3}H]MK801 binding, we examined the possibility that ifenprodil was binding to the polyamine binding site. However, the results of the analysis shown in Fig. 4 clearly are not consistent with a competitive interaction between spermine and ifenprodil. It is possible to explain these findings by proposing that ifenprodil reveals a previously suggested but as yet not fully substantiated heterogeneity of NMDA receptors (27). However, it seems unlikely that a radically different subset of receptors could exist

in view of the ability of glycine to convert the [^{3}H]MK801 binding from an ifenprodil-sensitive to an ifenprodil-insensitive state. Instead, we suggest that the binding of ifenprodil is sensitive to the extent to which the opening of the channel has been facilitated by either polyamines or glycine. Channel opening, per se, does not alter the action of ifenprodil. However, when the channel has been converted to a form that can, upon addition of glutamate or NMDA, exhibit prolonged opening (e.g., the glycine-enhanced state), high affinity ifenprodil binding cannot occur. Thus, ifenprodil preferentially binds to the inactivated state of the NMDA receptor. Recent electrophysiological studies have shown that in the absence of glycine NMDA responses rapidly desensitize. Glycine greatly slows the desensitization and produces more sustained effects of NMDA (13). These observations support the hypothesis that several states of the NMDA receptor can exist.

A previous study has demonstrated that ifenprodil and a structural analogue, SL 82.0715, are neuroprotective in a model of focal ischemia (21). [^{3}H]MK801 binding experiments performed with SL 82.0715 suggest that it has a similar mechanism of action to ifenprodil at the NMDA receptor.[1] Moreover, SL 82.0715 was less potent at the high affinity ifenprodil site but more potent at the low affinity site. Because ifenprodil was slightly more potent than SL 82.0715 when injected intraventricularly as a neuroprotective agent (21), these data suggest that the neuroprotective actions of ifenprodil are mediated by an action at its high affinity site.

These studies have confirmed earlier findings that showed that polyamines significantly increased [^{3}H]MK801 binding (9). Earlier studies have suggested that the actions of glutamate and glycine on [^{3}H]MK801 binding represent increased access of the ligand to its binding site rather than an actual change in affinity of the site for [^{3}H]MK801. This study has demonstrated that spermine also dramatically increases the rate of both binding and unbinding, suggesting that spermine also increases access of [^{3}H]MK801 to its binding site. As mentioned above, the kinetics of [^{3}H]MK801 binding are complex; indeed, ligands may bind to the phenyclidine site even when the NMDA channel is closed (28). This complexity makes it difficult to determine kinetically derived rate constants with confidence. For this reason, one cannot exclude the possibility that spermine also increases the affinity of [^{3}H]MK801 for its site. However, increased access of the ligand may be sufficient to explain the effects of spermine. Polylysine and lysyllysine also increased binding, suggesting that the cationic nature of amines may be responsible for the interaction with the receptor. An interesting question at this time regards the nature of the endogenous ligand, if any, for the site recognized by polycations. In this respect, spermine and/or spermidine represent better candidates as endogenous ligands than polylysine because polyamines are known to be present in the brain and their synthetic enzymes can be modulated by physiologically relevant stimuli (29). Clearly, more work is required to address these questions. We also examined the interaction between polyamines and several NMDA receptor antagonists that are believed to act at the MK801/phencyclidine binding site with a wide range of affinities. Spermine and spermidine increased the potency of [^{3}H]MK801, which is a phencyclidine-like channel blocker (30–32). In contrast, we found that spermine decreases the potency of ketamine, phencyclidine, and milacemide, which are also channel blockers (30, 33, 34). The effect of spermine on the potency of milacemide was somewhat greater than that observed with phencyclidine and ketamine (Table 3). This initially suggested that milacemide might bind to the polyamine binding site. However, the data in Fig. 3, indicating a noncompetitive interaction between milacemide and spermine, clearly revealed that this was not the case. Because milacemide does not alter the dissociation of [^{3}H]MK801 and glutamate and glycine have little effect on its apparent potency, these data are consistent with the suggestion that milacemide binds at the MK801/phencyclidine binding site within the NMDA-activated ion channel.

The basis of the differential effect of spermine on the apparent potency of the various phencyclidine-like channel blockers is not clear. Because the potency of the antagonists seems to depend somewhat on the state of activation of the channel, and the potency of MK801 increases while the others decrease, it is tempting to speculate that the difference may reflect dissimilar use-dependence properties of these compounds. However, at this time it is not possible to model use dependency using the [^{3}H]MK801 binding assay, so this suggestion must remain speculative.

From this and previous studies it is clear that polyamines may have a profound effect on the activity of the NMDA receptor. This is interesting in view of the putative modulation of the spermine synthetic pathways following cerebral ischemia (35–37). As with glycine and Zn^{2+}, however, it is difficult to determine the levels of polyamines available at the binding site *in situ*. If polyamines are indeed physiologically relevant activators of the NMDA receptor, their binding site is likely to be found inside the cell, because this is the site of polyamine production following activation of ornithine decarboxylase (29). This ostensibly precludes the simple addition of polyamine to the outside of cells in order to study polyamine effects on the NMDA receptor. It is also difficult to deplete spermine levels even if ornithine decarboxylase is inhibited (38). To determine whether polyamines might normally be activating NMDA receptors in cells in culture, we determined the potency of ifenprodil and milacemide in blocking NMDA and glycine-induced alterations in $[Ca^{2+}]_i$ in cultured rat brain neurons (Fig. 6). The potency of milacemide and the absence of a high affinity phase of inhibition with ifenprodil both suggested that polyamines may normally activate the NMDA receptor in cells in culture, because these profiles mimicked [^{3}H]MK801 binding when spermine had been added. However, it is necessary to interpret these results with caution because voltage-dependent inhibition, as is seen with MK801, phencyclidine, and ketamine (32, 33), for example, could produce similar shifts in potency. Further studies are needed to clarify this point.

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