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Polyamines antagonize *N*-methyl-D-aspartate-evoked depolarizations, but reduce Mg^{2+} block

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Abstract

This study utilized a grease-gap preparation to investigate the effects of polyamines on responses of CA1 hippocampal pyramidal cells to *N*-methyl-D-aspartate (NMDA) and on the block of the NMDA channel by Mg^{2+}. In the absence of added Mg^{2+}, 1,10-diaminodecane (0.1–1 mM) non-competitively antagonized NMDA-evoked depolarizations. Its antagonism slowly progressed to a stable value, was not use-dependent and did not reverse completely upon washout. Similar results were obtained with 100 μM spermine and 1 mM diethylenetriamine. Addition of 1 mM Mg^{2+} to the superfusion medium greatly reduced these effects. Conversely, the polyamines attenuated the blocking action of Mg^{2+}. Postnatal treatment with α-difluoromethylornithine reduced the total polyamine content of area CA1 in 10- to 15-day-old rats almost to the adult level (although spermine content was unaffected). Mg^{2+} less potently antagonized NMDA-evoked depolarizations in slices from 10- to 15-day-old rats than in slices from adult rats, and this difference was unaffected by the α-difluoromethylornithine treatment. These results suggest (1) that there are rapid and slow components to the antagonism of NMDA-evoked depolarizations by polyamines, both of which may involve permeation of the polyamine into or through the NMDA channel; (2) that polyamine release in brain could modulate the Mg^{2+} sensitivity of responses to NMDA; and (3) that changes in the total content of endogenous polyamine do not explain developmental differences in the sensitivity of NMDA-evoked depolarizations to Mg^{2+}.

Keywords: Polyamine; Spermine; NMDA (*N*-methyl-D-aspartate); Hippocampus; Mg^{2+}; Development

1. Introduction

The *N*-methyl-D-aspartate (NMDA) type of glutamate receptor appears to play a key role in the development of the brain, learning and memory, and a variety of neurologic and neuropathologic conditions (Choi, 1988; Collingridge and Singer, 1990; Bliss and Collingridge, 1993; Zorumski and Olney, 1993). The NMDA receptor is a ligand-gated cation channel composed of multiple glycopolypeptide subunits with binding sites for a number of positive and negative modulators. Two such modulators are Mg^{2+} and the polyamines spermine and spermidine.

The unique voltage-dependent block of the NMDA channel by physiological concentrations of Mg^{2+} limits the conditions under which the NMDA receptor can participate in normal synaptic function (Collingridge et al., 1988a,b). This is the major property of the NMDA receptor that allows it to function as a gating mechanism in long-lasting forms of plasticity. We demonstrated that Mg^{2+} less potently antagonized the NMDA-evoked depolarization of CA1 pyramidal cells in CA1-subiculum slices from 10- to 15-day-old rats than in slices from adult rats (Bowe and Nadler, 1990). The developmental pattern observed did not correlate with reported changes in NMDA receptor density. The Mg^{2+} sensitivity of the NMDA receptor can be regulated by the expression of NR_2 subunits (Kutsuwada et al., 1992; Monyer et al., 1994) and by protein kinase C-dependent phosphorylation (Chen and Huang, 1992). Some reports suggest that it may also be regulated by polyamines. Studies that utilized the membrane binding of [^{3}H](+)-5-methyl-10,11-dihydro-5*H*-dibenzo-

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[*a*,*d*]cyclohepten-5,10-imine maleate ([^{3}H]MK-801) or [^{3}H]*N*-(1-[thienyl]cyclohexyl)piperidine ([^{3}H]TCP) to assess the functional state of the NMDA receptor described interactions between Mg^{2+} and polyamines that suggested either an allosteric coupling between separate binding sites or agonist/partial agonist effects of Mg^{2+} upon a common stimulating site (Sacaan and Johnson, 1990a,b,1991; Rajdev and Reynolds, 1992). Both membrane binding studies (Williams et al., 1989, 1991; Bakker et al., 1991; Sacaan and Johnson, 1991) and electrophysiological studies of cultured neurons (Williams et al., 1990; Lerma, 1992; Rock and Macdonald, 1992a,b,c; Araneda et al., 1993; Benveniste and Mayer, 1993) or *Xenopus* oocytes injected with whole rat brain mRNA (Brackley et al., 1990; McGurk et al., 1990) demonstrated complex effects of polyamines on NMDA channel function. The endogenous polyamines can either enhance or block NMDA receptor function by acting at 2 or 3 sites that are distinct from the binding sites for glutamate or glycine. Potentiation of NMDA currents occurs at lower polyamine concentrations, can be observed at both positive and negative membrane potentials and results from a combination of increased affinity for glycine, increased maximal response in the presence of a saturating glycine concentration and reduced onset rate of receptor desensitization. Inhibition of NMDA currents occurs at higher polyamine concentrations, is only observed at negative membrane potentials and results from voltage-dependent channel block. Interactions between polyamines and Mg^{2+} have not been studied in intact tissue. Polyamine concentrations in the rat brain peak during early postnatal development and decline thereafter (Slotkin et al., 1982). In view of the interactions between Mg^{2+} and polyamines described in receptor binding studies, one consequence of elevated polyamine concentrations during development might be a change in the response of the NMDA receptor to Mg^{2+}. We therefore investigated the effect of polyamines on NMDA-evoked depolarizations and their interactions with Mg^{2+} in developing and adult rats.

2. Materials and methods

2.1. Grease-gap recording

Lactating female Sprague-Dawley rats with ten 9-day-old foster pups each and adult rats of either sex (60–100 days of age) were obtained from Zivic-Miller Laboratories (Allison Park, PA, USA). Rats were decapitated under ether anesthesia and CA1-subiculum slices were prepared as described in detail elsewhere (Martin et al., 1989; Martin and Nadler, 1991). Slices of 450-μm thickness were transferred to a two-compartment superfusion chamber and a grease barrier was formed at the CA1-subiculum border, such that the CA1 pyramidal cell bodies and dendrites lay in one compartment and their axons projected through the grease barrier to the subiculum in the other compartment. The compartments were independently superfused at 32° C with artificial cerebrospinal fluid comprised of (mM): 122 NaCl, 25 $NaHCO_3$, 3.1 KCl, 1.3 $CaCl_2$, 1.0 $MgSO_4$, 0.4 KH_2PO_4, 0.003 glycine and 10 D-glucose gassed continuously with 95% O_2-5% CO_2. Mg^{2+} was removed from the superfusion medium after about 40 min and the slice was equilibrated for another 40 min. At the end of the equilibration period, a test concentration of NMDA roughly equal to the expected EC_{50} was repeatedly applied until responses of constant amplitude were obtained. Responses were evoked by replacing the medium superfusing the CA1 compartment for 2 min with 5.7 compartment volumes of medium that contained NMDA. The negative deflection in the DC potential differentially recorded between the two compartments was taken to represent depolarization of the CA1 pyramidal cell population by NMDA. Presynaptic effects and intrinsic CA1 circuitry contributed little or nothing to the responses (Martin et al., 1992). There was no evidence of hysteresis.

Once response amplitudes stabilized, concentration-response curves were generated by applying a set of excitant concentrations in random order. At least 10 min was allowed for recovery between applications of the excitant. Then Mg^{2+} and/or polyamine was added to the medium superfusing the CA1 compartment and the excitant was retested at least 30 min later, whenever responses stabilized. Two or three concentration-response curves were obtained from each slice. Response amplitudes were normalized to the maximal response amplitude obtained without Mg^{2+} or polyamine in the same slice. The maximal concentration of NMDA chosen for testing was 25 μM for adults and 40 μM for 10- to 15-day-old rats (Bowe and Nadler, 1990). EC_{50} values were estimated by a least-squares regression of the linear portion of semilogarithmic concentration-response curves, with use of data points between about 20 and 80% of complete inhibition (Tallarida and Murray, 1987). Differences among slices in absolute response amplitude had no obvious effect upon the calculated EC_{50} values. Concentration ratios were calculated at the EC_{50} level. Because EC_{50} values and concentration ratios are not normally distributed, statistical analyses utilized the $\log_{10}$ transformations of these values (pEC_{50} and ΔpEC_{50}, respectively) (Gaddum, 1945; Fleming et al., 1972).

2.2. Polyamine assay

The polyamine content of area CA1 was determined by high-performance liquid chromatography (HPLC)

after derivatization with o-phthalaldehyde. Area CA1 was dissected from CA1-subiculum slices and all the CA1 slices from a single animal were pooled. Polyamines were extracted into 0.4 M $HClO_4$, 2 mM Na_2EDTA by homogenization for 30 s with a Polytron (Brinkmann Instruments, Westbury, NY, USA; setting of 6). After centrifugation at $26000 \times g$ for 15 min, the supernatant was stored at −30° C prior to assay. The pellet was dissolved in 1% (w/v) sodium dodecyl sulfate, 20 mM Na_2EDTA and protein content was determined (Hess et al., 1978).

Samples were adjusted to pH 5.4–5.6 by the addition of 2 M KOH and centrifuged to remove the $KClO_4$ precipitate. Each sample was applied to a freshly prepared 0.6×3 cm cellulose phosphate (coarse grade) column that had been equilibrated with 5 mM sodium phosphate, pH 6.2. To remove other amines, the columns were washed with 5 ml of 5 mM sodium phosphate, pH 6.2, and then with 5 ml of 100 mM boric acid-$NaHCO_3$ buffer, pH 8.5, that contained 25 mM NaCl. Polyamines were eluted with 3.5 ml of 200 mM boric acid-$NaHCO_3$ buffer, pH 9.4, that contained 600 mM NaCl. The first 0.5 ml of eluant (void volume) was discarded and the remainder was saved for HPLC. All the glassware was siliconized to reduce adsorption of polyamines. Spermine, spermidine and putrescine standards were recovered quantitatively by this procedure.

Portions of each polyamine sample were derivatized with o-phthalaldehyde, after addition of a 1,6-hexanediamine standard. Exactly 90 s after derivatization, 30 μl of the reaction mixture was applied to a 4.6×80 mm Zorbax 3-μm C18 reverse-phase column (Mac-Mod Analytical, Chadds Ford, PA, USA) equilibrated with Solvent A (0.1 M *N,N*-dimethylcyclohexylamine, 0.2 M phosphoric acid in 50% (v/v) methanol). o-Phthalaldehyde derivatives were separated with a methanol (Solvent B) elution gradient as follows: 3 min of 50% Solvent B, an exponential gradient of 50–80% Solvent B for 10 min and finally 8 min of 80% Solvent B. The flow rate was 1.5 ml/min. Heights of fluorescence peaks were corrected for variation in the height of the o-phthalaldehyde-internal standard signal and compared to external standards of known concentration. The limit of sensitivity was about 50 fmol.

2.3. α-Difluoromethylornithine treatment

Rat pups from several litters were taken from their natural mothers, randomized into saline-treated and α-difluoromethylornithine-treated groups and returned to foster mothers. Pups received daily subcutaneous injections of α-difluoromethylornithine (500 mg/kg in 0.9% (w/v) NaCl) or 0.9% (w/v) NaCl from the day of birth until 24 h before they were killed on postnatal days 10–15 (Slotkin et al., 1982; Bartolome et al., 1985). On each experimental day, two pups that had received the same treatment were randomly selected. One pup was used for grease-gap recording and the other for determination of CA1 polyamine content.

2.4. [^{3}H]TCP binding

Hippocampal membranes were prepared and [^{3}H]TCP binding assays were performed essentially as described by Bonhaus et al. (1990). Each experiment utilized both hippocampi from one adult or four 10- to 15-day-old rats. Hippocampal membranes from adult and 10- to 15-day-old rats were studied side-by-side. Membrane samples (50–75 μg of protein) were preincubated in 1 ml of 5 mM Tris-acetate, pH 7.2, with 100 μM NMDA, 10 μM glycine and various concentrations of spermine for 20 min at 25° C. Then 2.5 nM [^{3}H]TCP was added and binding was terminated by vacuum filtration through Whatman GF/C paper (presoaked for 4 h at 4° C in 0.1% (v/v) polyethylenimine) after either 5 or 500 min. A 5-min incubation period was used to investigate effects of spermine on the initial rate of binding, whereas a 500-min incubation period was used to test effects on equilibrium binding. Nonspecific binding was determined with 30 μM phencyclidine. Filters were washed twice for 3 s each with ice-cold 5 mM Tris-acetate, pH 7.2 (~ 12 ml total) and counted.

2.5. Materials

NMDA was purchased from Tocris Neuramin (Bristol, UK); (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) from Research Biochemicals (Natick, MA, USA); 1,6-hexanediamine dihydrochloride, diethylenetriamine (DET), 1,10-diaminodecane (DA10) and *N,N*-dimethylcyclohexylamine from Aldrich Chemical Co. (Milwaukee, WI, USA); o-phthalaldehyde from Pierce Chemical Co. (Rockford, Il, USA); [^{3}H]TCP (41–60 Ci/mmol) from DuPont/New England Nuclear Corp. (Boston, MA, USA); and other organic compounds from Sigma Chemical Co. (St. Louis, MO, USA). α-Difluoromethylornithine was kindly provided by Dr. T.A. Slotkin.

3. Results

3.1. Effects of polyamines on responses to NMDA

Spermine, 1,10-diaminodecane and diethylenetriamine were tested for their ability to potentiate or block the depolarizing action of NMDA on the CA1 pyramidal cell population. Polyamines had previously been classified as agonists, antagonists or inverse agonists, as determined from potentiation of [^{3}H]MK-801

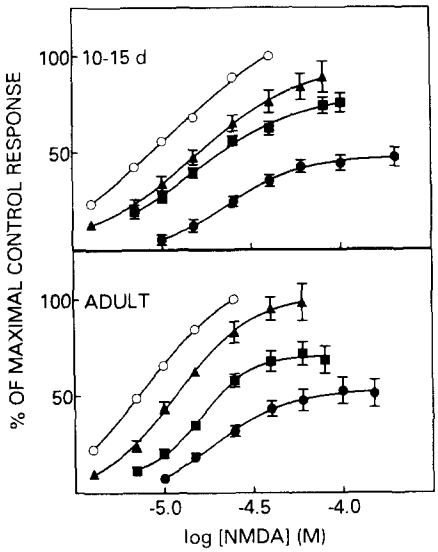

Fig. 1. 1,10-Diaminodecane non-competitively antagonized NMDA-evoked depolarizations of CA1 pyramidal cells. NMDA concentration-response curves were generated in the presence of 0 (○), 100 μM (▲), 316 μM (■) or 1 mM (●) 1,10-diaminodecane. Values are means ± S.E.M. for 6–12 slices. Where S.E.M. is not shown in this and subsequent figures, it was smaller than the symbol.

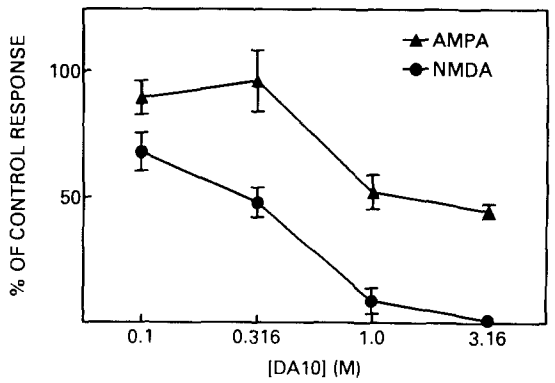

Fig. 2. 1,10-Diaminodecane (DA10) preferentially antagonized NMDA-evoked depolarizations. Each slice was exposed to 10 μM NMDA and 5 μM AMPA in the absence of 1,10-diaminodecane and in the presence of one or two concentrations of 1,10-diaminodecane. NMDA and AMPA were tested at equivalent concentrations relative to their EC_{50} values (Bowe and Nadler, 1990). The difference between antagonism of NMDA-evoked and AMPA-evoked depolarizations was significant at $P < 0.001$ (MANOVA). Values are means ± S.E.M. for 6 slices.

binding in the presence of saturating glutamate and glycine concentrations (Williams et al., 1989,1990). The endogenous polyamines spermine and spermidine were classed as agonists because they potentiated binding, 1,10-diaminodecane was considered an inverse agonist because it inhibited binding and diethylenetriamine was considered an antagonist because it blocked the effects of both spermine and 1,10-diaminodecane.

In nominally Mg^{2+}-free medium, 1,10-diaminodecane depressed the response of CA1 pyramidal cells to NMDA in a concentration-dependent manner (Fig. 1, Table 1). It both displaced the NMDA concentration-response curve to the right and reduced the maximal response to NMDA. This action is consistent with non-competitive antagonism in the presence of a receptor reserve (Kenakin, 1984). Similar results were obtained from adult and 10- to 15-day-old rats. However, all the polyamines we tested somewhat more effectively reduced NMDA potency in slices from adult rats. 1,10-Diaminodecane was relatively selective for NMDA-evoked depolarizations, compared with depolarizations evoked by AMPA (Fig. 2). Subsequent experiments utilized concentrations of 1,10-diaminodecane (100 and 316 μM) that antagonized NMDA, but not AMPA.

Table 1
Effects of polyamines on NMDA potency

Age	Polyamine	pEC_{50} (n) [a]	EC_{50} (μM)
Adult	Control	5.18 ± 0.02 (35)	6.6
	1,10-Diaminodecane, 100 μM	4.94 ± 0.02 (12)	12
	1,10-Diaminodecane, 316 μM	4.81 ± 0.02 (31)	16
	1,10-Diaminodecane, 1 mM	4.69 ± 0.04 (6)	20
	Diethylenetriamine, 1 mM	4.88 ± 0.04 (6)	13
	Spermine, 100 μM	5.00 ± 0.02 (7)	10
	1,10-Diaminodecane, 316 μM + spermine, 100 μM	4.82 ± 0.05 (6)	15
	1,10-Diaminodecane, 316 μM + diethylenetriamine, 500 μM	4.80 ± 0.04 (6)	16
10–15 days	Control	5.07 ± 0.01 (45)	8.5
	1,10-Diaminodecane, 100 μM	4.90 ± 0.04 (11)	13
	1,10-Diaminodecane, 316 μM	4.83 ± 0.02 (24)	15
	1,10-Diaminodecane, 1 mM	4.70 ± 0.06 (6)	20
	Diethylenetriamine, 1 mM	4.88 ± 0.05 (6)	13

[a] pEC_{50} is $-\log EC_{50}$. Values are means ± S.E.M. for n = number of slices tested. Two-way analysis of variance (age × polyamine) that utilized only treatment groups common to both ages demonstrated a significant reduction of NMDA potency by polyamine ($P < 0.01$). Polyamines were somewhat more effective in slices from adult rats ($P < 0.01$ for interaction between variables). All the polyamine treatments significantly reduced NMDA potency ($P < 0.01$, Dunnett's test).

Antagonism of NMDA by 1,10-diaminodecane developed gradually and the time to attainment of maximal antagonism was concentration-dependent. This process reached completion in 30–40 min during superfusion with 100 μM 1,10-diaminodecane, but with 3.16 mM 1,10-diaminodecane as long as 3 h of continuous application was required before depolarizations were reduced to a stable value. Repeated applications of NMDA during this period did not accelerate the approach to steady state. Thus there was no indication of use-dependent block. This slow time course could not be explained by 'rundown' of the slice, because NMDA concentration-response curves remained stable for the same periods in the absence of 1,10-diaminodecane and because responses to AMPA were unchanged. The action of 1,10-diaminodecane could not be fully reversed by washing with polyamine-free medium.

Low concentrations of spermine (1–10 μM) did not affect NMDA-evoked depolarizations in either adult or 10- to 15-day-old rats (not shown). Spermine remained ineffective in the presence of 5% (v/v) dimethyl sulfoxide (DMSO), which may overcome a diffusion barrier and allow spermine to reach a higher concentration within the slice (Sacaan and Johnson, 1990b). At concentrations of 100 μM or above, spermine reduced NMDA-evoked depolarizations (Table 1). At these concentrations, however, spermine also reduced AMPA-evoked depolarizations. As observed with 1,10-diaminodecane, spermine-induced antagonism approached steady state very slowly and the antagonism could not be fully reversed by washing. To determine if the slowly developing antagonism were masking an enhancement of NMDA-evoked depolarizations that developed more rapidly, NMDA was applied repeatedly soon after addition of 10 μM spermine to the superfusion medium. In 4 slices, no enhancement was observed. Co-administration of spermine (10–500 μM) with 1,10-diaminodecane did not reduce the antagonism by 1,10-diaminodecane, whether spermine was added to the superfusion medium simultaneously with or before 1,10-diaminodecane ($n = 12$). Addition of 5% (v/v) DMSO to the medium did not change this result. DMSO by itself did not affect the action of 1,10-diaminodecane.

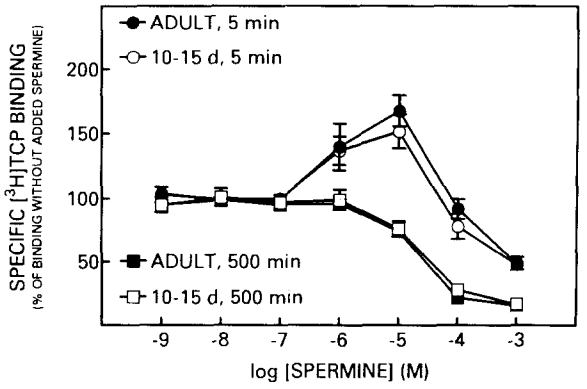

Fig. 3. Effects of spermine on the binding of [^{3}H]TCP to hippocampal membranes did not differ with age. Hippocampal membranes were incubated with 2.5 nM [^{3}H]TCP, 100 μM NMDA, 10 μM glycine and the indicated concentration of spermine for 5 or 500 min. The initial rate of binding was determined with a 5 min incubation and equilibrium binding with a 500 min incubation. Values are means ± S.E.M. for 3 experiments. Values obtained in the absence of spermine were as follows: adult, 5 min – 250 ± 14 fmol/mg protein; 10–15 days, 5 min – 256 ± 6 fmol/mg protein; adult, 500 min – 733 ± 5 fmol/mg protein; 10–15 days, 500 min – 632 ± 34 fmol/mg protein.

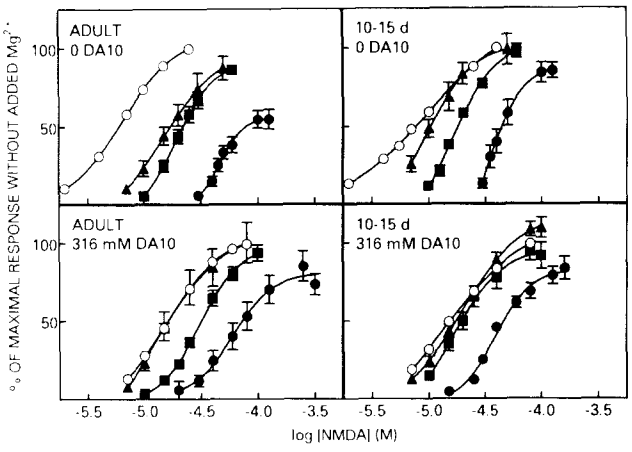

Fig. 4. 1,10-Diaminodecane (DA10) attenuated the blocking action of Mg^{2+}. NMDA concentration-response curves were generated in the presence or absence of 316 μM 1,10-diaminodecane with 0 (○), 0.316 (▲), 1.0 (■) or 10 (●) mM Mg^{2+}. Values are means ± S.E.M. for at least 6 slices. 1,10-Diaminodecane reduced the rightward shift of the NMDA concentration-response curve produced by Mg^{2+}. Conversely, the presence of Mg^{2+} largely blocked the inhibitory action of 1,10-diaminodecane ($P < 0.01$ in each case; three-way analysis of variance of pEC_{50} values).

As shown by other investigators (Sacaan and Johnson, 1990a), low concentrations of spermine (1–10 μM) increased the initial rate of [^{3}H]TCP binding to the NMDA receptor (Fig. 3). Concentrations of 10 μM or more reduced binding at equilibrium. No developmental difference was evident.

The putative polyamine antagonist diethylenetriamine (200 μM–1 mM) was tested for its ability to reverse the depression of NMDA-evoked depolarizations by 1,10-diaminodecane. No combination of diethylenetriamine and 1,10-diaminodecane concentrations demonstrated reversal of the 1,10-diaminodecane effect by diethylenetriamine, even when diethylenetriamine was applied to the slice first ($n = 16$). However, diethylenetriamine concentrations of 1 mM or higher attenuated NMDA-evoked depolarizations similarly to 1,10-diaminodecane (Table 1).

3.2. Interactions between polyamines and Mg^{2+}

Mg^{2+} shifted the NMDA concentration-response curve to the right, but only depressed the maximal

response to NMDA at a concentration of 10 mM (Fig. 4). As previously reported (Bowe and Nadler, 1990), Mg^{2+} more potently antagonized NMDA-evoked depolarizations in slices from adult rats than in slices from 10- to 15-day-old rats (Fig. 4, Table 2). Antagonism by Mg^{2+} and 1,10-diaminodecane was less than additive. In the presence of a physiological Mg^{2+} concentration (1 mM), responses to 1,10-diaminodecane were greatly diminished (Table 2). Conversely, 1,10-diaminodecane (100 or 316 μM), which itself shifted the NMDA concentration-response curve rightward, significantly attenuated the action of Mg^{2+} (Fig. 4, Table 2). With careful choice of 1,10-diaminodecane and Mg^{2+} concentrations, it was possible to demonstrate this interaction directly. For example, the addition of 316 μM 1,10-diaminodecane reversed the reduction in the NMDA-evoked depolarization produced by 10 mM Mg^{2+} (Fig. 5). Thus 1,10-diaminodecane and Mg^{2+} antagonized not only the action of NMDA, but also the actions of each other.

Diethylenetriamine and spermine interacted with Mg^{2+} similarly to 1,10-diaminodecane. A physiological concentration of Mg^{2+} greatly reduced antagonism of NMDA-evoked depolarizations by either compound (Table 2). Neither diethylenetriamine (1 mM) nor spermine (100 μM) diminished the ability of 1,10-diaminodecane to reverse Mg^{2+} antagonism. Instead, diethylenetriamine, like 1,10-diaminodecane, significantly attenuated the action of 1 mM Mg^{2+} (Table 2). Spermine also tended to reduce the Mg^{2+} block, although this effect did not reach statistical significance.

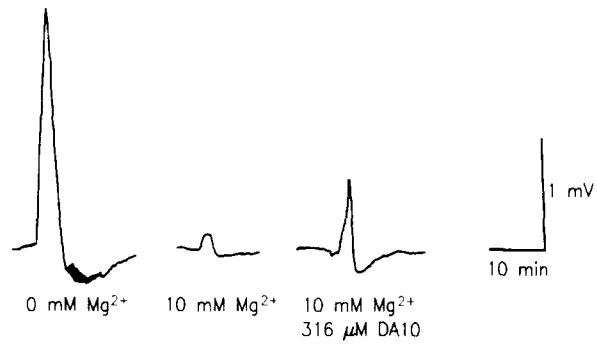

Fig. 5. Reversal of Mg^{2+} block by 1,10-diaminodecane (DA10). 1,10-Diaminodecane (316 μM), which by itself reduced by ~50% the depolarizations evoked by 25 μM NMDA, reversed the antagonism of NMDA produced by 10 mM Mg^{2+}. Similar results were obtained in 3 other slices.

Similar negative interactions between Mg^{2+} and polyamines were observed in adult and 10- to 15-day-old rats.

3.3. Effect of endogenous polyamine concentrations on Mg^{2+} antagonism of NMDA-evoked depolarizations

The concentrations of endogenous polyamines in area CA1 were significantly greater in 10- to 15-day-old rats than in adults (Table 3). Values in the developing rats exceeded those in adults by factors of 2.5, 2.2 and 10.8 for spermine, spermidine and putrescine, respectively. Daily treatment with α-difluoromethylornithine markedly reduced the levels of spermidine and pu-

Table 2
Effects of polyamines on the antagonism of NMDA-evoked depolarizations by 1 mM Mg^{2+}

Age	Polyamine	No added Mg^{2+}			1 mM Mg^{2+}		ΔpEC_{50} [b]
		pEC_{50} (n) [a]		EC_{50} (μM)	pEC_{50} [a]	EC_{50} (μM)	
Adult	Control	5.24 ± 0.03	(13)	5.8	4.71 ± 0.02	20	0.53 ± 0.03
	1,10-Diaminodecane, 100 μM	4.95 ± 0.03 [d]	(5)	11	4.62 ± 0.03	24	0.33 ± 0.05 [d]
	1,10-Diaminodecane, 316 μM	4.77 ± 0.03 [d]	(9)	17	4.52 ± 0.02 [d]	30	0.25 ± 0.03 [d]
	Diethylenetriamine, 1 mM	4.88 ± 0.04 [d]	(6)	13	4.54 ± 0.03 [d]	29	0.34 ± 0.03 [d]
	Spermine, 100 μM	5.00 ± 0.02 [d]	(7)	10	4.54 ± 0.03 [d]	29	0.46 ± 0.02
	1,10-Diaminodecane, 316 μM + spermine, 100 μM	4.82 ± 0.05 [d]	(6)	15	4.59 ± 0.05 [c]	26	0.22 ± 0.06 [d]
	1,10-Diaminodecane, 316 μM + diethylenetriamine, 500 μM	4.80 ± 0.04 [d]	(6)	16	4.63 ± 0.03	23	0.18 ± 0.03 [d]
10–15 days	Control	5.09 ± 0.02	(21)	8.1	4.76 ± 0.02	17	0.33 ± 0.02
	1,10-Diaminodecane, 100 μM	4.92 ± 0.03 [d]	(6)	12	4.73 ± 0.02	19	0.20 ± 0.03 [d]
	1,10-Diaminodecane, 316 μM	4.82 ± 0.03 [d]	(12)	15	4.71 ± 0.02	20	0.11 ± 0.03 [d]
	Diethylenetriamine, 1 mM	4.88 ± 0.05 [d]	(6)	13	4.69 ± 0.06	20	0.19 ± 0.05 [d]

For each slice, concentration-response curves were obtained in both the absence and presence of Mg^{2+}. [a] Values are means ± S.E.M. for n = number of slices tested. Two-way analysis of variance (age × polyamine) with repeated measures (± Mg^{2+}) that utilized only treatment groups common to both ages demonstrated a somewhat greater potency of NMDA in slices from 10- to 15-day-old rats ($P < 0.05$), a significant reduction of NMDA potency by both polyamine and Mg^{2+} ($P < 0.0001$ in each case) and significant interactions between age and polyamine ($P < 0.01$), age and Mg^{2+} ($P < 0.0001$) and polyamine and Mg^{2+} ($P < 0.0001$). The interaction between polyamine and Mg^{2+} did not differ significantly with age. [b] ΔpEC_{50} is the change in pEC_{50} of NMDA upon the addition of 1 mM Mg^{2+} to the superfusion medium (−log[concentration ratio]). [c] Experimental group differed from corresponding control at $P < 0.05$ (Dunnett's test). [d] Experimental group differed from corresponding control at $P < 0.01$ (Dunnett's test).

Table 3
Polyamine content (pmol/mg protein) of hippocampal area CA1 and the effects of daily treatment with α-difluoromethylornithine

Age	Spermine	Spermidine	Putrescine
Adult	2600 ± 200	2420 ± 60	44 ± 2
10–15 days (saline-treated)	6400 ± 700 [a]	5400 ± 300 [a]	470 ± 50 [a]
10–15 days (α-difluoromethylornithine-treated)	6100 ± 500	780 ± 70 [b]	36 ± 6 [b]

Values are means ± S.E.M. for 7–8 animals. [a] Significantly higher than the corresponding adult value at $P < 0.0001$ (Newman-Keuls test after one-way analysis of variance yielded $P < 0.0001$). Analyses were performed on log-transformed data to correct for the increase in variance with higher CA1 content. [b] Significantly lower than the corresponding value in saline-treated control rats at $P < 0.0001$ (Newman-Keuls test).

trescine measured during the 10- to 15-day period. However, the α-difluoromethylornithine treatment altered neither the potency of NMDA nor the ability of Mg^{2+} to inhibit NMDA-evoked depolarizations (Table 4). In other words, the reduction in total polyamine concentration did not make slices from 10- to 15-day-old rats look like adult slices with respect to the action of Mg^{2+}.

4. Discussion

4.1. Polyamines non-competitively antagonize NMDA-evoked depolarizations

In the absence of added Mg^{2+}, all the polyamines tested reduced the amplitude of NMDA-evoked depolarizations. 1,10-Diaminodecane was studied in greatest detail; its action was consistent with non-competitive antagonism in the presence of a receptor reserve. No concentration of diethylenetriamine or spermine attenuated the action of 1,10-diaminodecane. Indeed both polyamines acted much like 1,10-diaminodecane when superfused alone. These results do not correlate with the classification of 1,10-diaminodecane, diethylenetriamine and spermine as an inverse agonist, antagonist and agonist, respectively, at a stimulatory polyamine binding site (Williams et al., 1989, 1990). They are quite consistent, however, with reports that these and other polyamines act as voltage-dependent blockers of the NMDA channel (Donevan et al., 1992; Rock and Macdonald, 1992b,c; Subramaniam et al., 1992; Araneda et al., 1993; Benveniste and Mayer, 1993). The site(s) at which polyamines potentiate NMDA channel opening clearly differ pharmacologically from the site of voltage-dependent channel block.

In two respects our results differed from descriptions of polyamine-induced NMDA channel block in isolated cells. First, although some antagonism was observed soon after exposure of CA1 pyramidal cells to the polyamine, maximal block developed over an exceptionally long time course. Voltage-dependent channel block was reported to develop rapidly in cultured neurons or oocytes. However, several groups also found a slow component of block. In *Xenopus* oocytes injected with rat brain mRNA, 10–100 μM spermine initially potentiated NMDA-evoked currents, but after exposure for 30 min or more spermine inhibited these currents (Brackley et al., 1990). Benveniste and Mayer (1993) observed a slow component in the development and recovery from spermine-induced channel block in cultured hippocampal neurons at hyperpolarized membrane potentials. Finally, antagonistic effects of spermine and spermidine in the rat cortical wedge, another grease-gap preparation, were found not to reach a maximum until 30–60 min after their introduction into the superfusion medium (Robichaud and Boxer, 1993). In agreement with the present study, these effects could not be completely reversed by washing with polyamine-free medium. Robichaud and Boxer (1993) attributed the slow development of block to an action of spermine and spermidine at an intracellular site. The highly charged polyamines are unlikely to permeate lipid membranes, but they might gain access to the

Table 4
Postnatal treatment with α-difluoromethylornithine (DFMO) altered neither NMDA potency nor the antagonism of NMDA-evoked depolarization by Mg^{2+} in 10- to 15-day-old rats

$[Mg^{2+}]$	Treatment	pEC_{50} [a] (n) No added Mg^{2+}	EC_{50} (μM) No added Mg^{2+}	ΔpEC_{50} [a]	CR
0.316 mM	Saline	5.03 ± 0.04 (7)	9.3	0.14 ± 0.02	1.38
	DFMO	5.04 ± 0.02 (6)	9.1	0.13 ± 0.04	1.33
1.0 mM	Saline	5.09 ± 0.02 (21)	8.1	0.33 ± 0.02	2.15
	DFMO	5.11 ± 0.02 (4)	7.8	0.28 ± 0.02	1.90

Pups received daily injections of α-difluoromethylornithine (500 mg/kg s.c.) or saline from the day of birth until 24 h before they were killed. Values of EC_{50} and concentration ratio (CR) are geometric means. [a] Values are means ± S.E.M. for n = number of animals studied.

cell interior either through high affinity uptake (Harman and Shaw, 1981) or by penetrating the NMDA channel. The ability of Mg^{2+} to attenuate this slowly developing block is consistent with polyamine flux through the open channel (see below). Against this idea, however, is our finding that repeated application of NMDA failed to accelerate the rate of block. One would have expected repeated channel opening to facilitate the passage of polyamines through the channel, leading to a more rapid buildup on the intracellular surface of the receptor or within the pyramidal cell. Another difficulty with the hypothesis that polyamines act intracellularly is that the total intracellular concentration of polyamines in the adult CA1 area is about 500 μM, based on the results in Table 3. This value is already greater than the extracellular spermine concentrations needed to produce slowly developing block. It is difficult to understand how the inwardly directed transport or diffusion of polyamines could have had much additional effect, unless the intracellular polyamine concentration in the vicinity of the NMDA receptor is much lower than 500 μM.

Second, the slowly developing block was less selective for the NMDA receptor than rapidly developing voltage-dependent block. 1,10-Diaminodecane more potently reduced NMDA-evoked than AMPA-evoked depolarizations. However, spermine showed no such selectivity. Similarly, spermine and spermidine antagonized the depolarizing actions of both NMDA and AMPA in the rat cortical wedge (Robichaud and Boxer, 1993). The slowly developing block by polyamines may be exerted at a site or on a process common to NMDA and AMPA receptors. Thus the rapidly and slowly developing components of polyamine antagonism may involve different sites of action, even though the same polyamines appear to produce both effects.

Spermine did not potentiate NMDA-evoked depolarizations of CA1 pyramidal cells, even when low micromolar concentrations were tested and DMSO was used to facilitate penetration of spermine into the slice. Low micromolar concentrations of spermine accelerate the binding of [^{3}H]MK-801 and [^{3}H]TCP to the NMDA channel (Fig. 3; Ransom and Stec, 1988; Williams et al., 1989; Sacaan and Johnson, 1990a) and potentiate NMDA-evoked currents in isolated cells (Brackley et al., 1990; McGurk et al., 1990; Lerma, 1992; Rock and Macdonald, 1992a; Araneda et al., 1993; Benveniste and Mayer, 1993). However, except for a single report that spermine potentiates the maximal effect of NMDA on cGMP production in slices of immature rat cerebellum (Carter et al., 1990), spermine has only inhibited the actions of NMDA in slice preparations and in vivo (Carter et al., 1990; Rao et al., 1990; Robichaud and Boxer, 1993; Woodward and Cueto, 1993). At least three reasons may be suggested for the failure of spermine to potentiate NMDA-evoked depolarizations in the present study. First, polyamines act, in part, by enhancing the affinity of the receptor for the co-agonist glycine (Sacaan and Johnson, 1989; McGurk et al., 1990; Ransom and Deschenes, 1990; Benveniste and Mayer, 1993). In CA1-subiculum slices from adult rats, added glycine is without effect on NMDA-evoked depolarizations, suggesting that the glycine binding site is normally saturated (Bowe and Nadler, 1990). Because we observed stimulatory effects of glycine with some slices from immature rats, a near-saturating concentration of glycine (3 μM) was routinely included in the superfusion medium. Thus we could not have detected the component of spermine-induced potentiation that depends on enhancing glycine affinity in slices from either adult or immature rats. Second, spermine also potentiates responses to NMDA by slowing both the glycine-dependent and glycine-independent components of desensitization (Lerma, 1992; Benveniste and Mayer, 1993). The kinetics of NMDA-evoked responses in slice preparations, especially in grease-gap preparations, are much slower than the rate of NMDA receptor desensitization. Thus the peak response to NMDA almost certainly occurs at a time when the receptors have become maximally desensitized. Potentiation induced by slowing the onset of desensitization would likely be undetectable under these conditions. Third, at physiological membrane potentials the potentiating effect of polyamines on NMDA-evoked currents is offset or reversed by voltage-dependent block (Rock and Macdonald, 1992a; Benveniste and Mayer, 1993). Even at low concentrations of spermine, which accelerate the binding of [^{3}H]MK-801 and [^{3}H]TCP to the NMDA channel, there may be sufficient channel block to mask any potentiation that may occur. Moreover, the degree of polyamine-induced potentiation is known to vary markedly from one cultured hippocampal neuron to another (Benveniste and Mayer, 1993). Finally, by analogy with glycine, one might suspect that the stimulatory polyamine site in slice preparations is saturated with endogenous polyamines. The stimulatory site is probably located on the extracellular surface of the NMDA receptor (Lerma, 1992). It seems unlikely that the extracellular polyamine concentration would be high enough to saturate this site in vivo; the extracellular concentration of polyamines in brain is estimated to be ~ 1 μM (Gilad et al., 1993).

4.2. Negative interactions between polyamines and Mg^{2+}

Although both polyamines and Mg^{2+} inhibited responses to NMDA, their effects were less than additive. The three polyamines tested much less effectively antagonized the depolarizing action of NMDA in the presence of a physiological Mg^{2+} concentration (1 mM). Conversely, 1,10-diaminodecane and diethylene-

triamine diminished the blocking action of Mg^{2+} and spermine tended to do the same. This result differs somewhat from findings in membrane binding studies. Mg^{2+} was reported to antagonize effects of spermidine under certain conditions (Sacaan and Johnson, 1990a,b). However, spermidine and the putative polyamine antagonist arcaine affected the action of Mg^{2+} differently; spermidine enhanced (Reynolds, 1990; Sacaan and Johnson, 1991) and arcaine reduced (Sacaan and Johnson, 1990b) the ability of Mg^{2+} to inhibit [^{3}H]TCP or [^{3}H]MK-801 binding. This discrepancy would be understandable if the polyamine modulation of Mg^{2+} effects observed in binding studies were mediated by the stimulatory polyamine site. Actions mediated by this site apparently cannot be detected in grease-gap preparations.

In agreement with our findings, Williams et al. (1994) reported that inclusion of 1 mM Mg^{2+} in the bathing medium abolishes the inhibitory, but not the stimulatory, effect of spermine on the NMDA-evoked inward current through recombinant NR_{1A}/NR_{2B} receptors. Their result and ours suggest that the inhibitory effect of polyamines on NMDA currents would not be readily observed under physiological conditions. Mg^{2+} probably blocked polyamine-induced inhibition by preventing the access of polyamines to their site(s) of action. Mg^{2+} has also been shown to attenuate the block of NMDA currents by MK-801 (Huettner and Bean, 1988), another voltage-dependent channel blocker, although concentrations of Mg^{2+} greater than 1 mM were required. Mg^{2+} might prevent the action of polyamines through competition for a common or overlapping binding site within the NMDA channel, an allosteric interaction between distinct sites, physical blockade of the channel, repulsion of like charges and/or screening of surface charges at the mouth of the channel. This action of Mg^{2+} is consistent with the idea that the slowly developing antagonism of NMDA-evoked depolarizations involves the penetration of polyamines deeply into or through the NMDA channel. Polyamines would not be expected to permeate a Mg^{2+}-blocked channel.

The antagonism of Mg^{2+} by polyamines may occur by a mechanism similar to that of the antagonism of polyamines by Mg^{2+}. However, 1 mM Mg^{2+} more effectively attenuated the action of polyamines than polyamines attenuated the action of Mg^{2+}. At the concentrations tested, polyamines diminished, but did not abolish, the Mg^{2+} block of the NMDA channel. Polyamines can be released from brain by depolarizing stimuli and after activation of the NMDA receptor (Harman and Shaw, 1981; Gilad and Gilad, 1991; Fage et al., 1992). Our results suggest that, if polyamines reach high micromolar concentrations in the synaptic cleft, they could facilitate receptor activation by attenuating Mg^{2+} block.

4.3. Greater polyamine content of area CA1 does not explain the lower potency of Mg^{2+} during development

One objective of this study was to determine whether Mg^{2+} less potently antagonized the NMDA-evoked depolarization of CA1 pyramidal cells during development because of interference from endogenous polyamines. The polyamine content of area CA1 was considerably greater at 10–15 days of age than in adult rats, as expected from previous studies of the developing brain (Slotkin et al., 1982). α-Difluoromethylornithine treatment reduced the total polyamine content at this age almost to the level found in adults. Nevertheless, α-difluoromethylornithine treatment failed to increase the potency of Mg^{2+}. This result argues against the hypothesis that the elevated polyamine content of area CA1 in 10- to 15-day-old rats causes or contributes to the lesser Mg^{2+} block. It must be noted, however, that the various intracellular stores of polyamines may not have been affected equally by α-difluoromethylornithine treatment. We cannot exclude the possibility that polyamines in the vicinity of the NMDA receptor were not depleted.

Several other potential explanations for the developmental change in Mg^{2+} potency remain. The possibility that changes in receptor phosphorylation (Chen and Huang, 1992), resting membrane potential or the placement of NMDA receptors on the cell (Hamon and Heinemann, 1988) determine the developmental time course of Mg^{2+} sensitivity has yet to be explored. Interestingly, polyamines were also less potent NMDA receptor antagonists in slices from 10- to 15-day-old rats, suggesting a developmental modification of the NMDA receptor channel. A difference in the expression of NMDA receptor subunits appears not to be involved. The work of Monyer and coworkers (Monyer et al., 1994) suggests that CA1 pyramidal cells express Mg^{2+}-sensitive NMDA receptors throughout postnatal life; there is no evidence for a switch from a subtype of low Mg^{2+} sensitivity to a subtype of high Mg^{2+} sensitivity.

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