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Effects of Mono-, Di-, and Triamines on the *N*-Methyl-D-Aspartate Receptor Complex: A Model of the Polyamine Recognition Site

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SUMMARY

Systematic series of monoamines, diamines, and triamines were used to define the structural requirements for interaction at the polyamine recognition site of the *N*-methyl-D-aspartate receptor complex. Effects of amines on binding of [^{3}H]MK-801 to washed synaptic plasma membranes were measured in the presence of L-glutamate and glycine (100 μM each), in the absence or presence of spermine (10 μM). Linear aliphatic monoamines of methylene chain length up to 12 (dodecylamine) did not interact with the polyamine recognition site. Nonspecific inhibition of binding was observed at high concentrations of the longer monoamines. α,ω-Diamines of methylene chain length 2 (1,2-diaminoethane, DA2) through 12 (1,12-diaminododecane, DA12) had varying actions, depending on chain length. The shortest diamines (DA2 and DA3) acted as weak partial agonists, enhancing the binding of [^{3}H]MK-801. Intermediate-length diamines (DA4–DA7) were selective polyamine antagonists, having little or no effect on binding of [^{3}H]MK-801 measured in the absence of spermine but inhibiting binding measured in the presence of spermine. The longest diamines tested (DA8–DA12) acted as inverse agonists; they inhibited binding in the absence or presence of spermine, and this inhibition was blocked by the selective polyamine antagonist diethylenetriamine. Computer modeling of conformations of the diamines quantitatively documented that 1) these molecules are flexible and 2) long diamines may easily adopt conformations with inter-nitrogen distances mimicking those of short diamines. The *cis* and *trans* isomers of 1,4-diaminocyclohexane are inflexible, conformationally restricted diamines with markedly different actions. The *cis* isomer was a partial agonist and the *trans* isomer was an antagonist at the polyamine recognition site. Triamines of general structure $NH_2(CH_2)_3NH(CH_2)_xNH_2$ (TRI[3,*x*]), in which x = 3–12, were synthesized and tested for activity at the polyamine recognition site. Despite the large range of size, TRI[3,3] through TRI[3,9] were all full polyamine agonists of similar potency. TRI[3,10] was a partial agonist, whereas TRI[3,12] inhibited binding of [^{3}H]MK-801. Diethylenetriamine did not attenuate the effect of TRI[3,12]. Based on the results of the radioligand binding studies and the computer analysis, a model of the polyamine recognition site is proposed.

The NMDA subtype of excitatory amino acid receptor is a ligand-gated ion channel that is subject to modulatory control by endogenous and exogenous substances. The receptor complex contains distinct recognition sites for glutamate (or NMDA), glycine (1–3), Mg^{2+} (4–7), Zn^{2+} (7–10), polyamines (11–15), and open-channel blockers such as phencyclidine (16) and MK-801 (17). Binding of [^{3}H]MK-801 to the NMDA receptor complex on well washed membranes prepared from brain is greatly enhanced by agonists acting at the recognition sites for glutamate (18) and glycine (19). This is consistent with binding of [^{3}H]MK-801 to an activated or open-channel state of the NMDA receptor complex.

In the nominal absence of glutamate and glycine, binding of [^{3}H]MK-801 takes 24 hr or longer to reach equilibrium. In the presence of glutamate and glycine, the rate of binding of [^{3}H]MK-801 is increased and binding reaches equilibrium within 2–3 hr (7, 11–13, 19). Polyamines, including spermine and spermidine, have been shown to enhance the binding of [^{3}H]MK-801 (11–13) or [^{3}H]TCP (14) to levels above those seen in

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine; DET, diethylenetriamine; TCP, *N*-(1-[2-thienyl]cyclohexyl)-3,4-piperidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; cDAC, *cis*-1,4-diaminocyclohexane; tDAC, *trans*-1,4-diaminocyclohexane; DAx, diamines having the general structure $NH_2(CH_2)_xNH_2$; TRI[y,x], triamines having the general structure $NH_2(CH_2)yNH(CH_2)xNH_2$.

the presence of maximally effective concentrations of L-glutamate and glycine. The affinity for binding of [^{3}H]MK-801 is increased 2-fold by spermine in the presence of maximally effective concentrations of L-glutamate and glycine, with no change in the number of binding sites (12, 13). Results of kinetic analyses have shown that spermine increases the rates of both association and dissociation of binding of [^{3}H]MK-801 (12, 13), suggesting that spermine may function to increase the accessibility of the binding site for MK-801. Because spermine also increases the equilibrium affinity of binding, spermine may alter the properties of channel opening or the conformation of the receptor complex, such that the affinity of the binding site for MK-801, as well as its accessibility, is increased (15).

Polyamines like DET have no effect on the equilibrium binding of [^{3}H]MK-801 when assays are carried out in the presence of 100 μM L-glutamate and glycine, but selectively (12) and competitively (15, 20) attenuate the enhancement of binding caused by spermine or spermidine. These findings indicate that polyamines interact with a recognition site distinct from the amino acid recognition sites on the NMDA receptor complex. Polyamines like spermine and spermidine that enhance the binding of [^{3}H]MK-801 and increase NMDA-elicited currents have been termed "agonists" at the polyamine recognition site. Substances like DET that selectively block the effects of polyamine agonists on binding of [^{3}H]MK-801 and on NMDA-elicited responses have been termed "antagonists" at the polyamine recognition site (12, 15, 20).

Small modifications in the chemical structures of polyamine analogues result in marked differences in their effects on the binding of [^{3}H]MK-801 (12). For example, DET [$NH_2(CH_2)_2NH(CH_2)_2NH_2$] is a polyamine antagonist, but addition of a single methylene residue can produce a compound with partial agonist properties [*N*-(2-aminoethyl)-1,3-propanediamine, $NH_2(CH_2)_3NH(CH_2)_2NH_2$], and addition of two methylene residues results in a full agonist [3,3′-iminobispropylamine, $NH_2(CH_2)_3NH(CH_2)_3NH_2$]. This suggests that there are precise structural requirements for agonist action at the polyamine recognition site and that linear triamines below a certain chain length are not effective agonists (12).

Results of recent studies led to the suggestion that DA10 acts as an inverse agonist at the polyamine recognition site (20). DA10 decreased the binding of [^{3}H]MK-801 to the NMDA receptor complex when assays were carried out in the presence of glutamate and glycine, whether a polyamine agonist was present or not. This effect was not due to an action of DA10 at the recognition sites for glutamate, glycine, Mg^{2+}, Zn^{2+}, or MK-801. However, the inhibitory potency of DA10 was attenuated by the polyamine antagonist DET. These results led us to conclude that DA10 acts at the same site as spermine and DET but has an action opposite to that of spermine. By analogy to the action of β-carbolines like methyl-6,7-dimethyl-4-ethyl-β-carboline-3-carboxylate (DMCM) at the benzodiazepine site of the γ-aminobutyric acid$_A$ receptor (21), DA10 was termed an "inverse agonist" at the polyamine recognition site of the NMDA receptor complex (20).

Polyamines have been shown to modulate the properties of the NMDA receptor in electrophysiological as well as biochemical studies (20, 22, 23). Spermine enhanced NMDA-elicited currents in cultured hippocampal and striatal neurons (20, 22). Spermine was also reported to enhance NMDA-elicited currents in *Xenopus* oocytes expressing NMDA receptors after injection of mRNA prepared from rat brain (23).

The purpose of the present investigation was to define the structural features necessary for polyamine agonist, antagonist, and inverse agonist action. Systematic series of monoamines, diamines, and triamines were tested for effects on the binding of [^{3}H]MK-801. To have a sequential set of triamine analogues of spermidine, several were synthesized specifically for this study, completing the series described previously (24). The results of these studies have led to a model of the polyamine recognition site of the NMDA receptor complex.

Materials and Methods

Radioligand binding. Well washed synaptic plasma membranes were prepared from adult rat brain (Pel-Freez Biologicals, Rogers, AR) as previously described (12) and were stored at −80°. Membranes were thawed, washed by incubation at 32° for 30 min in 20 mM K-HEPES buffer (pH 7.0) containing 1 mM EDTA, centrifuged at 100,000 × *g* for 30 min, and resuspended in the same buffer. Assay tubes contained membranes (80–100 μg of protein), [^{3}H]MK-801 (7 nM), L-glutamate (100 μM), glycine (100 μM), and amines as indicated. Duplicate samples (200-μl total volume) were incubated at 32° for 3 hr. Nonspecific binding was determined with 10 μM MK-801 and represented 5–12% of total binding. Specific binding under control conditions (in the presence of 100 μM glutamate and glycine) varied among batches of membranes but was usually 450–600 fmol/mg of protein (1200–1400 cpm) and was never less than 300 fmol/mg protein (726 cpm). Binding reactions were terminated by rapid filtration, as previously described (12).

Synthetic chemistry. The triamines used in this study included a series of linear homologues of spermidine of structure $NH_2(CH_2)_3NH(CH_2)_xNH_2$, where x = 3 through 12 (for spermidine, x = 4). These compounds are referred to by the nomenclature TRI[3,x]. The synthesis of compounds in which x = 3, 5, 6, 9, 10, and 12 has been previously reported (24). To complete the series, compounds in which x = 7, 8, and 11 were prepared using a modification of published procedures, as illustrated in the following description for the preparation of TRI[3,7]. Acrylonitrile (5.48 ml, 0.083 mol) was added dropwise, with stirring, to 1,7-diaminoheptane (10.84 g, 0.083 mol) over a 3-min interval. The temperature was gradually raised to 100°, and the reaction was maintained at this temperature for 3 hr. The product was then distilled under high vacuum, to afford a center cut containing pure *N*-(2-cyanoethyl)-1,7-diaminoheptane (8.4 g, 55%). This material (8.2 g, 0.045 mol) was dissolved in absolute ethanol (80 ml), and the solution was saturated in the cold with dry ammonia. A small amount of Raney Nickel active catalyst (Aldrich) (50% slurry) was added, and the mixture was subjected to hydrogenation on a Parr apparatus (initial pressure, 60 psi) until the calculated amount of hydrogen was consumed (3–4 hr). The catalyst was removed by suction filtration, and the solvent was evaporated on a rotary evaporator. The residue (showing no nitrile absorption in the IR spectrum) was distilled under high vacuum to afford pure TRI[3,7] (5.64 g, 67%). The trihydrochloride salt was prepared by bubbling dry HCl gas through an ethanolic solution of the base. The resulting white solid was separated by filtration and dried by washing several times with ether. The resulting product (4.71 g, 56%) was homogeneous on thin layer chromatography (Analtech glass-backed silica gel GF plates, 250-μm layer; solvent, 1-butanol/acetic acid/water, 2:1:1, by volume; detection, 1% ninhydrin in ethanol spray, followed by oven heating at 60–70° for 15 min).

The physical properties of the cyanoethylated intermediates and the triamine products are given in Table 1. For these preparations, the starting diamines, 1,7-diaminoheptane and 1,8-diaminooctane, were purchased from Aldrich. 1,11-Diaminoundecane is not commercially available and was prepared from 1,9-dibromononane, as follows. 1,9-

TABLE 1
Physical properties of new synthetic linear triamines

Starting diamine, $NH_2(CH_2)_xNH_2$	Boiling point of cyanoethylated intermediate, $NH_2(CH_2)_xNHCH_2CH_2CN$	Triamine, $NH_2(CH_2)_xNHCH_2CH_2CH_2NH_2$			
		Free base		Trihydrochloride salt	
		Boiling point	MS[a]	Melting point	TLC, R_f[b]
$x = 7$	148–152° (2.0 mm)[c]	115–117° (0.5 mm)[c]	188	268–271°	0.17
$x = 8$	158–160° (0.3 mm)	114–118° (0.2 mm)	202	279–281°	0.22
$x = 11$	168–172° (0.3 mm)	160–162° (0.2 mm)	244	280–283°	0.42

[a] Mass spectrum, m/z (M + 1).
[b] TLC, thin layer chromatography. Sample spotted in aqueous methanol.
[c] Numbers in parentheses, pressure.

Dibromononane (Aldrich) (20 g, 0.07 mol), dissolved in acetonitrile (200 ml), was stirred with potassium cyanide (10.4 g, 0.16 mol) overnight at room temperature. The solvent was then removed on a rotary evaporator, the residue was taken up in chloroform (150 ml), and the chloroform solution was washed with water (3 × 50 ml). The solution was dried over sodium sulfate and evaporated to dryness. The residue (12.5 g) was dissolved in ethanol (120 ml) saturated with dry ammonia. A small amount of Raney Nickel active catalyst was added, and the mixture was subjected to hydrogenation on the Parr apparatus until the calculated amount of hydrogen was consumed. The catalyst was removed by filtration, the solvent evaporated on a rotary evaporator, and the residue distilled under high vacuum, to yield pure 1,11-diaminoundecane (9.8 g, 77%).

Computer modeling. Structures of DA2–DA12, cDAC, and tDAC were constructed using SYBYL 5.32 (TRIPOS and Associates, St. Louis, MO), running on an IRIS 4D/80 computer. The structures were generated from a fragment database by using standard bond lengths and bond angles. The geometries of each molecule were optimized using the MAXIMIN option within SYBYL and the Tripos force field (25), with a termination criterion of a root mean square conjugate gradient change of 0.01 kcal/mol-Å^2 and a distance-dependent dielectric of $1/r$. Atomic charges were calculated by using the Gasteiger and Marsili method, as implemented within SYBYL. A systematic conformational search was performed for each molecule, using the SEARCH option. All rotatable bonds were scanned in 10° increments. Conformations were rejected if the distance between any two atoms was less than the sum of their van der Waals radii. The rings of cDAC and tDAC were searched allowing for a variance of 0.1 Å and 5° in the ring-closure bond.

Additional materials. (+)-[3-^{3}H]MK-801 (specific activity, 18–28 Ci/mmol) was purchased from New England Nuclear (Boston, MA). (+)-MK-801 was a gift from Merck & Co., Inc. (West Point, PA). L-Glutamate and glycine were purchased from Sigma Chemical Co. (St. Louis, MO). Homospermidine was kindly supplied by Dr. Carl W. Porter (Roswell Park Memorial Institute, Buffalo, NY). Spermine, spermidine, DET, monoamines, diamines, and diaminocyclohexanes were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Results

Screening for activity at the polyamine recognition site. Glutamate (18), glycine (19), and micromolar concentrations of Mg^{2+} (7), as well as polyamines, increase the binding of [^{3}H]MK-801 to the NMDA receptor. To monitor the agonist activity of test compounds at the polyamine recognition site, assays contained maximally effective concentrations of glutamate and glycine (Fig. 1A). Under these conditions, addition of other agonists acting at the glutamate or glycine sites, or addition of Mg^{2+}, does not further enhance the equilibrium binding of [^{3}H]MK-801. Addition of polyamine agonists does, however, increase binding of [^{3}H]MK-801. In the presence of 10 μM spermine, agonists at the polyamine site do not further enhance the binding of [^{3}H]MK-801 (Fig. 1A).

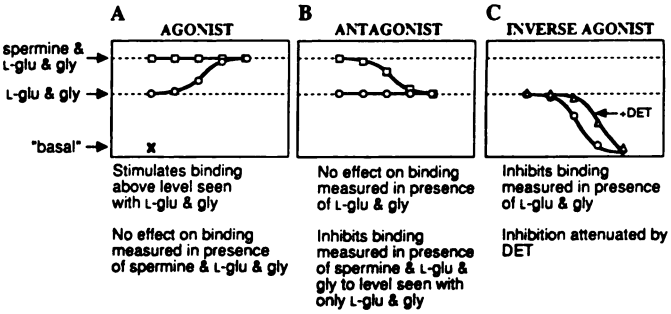

Fig. 1. Prototypical behavior of agonists, antagonists, and inverse agonists at the polyamine recognition site. Symbols show idealized representations of the binding of [^{3}H]MK-801 to well washed rat brain membranes measured in the absence of added modulators (×), in the presence of maximally effective concentrations of L-glutamate and glycine (○), in the presence of maximally effective concentrations of L-glutamate, glycine, and spermine (□), or in the presence of maximally effective concentrations of L-glutamate and glycine, with DET (△). A, Concentration-response of a prototypical agonist; B, concentration-response of a prototypical antagonist; C, concentration-response of a prototypical inverse agonist.

To test compounds for activity as polyamine antagonists, assays were carried out in the presence of maximally effective concentrations of glutamate and glycine, in the presence or absence of 10 μM spermine. Selective polyamine antagonists inhibit binding in the presence of spermine down to the level observed in the absence of spermine (Fig. 1B), while having no effect on binding measured in the absence of spermine (Fig. 1B).

Inverse agonists at the polyamine recognition site inhibit the binding of [^{3}H]MK-801 when assays are carried out in the presence of L-glutamate and glycine in the absence of spermine (Fig. 1C). A similar pattern of inhibition is seen when assays are carried out with antagonists that are selective for the glutamate or glycine sites, with open-channel blockers that act competitively at the binding site for [^{3}H]MK-801, with Zn^{2+}, with millimolar concentrations of Mg^{2+}, or with nonspecific inhibitors (e.g., detergents or extremes of pH). However, the distinguishing characteristic of inverse agonists at the polyamine site is that their effects are attenuated by the polyamine antagonist DET. Therefore, to determine whether polyamine analogues that inhibited the binding of [^{3}H]MK-801 in the absence of spermine were inverse agonists, assays were carried out in the absence and presence of DET (Fig. 1C).

Monoamines. A series of aliphatic amines were tested for activity at the polyamine recognition site. Monoamines of methylene chain length 0–4 had no effect at concentrations up to 1 mM (Fig. 2, A and B). Monoamines of methylene chain length 5–8 inhibited the binding of [^{3}H]MK-801 when present at concentrations of 1 mM, whether assays were carried out in

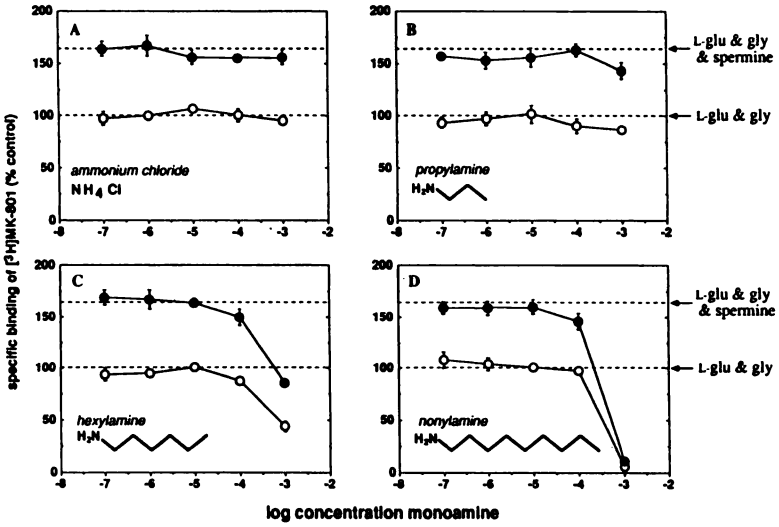

Fig. 2. Effects of monoamines on the binding of [^{3}H]MK-801. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine (○) and in the presence of 100 μM L-glutamate and glycine plus 10 μM spermine (●). *Dashed lines* in this and subsequent figures show control binding in the presence of 100 μM L-glutamate and glycine (100%) or in the presence of 100 μM L-glutamate and glycine plus 10 μM spermine. Values are means ± standard errors from three experiments. A, Ammonium chloride; B, propylamine; C, hexylamine; D, nonylamine.

the absence or presence of 10 μM spermine (Fig. 2C). Compounds with 9–12 methylenes caused little or no inhibition of binding at concentrations up to 0.1 mM, but complete inhibition was seen when assays were carried out in the presence of 1 mM monoamine (Fig. 2D).

To determine whether the inhibition observed with high concentrations of long-chain monoamines was due to inverse agonism at the polyamine recognition site, assays were carried out in the presence of DET (300 μM and 1 mM) and the long-chain monoamines (Fig. 3). DET had no effect on the binding of [^{3}H]MK-801 in the absence of added polyamines but reversed the inhibition caused by DA12 (30 μM), an inverse agonist at the polyamine recognition site (see below). Equivalent inhibition caused by the monoamines hexylamine and nonylamine was not reversed by DET.

Diamines. A series of unbranched aliphatic diamines were tested for activity at the polyamine recognition site. Compounds of methylene chain lengths ranging from 2 (DA2) to 12 (DA12) were tested. The shortest diamines, DA2 and DA3, had partial agonist properties (Fig. 4A), enhancing the binding of [^{3}H]MK-801 to the NMDA receptor complex. Diamines with methylene chain length of 4–7 had little or no effect on the

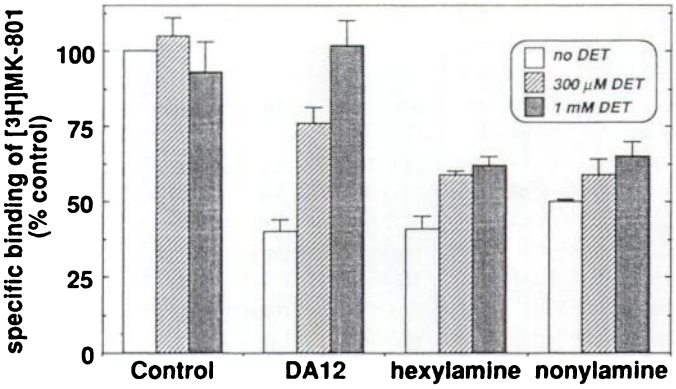

Fig. 3. Effect of DET on the inhibition of binding of [^{3}H]MK-801 by the monoamines hexylamine and nonylamine and by the diamine DA12. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine. DA12 (30 μM), hexylamine (1 mM), or nonylamine (300 μM) was present in concentrations sufficient to provide a 40–50% inhibition of the binding of [^{3}H]MK-801. DET was also present at 0.3 or 1 mM where indicated. Values are means ± standard errors from three experiments.

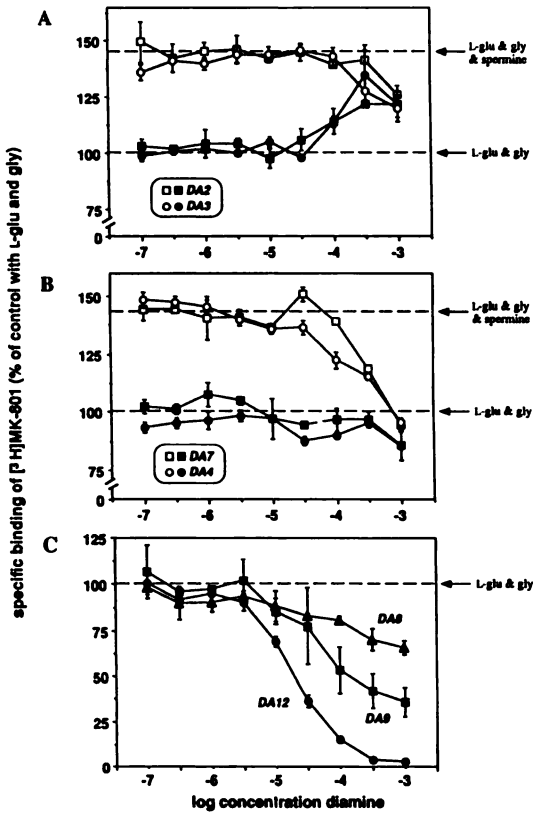

Fig. 4. Effects of diamines on the binding of [^{3}H]MK-801. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine and in the presence of 100 μM L-glutamate and glycine plus 10 μM spermine. A, Concentration-response curves for DA2 and DA3; B, concentration-response curves for DA4 and DA7; C, concentration-response curves for DA8, DA9, and DA12. Values are means ± standard errors from at least three experiments, except for DA7, which is the mean ± range of two experiments.

binding of [^{3}H]MK-801 when assays were carried out in the absence of spermine. However, DA4–DA7 inhibited the binding of [^{3}H]MK-801 in the presence of spermine down to the level observed in the absence of spermine (Fig. 4B). Antagonism of the effects of spermine by diamines of intermediate chain length is consistent with an interaction at the polyamine rec-

ognition site and suggests that DA4–DA7 are polyamine antagonists.

To investigate the mechanism of inhibition by diamines with intermediate chain length, concentration-effect curves for the polyamine agonist TRI[3,6] were carried out in the presence of DA5 (Fig. 5). The concentration-effect curve for TRI[3,6] was shifted to the right, consistent with competitive antagonism. At high concentrations, DA5 decreased the maximum response to the agonist, indicating a weak noncompetitive or nonspecific action.

Diamines of methylene chain length 8–12 inhibited the binding of [^{3}H]MK-801 when assays were carried out in the absence of spermine (Fig. 4C). The potency and efficacy of diamines as inhibitors of the binding of [^{3}H]MK-801 increased monotonically with increasing chain length. DA8 at a concentration of 1 mM inhibited binding by 34%, whereas DA12 caused nearly complete inhibition at 300 μM. We have previously suggested that DA10 acts as an inverse agonist at the polyamine recognition site (20). To determine whether DA12 has a similar mechanism of action, concentration-effect curves for DA12 were carried out in the absence or presence of the polyamine antagonist DET. DET caused a concentration-dependent, parallel shift in the inhibitory concentration-effect curve of DA12 (Fig. 6). This suggests that DA12 acts as an inverse agonist at the polyamine recognition site.

All the diamines tested were linear molecules that are predicted to be flexible in solution and capable of adopting many conformations. Because the length of the methylene chain is a critical determinant of the activity of diamines at the polyamine recognition site, computerized molecular modeling was utilized to determine the internitrogen distances and to obtain a more quantitative understanding of the conformations these diamines may assume (Table 2). The SEARCH option within SYBYL was used to search systematically the conformational space available to the diamines. Global energy minimum conformations were obtained for each diamine. The N-N distances for conformations that have energies within 5 or 20 kcal of the global minimum were determined, as were the ranges of N-N distances for these conformations. For linear diamines, the maximum N-N distances corresponded to the global energy minima, i.e., the fully extended conformations, which minimize electrostatic interactions between the charged nitrogens and

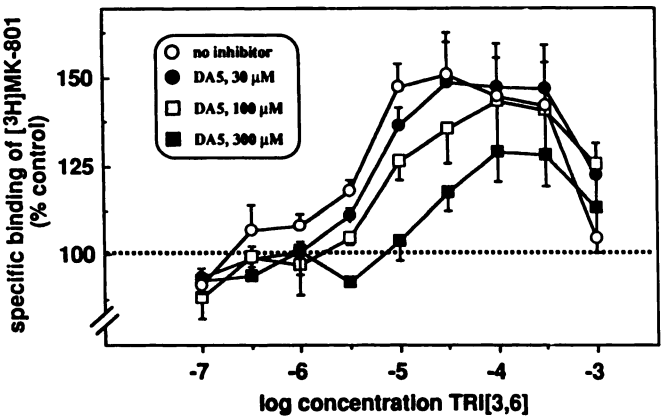

Fig. 5. Effect of DA5 on the concentration-response curve of TRI[3,6]. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine and increasing concentrations of TRI[3,6]. DA5 was present as indicated. Values are means ± standard errors from three experiments.

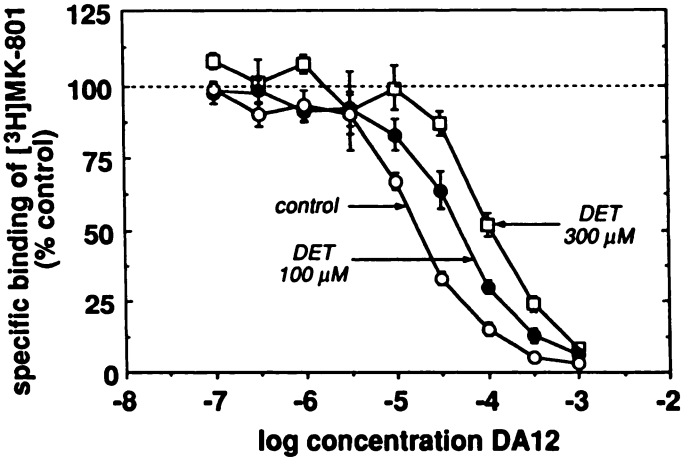

Fig. 6. Effect of DET on the concentration-response curve of DA12. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine and increasing concentrations of DA12. DET was also present as indicated. Values are means ± standard errors from three experiments.

are energetically the most favorable. With increasing chain length there was a geometric increase in the number of conformations within 5 or 20 kcal of the global energy minima. The range of N-N distances also increased with increasing chain length (Table 2).

Conformationally restricted agents are potentially more useful than flexible compounds in testing models of the structures of the active sites of receptors. To determine whether conformationally restricted diamines have activity at the polyamine recognition site of the NMDA receptor complex, cDAC and tDAC were tested (Fig. 7). cDAC was a partial agonist (Fig. 7A), whereas tDAC behaved as a polyamine antagonist (Fig. 7B). To test the hypothesis that differences in the N-N distances of the isomers may account for differences in activity, computerized molecular modeling was used to determine the global minimum energy conformations of the isomers. Both cDAC and tDAC are in chair conformations at their minimum energy, with tDAC having amino groups oriented diaxially. However, the N-N distances are equivalent (Table 2) and closely match the N-N distance of DA3, a partial agonist at the polyamine site.

Triamines. Linear triamines of structure $NH_2(CH_2)_3$-$NH(CH_2)_xNH_2$, in which x = 3–12, were tested for activity at the polyamine recognition site. Triamines in which x = 3–9 enhanced the binding of [^{3}H]MK-801, behaving as polyamine agonists (Fig. 8). With these triamines, half-maximal stimulation occurred at concentrations between 3 and 10 μM. No differences in the extent of stimulation were observed. TRI[3,10] behaved as a partial agonist (Fig. 8). TRI[3,11] and TRI[3,12] inhibited the binding of [^{3}H]MK-801 (Fig. 8). To determine whether the inhibition caused by TRI[3,12] was due to inverse agonism at the polyamine recognition site, concentration-response curves for TRI[3,12] were carried out in the absence and presence of the polyamine antagonist DET. The inhibitory potency of TRI[3,12] was unaffected by DET (300 μM) (Fig. 9). The effect of this concentration of DET on the stimulatory action of the polyamine agonist spermidine (TRI[3,4]) was measured in the same experiments and is illustrated for comparison (Fig. 9). The lack of effect of DET on the inhibition caused by TRI[3,12] contrasts with the effect of

TABLE 2
Computer modeling of diamines

Molecule	N-N distance in global minimum energy conformation	Values within 5 kcal of global minimum		Values within 20 kcal of global minimum	
		Number of conformations (10° scan)	N-N range	Number of conformations (10° scan)	N-N range
	Å		Å		Å
DA2	3.9	24	2.7–3.9	36	2.7–3.9
DA3	5.1	139	4.5–5.1	603	3.3–5.1
DA4	6.4	805	5.3–6.4	12,263	3.8–6.4
DA5	7.6	4,667	6.4–7.6	223,115	4.2–7.6
DA6	9.1	95,777	5.7–9.1[a]	>500,000	4.2–9.1
DA7	10.2	112,349	7.6–10.2	>500,000	4.9–10.2
DA8	11.5	>500,000	8.4–11.5	>500,000	5.6–11.5
DA9	12.7	>500,000	8.6–12.7	>500,000	6.0–12.7
cDAC	4.9	1	4.9	6	4.6–5.8
tDAC	4.9	3	4.9–5.5	9	4.7–5.5

[a] Within the energy ranges specified, DA6 can assume conformations in which the N-N distances are closer than those in DA5, despite the larger size of DA6. This is analogous to the enhanced stability of cyclohexane, compared with cyclopentane, and is due to the energy necessary for stretching bond angles further from the optimal value of 109.5° for an sp_3 hybridized carbon.

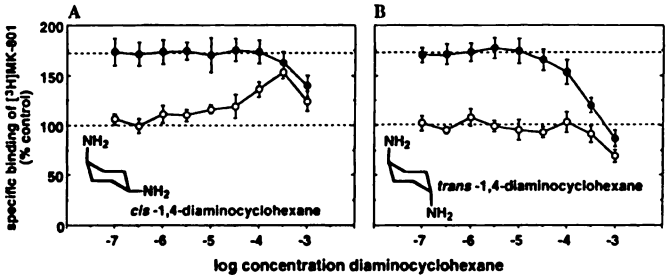

Fig. 7. Effects of cDAC and tDAC on the binding of [^{3}H]MK-801. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine (○) or in the presence of 100 μM L-glutamate and glycine plus 10 μM spermine (●), with cDAC (A) or tDAC (B). Values are means ± standard errors from three experiments.

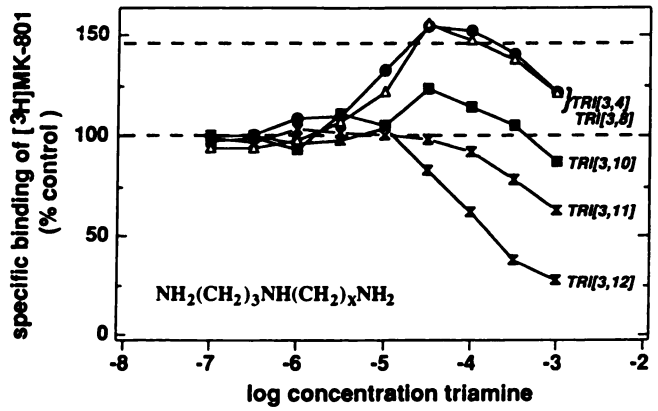

Fig. 8. Effects of triamines on the binding of [^{3}H]MK-801. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine and increasing concentrations of the indicated triamine. Values are the means of at least three experiments.

DET on stimulation caused by polyamine agonists such as spermidine (TRI[3,4]) (Fig. 9) and on the inhibition caused by inverse agonists such as DA10 and DA12 (Fig. 6) (20).

To test whether agonism at the polyamine site requires an aminopropyl group, experiments were carried out using homospermidine (TRI[4,4]). This compound was found to be a full polyamine agonist, with effects similar to those of spermidine (Fig. 10).

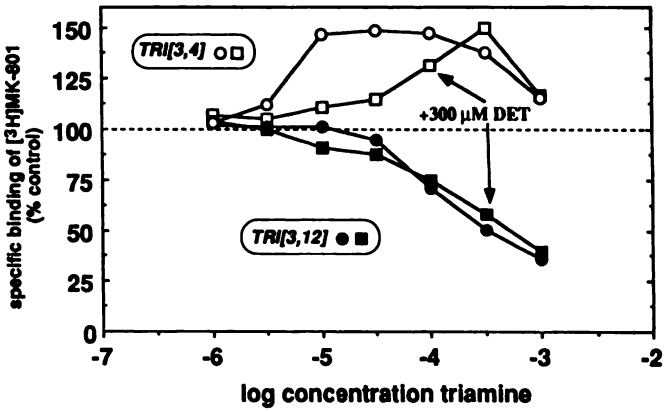

Fig. 9. Effect of DET on concentration-response curves of TRI[3,4] and TRI[3,12]. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine and increasing concentrations of TRI[3,4] or TRI[3,12]. DET was also present as indicated. Values are means of three experiments.

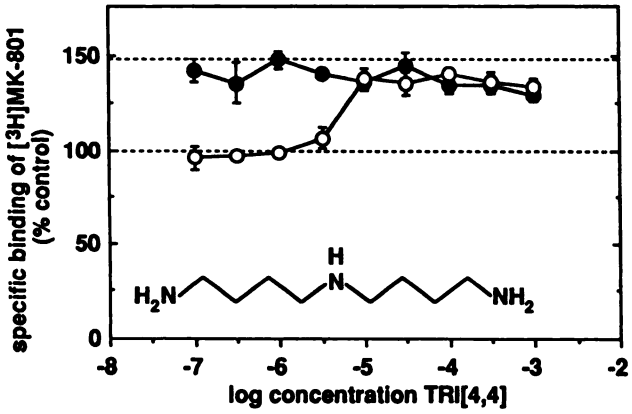

Fig. 10. Effect of TRI[4,4] on the binding of [^{3}H]MK-801. The binding of [^{3}H]MK-801 was measured in the presence of 100 μM L-glutamate and glycine (○) or in the presence of 100 μM L-glutamate and glycine plus 10 μM spermine (●), with increasing concentrations of TRI[4,4]. Values are means ± standard errors from three experiments.

Discussion

Polyamines are simple flexible molecules composed of primary and secondary amines linked by methylene residues. The

number of amines and the length of the methylene chains were systematically varied, in an attempt to define the structural requirements for activity at the polyamine recognition site of the NMDA receptor. The receptor complex must contain functional groups that interact with the amine residues in the polyamines. These groups could be negatively charged, serve as hydrogen-bond acceptors or donors, or be electron-rich aromatic residues.

There is no evidence for a selective interaction of aliphatic monoamines with the polyamine site. These compounds were either inert or caused nonselective inhibition of the binding of [^{3}H]MK-801. The inhibition caused by the long-chain monoamines was not blocked by the polyamine antagonist DET. The amphipathic, detergent-like nature of these monoamines may result in nonspecific disruption of membrane structure and function. Therefore, linear aliphatic monoamines do not contain the minimum structural requirements for interaction at the polyamine site.

The diamine series included compounds that behave as agonists, antagonists, and inverse agonists at the polyamine recognition site. The shortest diamines, DA2 and DA3, were weak partial agonists; the intermediate-chain-length diamines, DA4–DA7, acted as selective antagonists; and the longest members of this series, DA8–DA12, behaved as inverse agonists. Thus, two amine groups are sufficient to allow interaction with, but not full agonism at, the polyamine site. The ability of diamines of widely varying chain length to affect the NMDA receptor may be due in part to the highly flexible nature of these molecules. It is not energetically costly for long-chain diamines to assume conformations in which the N-N distances are similar to those of shorter-chain diamines. The observation that DA8–DA12 have activities different from those of shorter diamines suggests that DA8–DA12 exist, in solution, in an extended form that accounts for their inverse agonist effects.

The rigid diamines cDAC and tDAC have relatively fixed N-N distances but markedly different activities. Because the distances between the amines in the two isomers are nearly identical, some other aspect of the shapes of these molecules must account for the difference in their activities. In the agonist cDAC the amines are on the same side of the cyclohexane ring, but in the antagonist tDAC they are on opposite sides of the ring. The polyamine recognition site must have a structure that can distinguish between these two shapes.

Based on these findings, it is reasonable to propose that agonist effects require simultaneous interaction of at least two amines with interaction points on the receptor that are separated by about 5 Å. These points can accommodate interaction with DA3 and cDAC but not tDAC. tDAC is not inert but, instead, acts as a selective polyamine antagonist. These findings illustrate the marked specificity of the interaction of polyamines with the NMDA receptor complex.

The most striking result of studies of triamines is the marked similarity in activity, potency, and efficacy of TRI[3,3] through TRI[3,9]. The lack of dependence of activity on chain length in this series may be due to the flexibility of these molecules. Not all triamines are polyamine agonists; the polyamine antagonist DET is a triamine, TRI[2,2]. We have previously shown that, whereas TRI[2,2] is a polyamine antagonist, TRI[3,2] has partial agonist properties and TRI[3,3] is a full agonist (12). These results indicate that full agonism at the polyamine recognition site requires triamines at least as long as TRI[3,3], although smaller triamines do interact at the site.

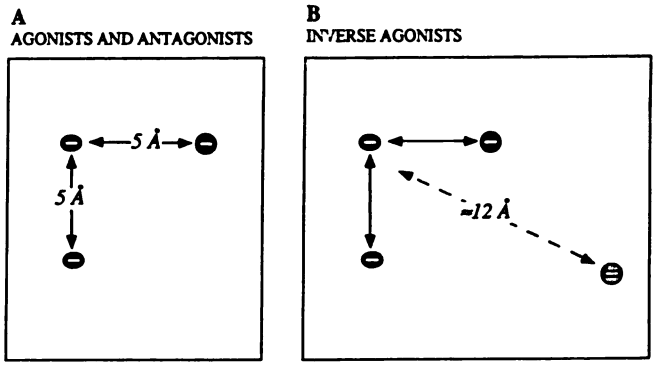

Fig. 11. Modeling of the polyamine recognition site. A, Three amine interaction points are indicated by *solid circles*. Polyamines capable of interacting simultaneously at three points are agonists and at only two points are antagonists. B, An additional amine interaction point, at a distance of about 12 Å from the other points, is required to form the interaction site for inverse agonists.

The longest triamine tested, TRI[3,12], inhibits the binding of [^{3}H]MK-801 in the absence of spermine. Because this inhibition is not blocked by DET, the mechanism of inhibition by TRI[3,12] is presumably different from that of inverse agonists such as DA10 and DA12. The mechanism of action of TRI[3,12] has not been determined, but it could be acting as a channel blocker. This inhibition may be mechanistically similar to the inhibition of [^{3}H]MK-801 binding seen with high (>100 μM) concentrations of spermine and spermidine and with concentrations of DET above 300 μM (12, 20, 27). At concentrations above 100 μM, spermine blocks NMDA-induced currents in a voltage-dependent manner in *Xenopus* oocytes expressing NMDA receptors after injection of rat brain RNA,[2] possibly by an open-channel block mechanism. Thus, high concentrations of some polyamines (e.g., spermine and DET) may have effects not only at the polyamine recognition site but also at an ion channel binding site. On the other hand, some long-chain triamines (e.g., TRI[3,12]) that have little or no activity at the polyamine recognition site may act preferentially as open-channel blockers.

DA2 and DA3 have partial agonist properties, whereas DA4 (putrescine) and longer-chain diamines are antagonists. It might be postulated from these results that only compounds containing a diaminopropyl (or smaller) group can be agonists at the polyamine recognition site. If so, this would be a constraint on models of the site. However, the triamine TRI[4,4] (homospermidine) was tested and found to be a full agonist. This indicates that molecules containing diaminobutyl residues can act as agonists at the polyamine recognition site, and models of the site must accommodate this fact.

A model of the polyamine recognition site that is consistent with most of the findings described above is shown in Fig. 11A. The main feature of this model is the presence of three amine interaction points. Two points are about 5 Å apart, whereas the third is 5–6 Å distant from one or both of the first two points. These distances were chosen primarily on the basis of the results of the conformational analysis that indicated that cDAC and tDAC have N-N distances of about 5 Å. This site can accommodate interactions with diamines and triamines,

[2] K. Williams and P. B. Molinoff, unpublished observations.

but we postulate that full agonism at the polyamine site requires simultaneous interaction with all three amine interaction points. Triamines like spermidine can interact at the three points simultaneously. Tetraamines such as spermine can also interact at three points simultaneously and so are agonists. Diamines such as DA4 or DA5 interact with the site but are unable to bind simultaneously to more than two points and, therefore, act as antagonists. DET, although it is a triamine, is too short to bind simultaneously to the three amine interaction points and so behaves as an antagonist. The agonism of the shortest diamines (DA2 and DA3) may be explained by the concerted action of two molecules of these compounds. One would necessarily have to postulate that there are steric constraints that allow two molecules of DA2 or DA3, but not DA4, to have access to the three points simultaneously.

Results of recent studies led to the suggestion that polyamines and Mg^{2+} may interact at a common recognition site on the NMDA receptor complex to enhance the binding of open-channel blockers (26, 27). Inorganic divalent cations, including Mg^{2+} and Sr^{2+}, enhance the binding of [^{3}H]MK-801 to the NMDA receptor in the nominal absence of glutamate and glycine (7), as do spermine and spermidine. The polyamine analogue arcaine blocks the enhancing effects of the inorganic divalent cations and of spermidine on the binding of [^{3}H]MK-801 (26, 27). The model of the polyamine recognition site described above could accommodate interactions with inorganic cations such as Mg^{2+}.

An explanation of inverse agonism requires a more elaborate model. Because the effects of both agonists and inverse agonists are attenuated by DET, it is reasonable to propose that these effects are mediated by sites with overlapping domains (Fig. 11B). Because inverse agonism is a property of long-chain diamines, we postulate that there may be a fourth amine interaction point at some distance from the other three points. In this model, competitive antagonism by DET of the effects of inverse agonists can be accounted for by the same mechanism by which DET blocks the actions of polyamine agonists. Alternatively, it is possible that inverse agonists inhibit the binding of [^{3}H]MK-801 by interacting at a site distinct from that at which polyamine agonists act. In this case, it would be coincidental that DET competitively inhibits the effects of both agonists and inverse agonists.

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