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An Acidic Amino Acid in the *N*-Methyl-D-Aspartate Receptor that Is Important for Spermine Stimulation

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SUMMARY

The polyamine spermine has multiple effects on *N*-methyl-D-aspartate (NMDA) receptors, including "glycine-independent" stimulation, which is seen in the presence of saturating concentrations of glycine; "glycine-dependent" stimulation, which is due to an increase in the affinity of the receptor for glycine; and voltage-dependent block. These effects may involve three separate polyamine binding sites on the receptor. To identify amino acid residues that are important for spermine binding, we used site-directed mutagenesis to alter amino acids in and around a region of the NR1 subunit of the NMDA receptor that shows homology with PotD, a polyamine binding protein from *Escherichia coli*. Mutated subunits, expressed in heteromeric and homomeric NMDA receptors, were studied by voltage-clamp recording in *Xenopus* oocytes. Mutation of two acidic residues (E339 or E342) to neutral amino acids reduced or abolished glycine-independent stimulation by spermine without affecting glycine-dependent stimulation or voltage-dependent block by spermine. Mutation of these residues also had modest effects on sensitivity to protons and to ifenprodil but did not alter sensitivity to glutamate and glycine or to voltage-dependent block by Mg^{2+}. Residue E342 in NR1 appears to be critical for glycine-independent spermine stimulation. Mutations at equivalent positions in NR2A(E352Q) or NR2B(E353Q) had no effect on sensitivity to spermine, pH, or ifenprodil. Residue E342 in NR1 may form part of a discrete spermine binding site on the NMDA receptor or be involved in the mechanism of modulation by polyamines. This residue may also be involved in modulation by protons and ifenprodil.

Numerous endogenous substances have been found to modulate the properties of NMDA receptors. Some of these substances, which include polyamines, neurosteroids, protons, and Zn^{2+}, may alter the activation of NMDA receptors *in vivo* (1) and could influence processes such as the induction of long-term potentiation and excitotoxicity that are mediated by NMDA receptors. With the cloning of cDNAs encoding subunits of NMDA receptors (2, 3), it has become possible to investigate the site and mechanism of action of these modulators at a molecular level. In some cases, the effects of modulators have been found to be dependent on the subunit composition of the receptor. For example, effects of the polyamine spermine are dependent of the type of NR1 splice variant and the type of NR2 subunit present in homomeric NR1 receptors and in heteromeric NR1/NR2 receptors (4–8).

There is only limited information concerning structure-function relationships of NMDA receptor subunits and the possible localization of binding sites. Site-directed mutagenesis has been used to identify amino acid residues in the NR1 subunit that form a glycine-binding site (9, 10). Mutation of amino acid residues in the putative channel-forming regions of NR1 and NR2 subunits alters divalent cation permeability and sensitivity to block by Mg^{2+} and MK-801 (11–14). Cysteine residues (C744 and C798) that are involved in the effects of redox reagents have also been identified (15). These residues also influence sensitivity to pH and spermine and may be involved in channel gating (15). Sensitivity to polyamines is controlled by the presence or absence of a 21-amino acid insert, encoded by exon 5, in the NR1 subunit, although this insert does not appear to form part of a spermine binding site (4, 16).

Spermine has four macroscopic effects on recombinant NMDA receptors: (1) "glycine-independent" stimulation, which is seen in the presence of saturating concentrations of glycine; (2) "glycine-dependent" stimulation, which is seen with subsaturating concentrations of glycine and involves an increase in the affinity of the receptor for glycine; (3) voltage-dependent inhibition, which is more pronounced at hyperpolarized membrane potentials; and (4) a decrease in agonist affinity, which can mask stimulatory effects of spermine when low concentrations of glutamate or NMDA are used to activate NMDA receptors (4–8). All four effects of spermine are seen at homomeric NR1A receptors and at heteromeric

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; kb, kilobase(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

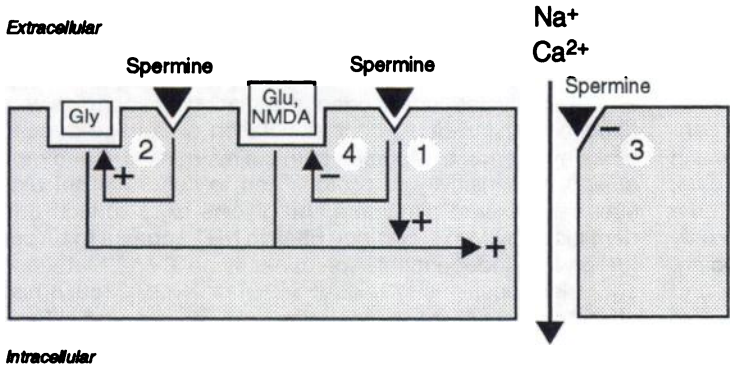
NR1A/NR2B receptors, but only glycine-dependent stimulation and voltage-dependent block are seen at NR1A/NR2A receptors (Fig. 1A). Spermine has no effect at NR1A/NR2C or NR1A/NR2D receptors (7). We have suggested that the effects of spermine may involve at least three discrete spermine binding sites (7, 8) (Fig. 1A).

Glutamate receptor subunits, including NR1, have homology with bacterial periplasmic amino acid binding proteins (2, 9, 17–19). In the present study, we used site-directed mutagenesis to attempt to identify amino acid residues that contribute to spermine binding sites on the NMDA receptor. The strategy that we adopted is centered around a region of NR1 that shares amino acid sequence homology with PotD, a periplasmic polyamine binding protein from *Escherichia coli* that is a component of the *E. coli* polyamine transport system (20, 21). Mutant NR1 subunits, expressed together with NR2B or NR2A in heteromeric NR1/NR2 receptors and expressed as homomeric NR1 receptors, were studied by voltage-clamp recording in *Xenopus* oocytes. We identified amino acid residues in NR1 that selectively influence the glycine-independent form of spermine stimulation; these residues may form part of a discrete polyamine binding site.

Materials and Methods

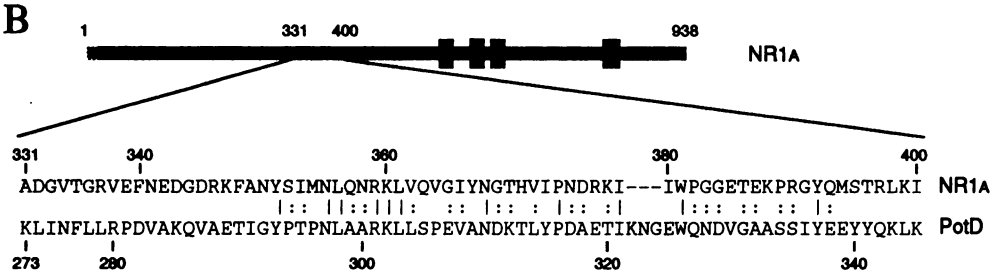
Sequence comparisons. Analysis of the sequence homology of NR1A and PotD was carried out according to the methods of Needle-

A



Effect of Spermine	NR1A	NR1A/ NR2A	NR1A/ NR2B
1. "Glycine-independent" stimulation	yes	no	yes
2. "Glycine-dependent" stimulation (increase affinity for glycine)	yes	yes	yes
3. Voltage-dependent inhibition	yes	yes	yes
4. Decrease affinity for agonists	yes	no	yes

B



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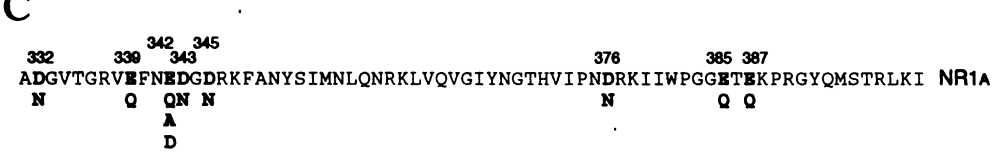

Fig. 1. Properties of NMDA receptors and design of NR1 mutants. A, Schematic model of the NMDA receptor illustrating the four macroscopic effects of spermine and the manifestation of these effects at homomeric NR1A and heteromeric NR1A/NR2 receptors. B, Schematic of the NR1A subunit cDNA and a region showing homology (amino acids 351–393) with PotD. *Vertical lines*, identical amino acids. *Colons*, favored substitutions. *Gaps* in NR1A were introduced to maximize homology. Amino acid residues in NR1A are numbered from the initiator methionine (2). C, Amino acid mutations in NR1A described in this study.

man and Wunsch (22) with the program DNASIS (Hitachi Software Engineering Co. Ltd.).

Mutagenesis of NR1 and NR2. To prepare NR1A mutants, a 2.6-kb *Sph*I/*Sal*I fragment of plasmid pN60 (2) was inserted into the same sites of M13 mp18 or M13 mp19 (23). Similarly, to prepare NR2 mutants, a 2.2-kb *Bam*HI/*Xma*I fragment of pBSNR2A and a 2.1-kb *Bam*HI/*Sph*I fragment of pBSNR2B were inserted into the same sites of M13 mp18 and M13 mp19, respectively. Site-directed mutagenesis (24) was carried out using the oligonucleotides shown in Table 1. The mutated DNA fragments were isolated from the replicative form of M13 and religated into the same sites of pN60, pBSNR2A, or pBSNR2B. Mutations were confirmed by DNA sequencing (25) using the M13 phage system (23) with commercial and synthesized primers. To prepare NR1B mutants, the pBSNR1B clone was digested with *Bgl*II, and the 5.7-kb was fragment isolated. The 1.6-kb *Bgl*II fragment of each NR1A mutant was then ligated with the 5.7-kb fragment of pBSNR1B to yield the appropriate NR1B mutant. For example, the fragment from NR1A(E342Q), when ligated into pBSNR1B, produces NR1B(E363Q). The orientation of the inserts was verified by digestion with *Xho*I. Plasmids were propagated in *E. coli* DH5α and isolated using standard procedures.

Expression in oocytes and voltage-clamp recording. Plasmids were linearized by using *Not*I (NR1 clones) or *Eco*RI (NR2 clones). Capped cRNAs were synthesized from linearized cDNA templates using an *in vitro* transcription kit (mMessage mMachine; Ambion Inc., Austin, TX) with T7 (NR1) or T3 (NR2A, NR2B) RNA polymerase (8, 26).

Defolliculated stage V–VI oocytes were prepared from *Xenopus laevis* (Nasco, Fort Atkinson, WI) as described previously (27). Oocytes were injected with NR1A or NR1B plus NR2A or NR2B cRNAs in a ratio of 1:5 (1–2 ng of NR1 plus 5–10 ng of NR2). For homomeric NR1A receptors, oocytes were injected with 10 ng of NR1A cRNA. After injection, oocytes were maintained in a saline solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Na-HEPES, 2.5 mM sodium pyruvate, 50 μg/ml gentamycin, pH 7.5) at 18° for 1–4 days before recording. The saline solution was replaced daily.

Macroscopic currents were recorded with a two-electrode voltage-clamp using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA) as described previously (8, 26). Electrodes were filled with 3 M KCl and had resistances of 0.4–3 MΩ. Oocytes were continuously superfused (~5 ml/min) with a Mg^{2+}-free saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, 10 mM Na-HEPES, pH 7.5). The extracellular solution contained $BaCl_2$ instead of $CaCl_2$ to minimize Ca^{2+}-activated Cl^- currents (26, 28). In most experiments, oocytes were injected with K^+-BAPTA (100 nl; 40 mM, pH 7.4) on the day of recording to eliminate a slowly activating Cl^- current that is seen even in the presence of extracellular Ba^{2+} (26). Data were digitized and recorded using an MP-100 interface with AcqKnowledge software (Biopac Systems, Goletta, CA) on a Macintosh computer. All reported values are mean ± standard error from *n* oocytes, and all mutants were examined in oocytes from at least two different donor frogs.

TABLE 1

Oligonucleotides for production of NR1 and NR2 mutants

The mutated nucleotides are underlined. u = untranslated strand.

Mutant	Oligonucleotide	M13 clone used
NR1 mutants		
D332N	TAA GTA TGC GAA CGG AGT GAC	M13mp19
E339Q	TGG CCG TGT GCA ATT CAA TGA	M13mp19
E342Q	GGA ATT CAA TCA GGA TGG GGA	M13mp19
E342Au	GTC CCC ATC CGC ATT GAA TTC	M13mp18
E342Du	GGT CCC CAT CGT CAT TGA ATT	M13mp18
D343N	ATT CAA TGA GAA TGG GGA CCG	M13mp19
D345Nu	AAC TTC CGG TTC CCA TCC TCA	M13mp18
D376Nu	ATC TTC CTG TTA TTT GGG ATG	M13mp18
E385Qu	TTT CTC TGT CTG TCC TCC TGG CC	M13mp18
E387Q	AGG AGA GAC ACA GAA ACC TCG	M13mp19
NR2 mutants		
NR2A (E352Q)	GTC CTT CAG TCA GGA AGG TTA	M13mp18
NR2B (E353Q)	GTC CTT CAG CCA AGA TGG CTA	M13mp19

Materials. L-Glutamate and glycine were purchased from Sigma Chemical Co. (St. Louis, MO). Spermine tetrahydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ifenprodil was a gift from Synthélabo Recherche (Bagneux, France). The rat brain NR1A and NR1B clones (2, 29) were a gift from Dr. S. Nakanishi (Kyoto University, Japan). The NR2A and NR2B clones (30) were a gift from Dr. P. H. Seeburg (University of Heidelberg, Germany).

Nomenclature. NR1 variants are referred to by the nomenclature of Sugihara *et al.* (29). Thus, NR1A lacks the amino-terminal 21-amino acid insert, whereas NR1B contains this insert (29). Mutations are referred to in parentheses after the subunit name, with the wild-type amino acid, its position, and the mutation. Thus, NR1A(E342Q) contains a glutamine (Q) residue at position 342 instead of glutamate (E), as found in the wild-type NR1A subunit. Amino acids are numbered from the initiator methionine residues in NR1 and NR2 subunits (2, 29, 30).

Results

Strategy and comparison of NR1 with PotD. The NR1 subunit is expressed as at least eight alternatively spliced mRNAs (29, 31). Sensitivity to glycine-independent stimulation by spermine is controlled by the presence or absence of a 21-amino acid insert in the large amino-terminal domain of NR1, which is presumed to be extracellular (4). Stimulation is seen with variants such as NR1A that lack the amino-terminal insert but not with variants such as NR1B that contain the insert (4, 5). In the present study, we first made mutations in the NR1A variant (2, 29), which lacks the amino-terminal insert and is sensitive to spermine stimulation.

Because effects of spermine are seen at homomeric NR1A receptors as well as at some heteromeric NR1A/NR2 receptors (Fig. 1A), we hypothesized that the spermine binding sites are located on the NR1A subunit or can be formed by the interaction of two or more NR1A subunits in homomeric NR1A receptors. To identify regions of NR1A that may contribute to spermine binding sites, the amino acid sequence of NR1A was compared with that of PotD, a polyamine binding protein from *E. coli* (20). A region of NR1A (residues 351–393) was found to share homology with a region of PotD (residues 293–338) (Fig. 1B). This region of NR1 shows 24% identity in a 46-amino acid overlap. This is similar to the levels of identity (25–31%) seen in regions of NR1 that show similarity with the glutamine binding protein from *E. coli* (2). The region of interest is upstream of segments of NR1A (residues 398–425, 457–543, and 655–792) that show similarity with the glutamine binding protein and the lysine/arginine/ornithine binding protein (2, 9). The region of homology includes a conserved RKL motif (residues 359–361 in NR1A). An RKL motif is also found in spermidine acetyltransferase from *E. coli* in a region that shows some similarity with PotD and may be close to the active center of the enzyme (32). In PotD, three acidic amino acids have been found to form part of a polyamine binding site.[1] To attempt to identify residues that may be important for polyamine binding in NR1A, we carried out site-directed mutagenesis to neutralize acidic amino acid

[1] K. Kashiwagi, R. Pistocchi, S. Shibuya, S. Sugiyama, K. Morikawa, and K. Igarashi, unpublished observations.

residues in and around the region of NR1A that shows homology with PotD (Fig. 1B). The mutations included changing the codons for glutamate residues (E) to codons for glutamine (Q) and codons for aspartate residues (D) to those for asparagine (N) (Fig. 1C). The E342Q mutation had the most dramatic effects on spermine stimulation, and we therefore tested other mutations (i.e., E342A, E342D) at this position.

Spermine sensitivity at heteromeric NR1/NR2 receptors. Heteromeric NR1A/NR2B receptors are sensitive to modulation by spermine (Fig. 1) and gate much larger macroscopic currents than do homomeric NR1A receptors. Thus, the properties of mutant NR1A subunits were first examined in NR1A/NR2B receptors, which provide a sensitive and robust system to test the multiple effects of spermine. The experimental paradigms are illustrated in Fig. 2. In oocytes voltage-clamped at −20 mV to minimize the voltage-dependent block by spermine, and with 10 μM glutamate and 10 μM glycine to activate the receptors, spermine produces glycine-independent stimulation at wild-type NR1A/NR2B receptors (Fig. 2A). In oocytes voltage-clamped at −20 mV but with 10 μM glutamate and 0.1 μM glycine to activate the receptors, spermine produces both glycine-independent and -dependent stimulation (Fig. 2B). Because spermine increases the affinity of NR1A/NR2B receptors for glycine, a larger potentiation by spermine is seen with 0.1 μM glycine than with 10 μM glycine (compare traces in Fig. 2, A and B, for wild-type receptors). In oocytes voltage-clamped at −100 mV and with 10 μM glutamate and 10 μM glycine to activate the receptors, spermine produces glycine-independent stimulation followed by a slowly developing block (i.e., voltage-dependent inhibition) at wild-type NR1A/NR2B receptors (Fig. 2C). These three paradigms were used to study the effects of spermine at NR1A/NR2B receptors containing wild-type and mutant NR1A subunits (Figs. 2 and 3). Wild-type and mutant subunits were compared in all experiments; in Fig. 3, the broken lines in A, B, and D correspond to the effects of spermine at

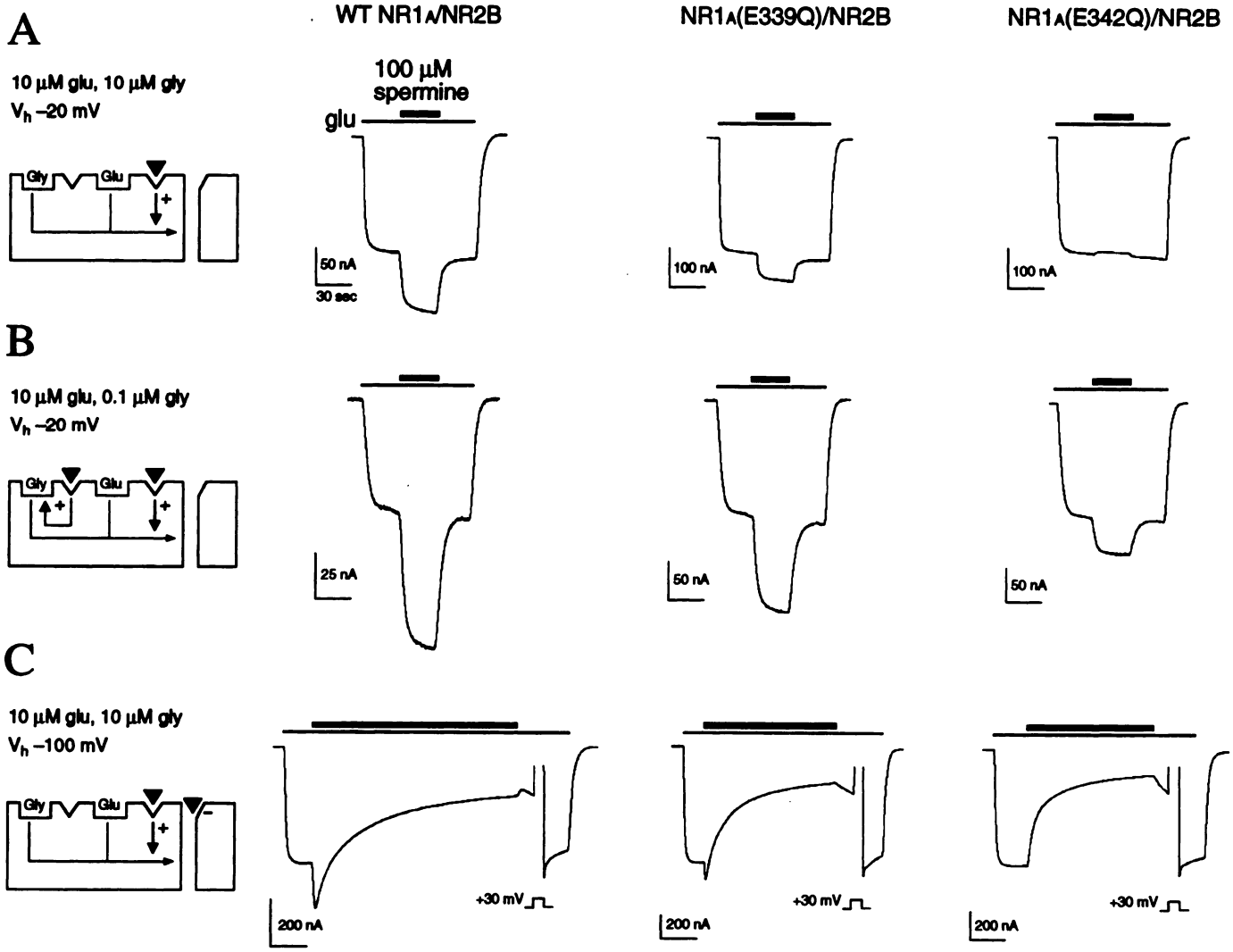

Fig. 2. Screening for effects of spermine at mutant NR1A/NR2B receptors. Traces are inward currents induced by glutamate in the absence and presence of 100 μM spermine in oocytes expressing NR1A/NR2B receptors containing wild-type (*WT*), E339Q, and E342Q NR1A subunits. Traces are from the same oocyte for each subunit combination, and oocytes were from the same frog. The concentrations of glutamate and glycine and the holding potential are given next to each row. *Schematics* of the NMDA receptor (see Fig. 1A) show the effects being measured: glycine-independent stimulation (A), glycine-independent and glycine-dependent stimulation (B), and glycine-independent stimulation and voltage-dependent block (C). In oocytes voltage-clamped at −100 mV (C), the oocyte was depolarized to +30 mV for 6 sec after washout of spermine to speed recovery of the response to glutamate. *Horizontal calibration bars*, 30 sec for all traces.

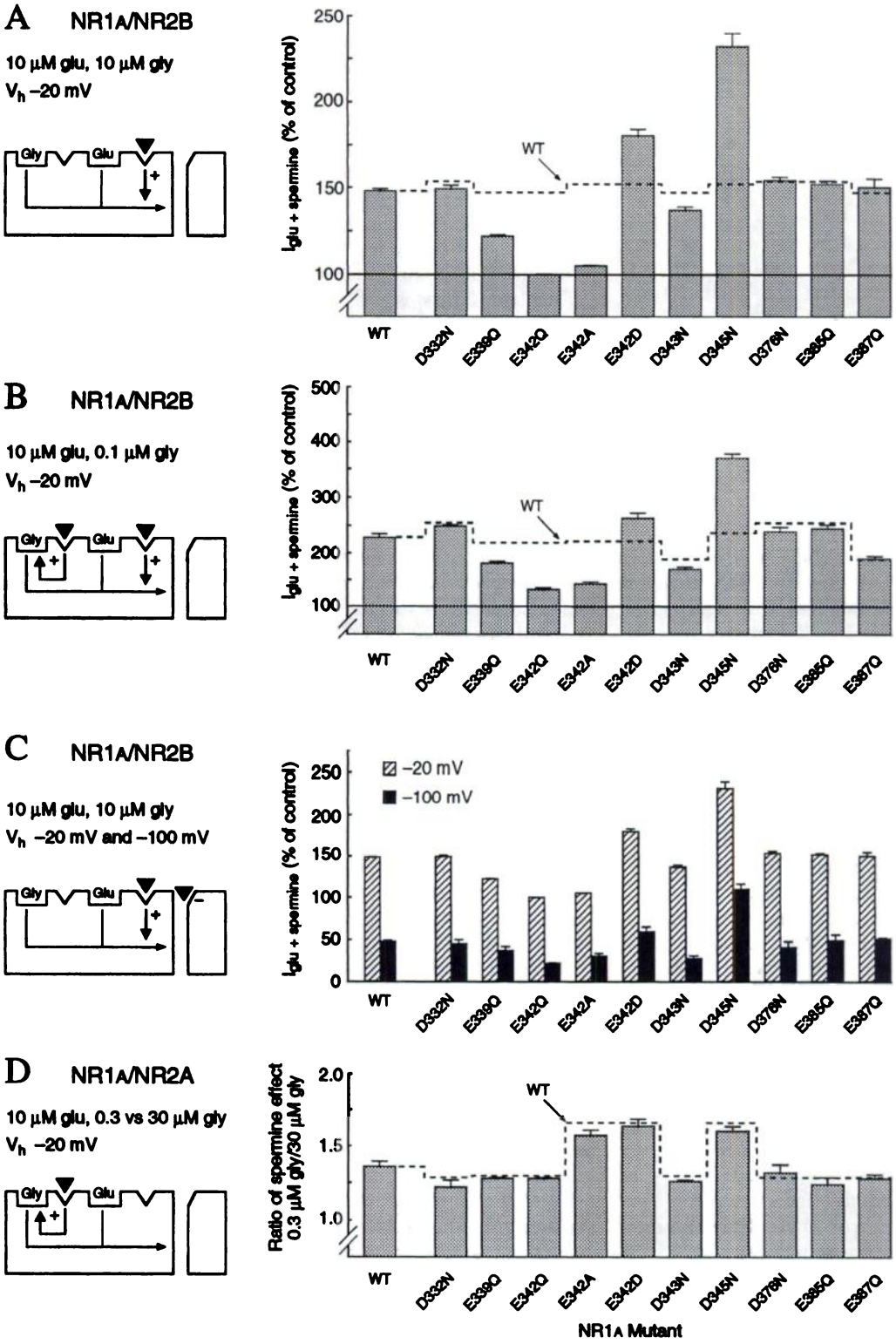

Fig. 3. Properties of heteromeric NR1A/NR2 receptors. The effects of 100 μM spermine on responses to glutamate and glycine were measured at NR1A/NR2B receptors (A, B, and C) and NR1A/NR2A receptors (D) containing wild-type (*WT*) and mutant NR1A subunits. The concentrations of glutamate and glycine and the holding potential are shown next to each panel together with a schematic of the NMDA receptor (see Fig. 1A) to show the effects being measured: glycine-independent stimulation (A), glycine-independent and glycine-dependent stimulation (B), glycine-independent stimulation and voltage-dependent block (C), and glycine-dependent stimulation (D). In A, B, and C, responses measured in the presence of spermine are expressed as a percentage of the control response to glutamate measured under the same experimental conditions. In D, the results are expressed as the ratio of the effect of spermine measured with 0.3 μM glycine to the effect of spermine measured with 30 μM glycine. *Broken lines* in A, B, and D represent responses in wild-type receptors measured in the same batches of oocytes as the respective mutants. Data are mean ± standard error from 5–14 oocytes for each mutant and from 25–36 oocytes for wild-type receptors.

wild-type receptors measured in the same batches of oocytes as the mutant receptors.

All of the mutant NR1A subunits, when coexpressed with NR2B, generated receptors that gate large macroscopic currents (100–1000 nA at −20 mV; Table 2) similar to wild-type NR1A/NR2B receptors. A number of mutations had effects on glycine-independent stimulation by spermine (Figs. 2A and 3A). In particular, stimulation was reduced with NR1A(E339Q) and abolished with NR1A(E342Q). Mutation of E342 to alanine (E342A) greatly reduced spermine stimulation, whereas changing this residue to aspartate (E342D), which retains a negative charge at this position, produced an increase in stimulation by spermine (Fig. 3A). Stimulation by another polyamine, spermidine, was also reduced at NR1A/NR2B receptors containing E339Q, E342Q, and E342A (data not shown). A larger stimulation by spermine was seen at

TABLE 2

Properties of NR1/NR2B receptors containing mutant NR1A subunits

I_{glu} is the response to glutamate (10 μM) + glycine (10 μM) in oocytes voltage-clamped at −20 mV. EC_{50} values for glutamate were derived from glutamate concentration-response curves measured in the presence of 10 μM glycine. EC_{50} values for glycine were derived from glycine concentration-response curves measured in the presence of 10 μM glutamate.

NR1 A mutant	I_{glu}		Glycine EC_{50}		Glutamate EC_{50}	
	nA	n	μM	n	μM	n
Wild-type	346 ± 26	70	0.17 ± 0.02	10	1.4 ± 0.1	4
E339Q	448 ± 44	40	0.19 ± 0.02	6	1.9 ± 0.1	4
E342Q	393 ± 42	42	0.20 ± 0.02	6	2.2 ± 0.1	4
E342A	527 ± 68	21	0.11 ± 0.01	4	2.1 ± 0.2	4
D345N	172 ± 15	39	0.28 ± 0.03	6	1.3 ± 0.1	4

NR1A(D345N) than at wild-type receptors (Fig. 3A). A similar effect was seen with aspartate to alanine (D to A) and aspartate to glutamate (D to E) mutations at D345. Spermine stimulation was 195 ± 3% (six oocytes) at NR1A(D345A)/NR2B receptors and 254 ± 5% (six oocytes) at NR1A(D345E)/NR2B receptors compared with 143 ± 3% at wild-type NR1A/NR2B receptors. Thus, the effects seen with D345 mutations did not appear to be due simply to the loss of an acidic group at this position. Mutations that neutralize other acidic residues in this region of the NR1A subunit (D332N, D376N, E385Q, and E387Q) had no effect on stimulation by spermine (Fig. 3A).

In the presence of 0.1 μM glycine, a condition in which spermine produces both glycine-independent and glycine-dependent stimulation, the effect of spermine was reduced at NR1A/NR2B receptors containing E339Q, E342Q and E342A and increased at receptors containing E342D and D345N (Fig. 3B). Importantly, however, stimulation by spermine does occur at E342Q and E342A with 0.1 μM glycine (Figs. 2 and 3B), suggesting that these mutations, which abolish glycine-independent stimulation (Fig. 3A), do not affect glycine-dependent stimulation (Fig. 3B). This conclusion is supported by results with NR1A/NR2A receptors (see below).

In oocytes voltage-clamped at −100 mV, voltage-dependent block by spermine was seen at NR1A/NR2B receptors containing wild-type and mutant NR1A subunits. The mutations did not affect voltage-dependent block by spermine (Figs. 2C and 3C). The effects of spermine on responses to glutamate at wild-type and NR1A(E342Q)/NR2B receptors were also studied by using voltage ramps. At wild-type receptors, spermine (100 μM) had dual effects involving a net inhibition at hyperpolarized membrane potentials and stimulation at positive potentials as previously reported (5). At NR1A(E342Q)/NR2B receptors, spermine (100 μM) inhibited the response to glutamate at membrane potentials more negative than ~−20 mV but had no effect at positive potentials up to +40 mV (data not shown).

Spermine produces voltage-dependent block and glycine-dependent stimulation but not glycine-independent stimulation at NR1A/NR2A receptors (Fig. 1A). Thus, glycine-dependent stimulation can be studied in relative isolation at NR1A/NR2A receptors in oocytes voltage-clamped at −20 mV. Effects of spermine on responses to glutamate at NR1A/NR2A receptors were studied with 0.3 μM and 30 μM glycine (each with 10 μM glutamate). With 30 μM glycine (a saturating concentration), spermine produced a small inhibition at NR1A/NR2A receptors, and with 0.3 μM glycine, spermine potentiated the response to glutamate. The difference between the effects of spermine seen with 30 μM and 0.3 μM glycine thus represents glycine-dependent stimulation. For example, at NR1A/NR2A receptors containing NR1A(E342Q), currents measured in the presence of 100 μM spermine were 89 ± 1% of control (30 μM glycine) and 114 ± 1% (0.3 μM glycine; seven oocytes; ratio of spermine effect at 0.3 μM glycine/30 μM glycine = 1.28 ± 0.01). At wild-type NR1A/NR2A receptors in the same batches of oocytes, currents measured in the presence of 100 μM spermine were 88 ± 2% of control (30 μM gly) and 113 ± 3% (0.3 μM glycine; seven oocytes; ratio of spermine effect at 0.3 μM glycine/30 μM glycine = 1.29 ± 0.02). In Fig. 3D, the ratios of the effects of spermine on responses to 0.3 μM and 30 μM glycine are plotted for NR1A/NR2A receptors containing wild-type and mutant NR1A subunits. None of the mutations altered glycine-dependent stimulation at NR1A/NR2A receptors (Fig. 3D). Again, this suggests that mutations at E339 and E342 selectively affect the glycine-independent form of spermine stimulation.

At NR1A/NR2B receptors, glycine-independent stimulation is influenced by the concentration of NMDA or glutamate used to activate the receptors (8). Thus, 100 μM spermine potentiates responses to 10 μM glutamate but has little or no effect on responses to 1 μM glutamate (8). This difference is due to a decrease in agonist affinity that masks spermine stimulation at low concentrations of glutamate (effect No. 4 in Fig. 1A). If mutations in NR1A alter the affinity of NR1A/NR2B receptors for glutamate, 10 μM glutamate may not be saturating and an effect of spermine on agonist affinity could then mask spermine stimulation at receptors containing these mutants. To test this possibility, we compared the effects of spermine on responses to 10 μM and 100 μM glutamate (each with 10 μM glycine) at NR1A(E342Q)/NR2B receptors. Spermine (100 μM) had no effect on responses 10 μM glutamate (100 ± 1% of control) or 100 μM glutamate (101 ± 1% of control; five oocytes). Thus, a change in agonist affinity cannot account for the lack of spermine stimulation at NR1A(E342Q)/NR2B receptors.

The ability of spermine to decrease agonist affinity may be mediated through the same site at which spermine produces glycine-independent stimulation (Fig. 1A). To determine whether the effect of spermine on agonist affinity is also altered by mutations in NR1A, we compared the effects of 100 μM spermine on responses to 1 μM and 10 μM glutamate at NR1A/NR2B receptors. In receptors containing mutants that reduce or abolish glycine-independent stimulation, the effect of spermine on agonist affinity was also reduced, consistent with the model shown in Fig. 1A. Thus, at wild-type NR1A/NR2B receptors, responses in the presence of spermine were 148 ± 1% of control (10 μM glutamate) and 104 ± 1% (1 μM glutamate) (29 oocytes; ratio of spermine effect with 0.1 μM glutamate/10 μM glutamate = 0.70 ± 0.01), whereas at NR1A(E342Q)/NR2B receptors, responses in the presence of spermine were 99 ± 1% (10 μM glutamate) and 87 ± 2% (1 μM glutamate) (nine oocytes; ratio of spermine effect with 0.1 μM glutamate/10 μM glutamate = 0.88 ± 0.02).

We examined concentration-response relationships for spermine at NR1A/NR2B receptors (Fig. 4). A complication in these experiments is that the inhibitory effect of spermine becomes pronounced at concentrations above 100 μM using

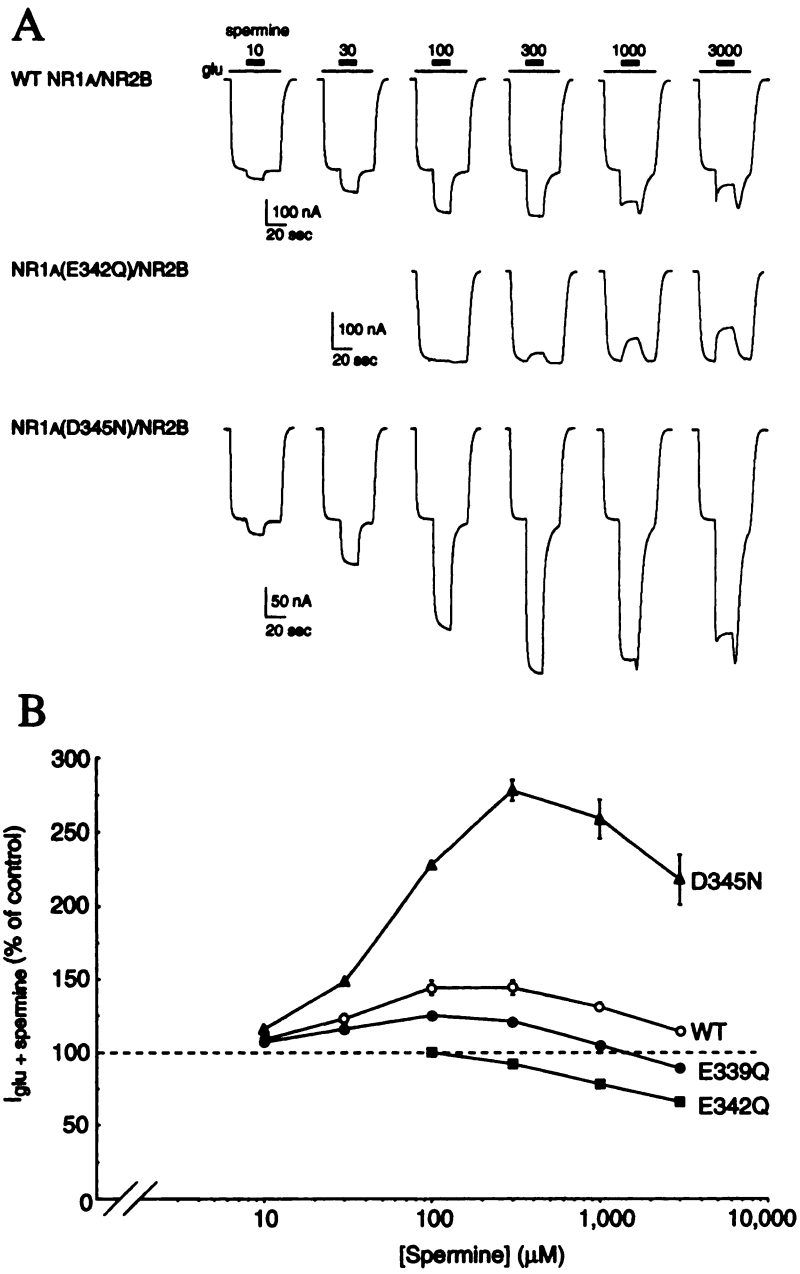

Fig. 4. Concentration-responses analyses for spermine. A, Representative traces showing the effects of spermine (10–3000 μM) on responses to glutamate (10 μM with 10 μM glycine) in oocytes expressing NR1A/NR2B receptors containing wild-type (*WT*), E342Q, and D345N NR1A subunits. B, The effects of various concentrations of spermine on responses to glutamate were measured using the protocols shown in A. Data are steady state responses measured in the presence of spermine and are expressed as a percentage of the control response to glutamate from four to six oocytes for each subunit combination. Oocytes were voltage-clamped at −20 mV.

oocytes voltage-clamped at −20 mV. Thus, at wild-type NR1A/NR2B receptors, the degree of steady state potentiation by spermine is actually reduced at high concentrations of spermine (Fig. 4). Nevertheless, one can detect both the stimulatory and inhibitory components with high concentrations of spermine at NR1A/NR2B receptors (see traces for WT NR1A/NR2B in Fig 4A). With NR1A(E339Q), the magnitude of the response to spermine rather than the potency appeared to be altered (Fig. 4B). No stimulation by spermine was seen at NR1A(E342Q)/NR2B receptors at concentrations of spermine up to 3 mM (Fig. 4, A and B). With NR1A (D345N), the magnitude of spermine stimulation was increased, but the potency of spermine appeared to be unaltered (Fig. 4, A and B).

Properties of homomeric NR1A receptors. The amino acids in NR1A that affect spermine stimulation at NR1A/NR2B receptors could form part of a spermine binding site or influence the mechanism of action of spermine or the interaction of NR1A and NR2B subunits in a heteromeric complex. If the amino acids of interest are part of a polyamine binding site, then these residues would be predicted to influence glycine-independent stimulation in homomeric NR1A receptors. To test this hypothesis, we studied the effects of spermine on responses to glutamate (10 μM; with 10 μM glycine) at homomeric NR1A receptors (Fig. 5A). Because homomeric NR1 receptors produce very small macroscopic currents, responses were measured in an extracellular solution containing $CaCl_2$ using oocytes voltage-clamped at −70 mV. Under these conditions, there is an initial spike that represents a Ca^{2+}-activated Cl^- conductance (28) followed by a plateau phase. Effects of spermine were measured during the plateau phase. Similar to results seen at NR1A/NR2B receptors, glycine-independent stimulation by spermine was reduced or abolished at E339Q, E342Q, and E342A receptors but not at

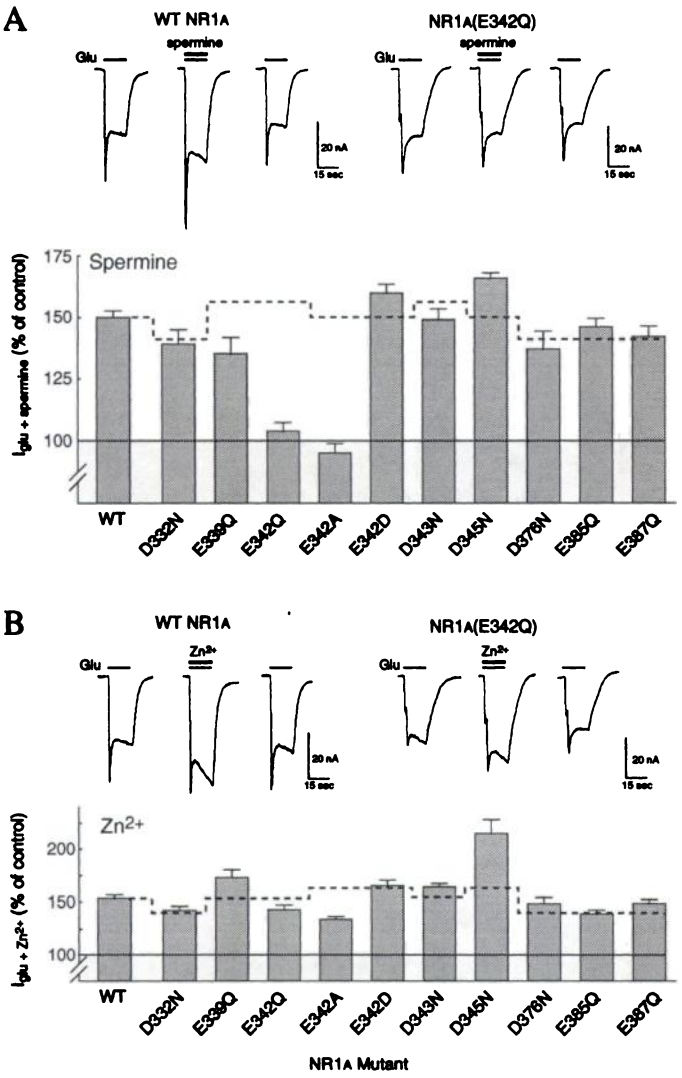

Fig. 5. Properties of homomeric NR1A receptors. The effects of 100 μM spermine (A) and 1 μM Zn^{2+} (B) on responses to glutamate and glycine (10 μM each) were measured at homomeric NR1A receptors expressed from wild-type (*WT*) and mutant NR1A subunits in oocytes voltage-clamped at −70 mV in an extracellular solution containing $CaCl_2$. Representative traces showing the effects of spermine and Zn^{2+} at wild-type and E342Q receptors are from the same oocyte for each subunit combination in A and B. Data, expressed as a percentage of the control response to glutamate, are mean ± standard error from five to nine oocytes for each mutant and from 20 oocytes for wild-type receptors. *Broken lines*, responses in wild-type receptors measured in the same batches of oocytes as the respective mutants.

E342D receptors (Fig. 5A). Other E-to-Q and D-to-N mutations in this region of NR1A had no effect on spermine stimulation.

Low concentrations of Zn^{2+} have been reported to potentiate responses at some types of homomeric NR1 receptors (31, 33). Potentiation by Zn^{2+} is seen at homomeric NR1 receptors expressed from variants such as NR1A that lack the amino-terminal insert (31), similar to potentiation by spermine. Thus, spermine and Zn^{2+} may share a common binding site or mechanism of action. We studied the effects of Zn^{2+} on homomeric NR1A receptors to determine whether mutations that affect sensitivity to spermine also affect sensitivity to Zn^{2+} (Fig. 5B). Mutations at E339 and E342 had little or no effect on potentiation by Zn^{2+}.

Sensitivity of NR1A/NR2B receptors to other modulators. To determine the specificity of mutations at E339, E342, and D345, we examined the sensitivity of NR1A/NR2B receptors to glutamate and glycine and to Mg^{2+}, Zn^{2+}, protons, and ifenprodil. The mutations had no effect on sensitivity to glutamate or glycine (Table 2) or on voltage-dependent block by Mg^{2+} (Fig. 6A). Block by Zn^{2+} was slightly reduced in receptors containing NR1A(E339Q), (E342Q), and (E342A) and increased in receptors containing NR1A (D345N) (Fig. 6B). Proton sensitivity was determined by measuring responses to glutamate at pH 8.5, 7.5, and 6.5. In Fig. 6C, currents measured at pH 7.5 and 6.5 are expressed relative to currents at pH 8.5 in the same oocytes. Thus, as previously reported (16), protons markedly inhibited responses at wild-type NR1A/NR2B receptors. Proton inhibition was reduced somewhat by the E339Q, E342Q, and E342A mutations and increased by the D345N mutation (Fig. 6C). Inhibition by ifenprodil was reduced in receptors containing NR1A(E342Q) and, to a lesser extent, NR1A(E342A) (Fig. 6D). Other E-to-Q and D-to-N mutations in NR1A had no effect on sensitivity to ifenprodil.

Properties of mutant NR1B subunits. The NR1B variant of NR1 is identical to NR1A in its carboxyl terminus but contains a 21-amino acid insert in the large amino-terminal domain (29). This insert has been found to influence sensitivity to polyamines and protons (4, 5, 16). Therefore, we examined mutations at acidic residues in NR1B corresponding to those that influence spermine stimulation in NR1A. We studied the following mutations in NR1B: E360Q, E363Q, D364N, and D366N, which correspond to E339Q, E342Q, D343N, and D345N, respectively, in NR1A. Mutant NR1B subunits were coexpressed with NR2B, and we studied wild-type NR1B/NR2B and wild-type NR1A/NR2B receptors in the same batches of oocytes (Fig. 7). As previously reported (5, 6) wild-type NR1A/NR2B but not NR1B/NR2B receptors were sensitive to glycine-independent spermine stimulation (Fig. 7). Mutations E360Q, E363Q, and D364N in NR1B did not alter sensitivity to spermine, whereas a small potentiation was seen with NR1B (D366N) (Fig. 7).

Wild-type NR1B/NR2B receptors were less sensitive to inhibition by protons than were NR1A/NR2B receptors (Fig. 7). Mutations E360Q and E363Q decreased inhibition by protons, whereas D366N increased proton inhibition (Fig. 7). The effects on proton sensitivity are very similar to changes in pH sensitivity seen with the corresponding mutations in NR1A(E339Q, E342Q, and D345N; see Fig. 6C). Thus, the changes in pH sensitivity seen with these mutations appear to be independent of the presence of the 21-amino acid amino-terminal insert.

Properties of mutant NR2A and NR2B subunits. E342 in NR1A appears to be critical for glycine-independent spermine stimulation in homomeric NR1A and heteromeric NR1A/NR2B receptors. This residue also influences sensitivity to pH and ifenprodil in NR1A/NR2B receptors. The corresponding amino acids in the NR2A and NR2B subunits are also glutamate residues: E352 in NR2A and E353 in NR2B (Fig. 8, *top*). To determine whether acidic residues at these positions in NR2A and NR2B influence sensitivity to spermine, protons, and ifenprodil, we constructed mutants in which these glutamate residues were neutralized: NR2A(E352Q) and NR2B(E353Q). The properties of these mutants were examined after coexpression with wild-type NR1A (Fig. 8).

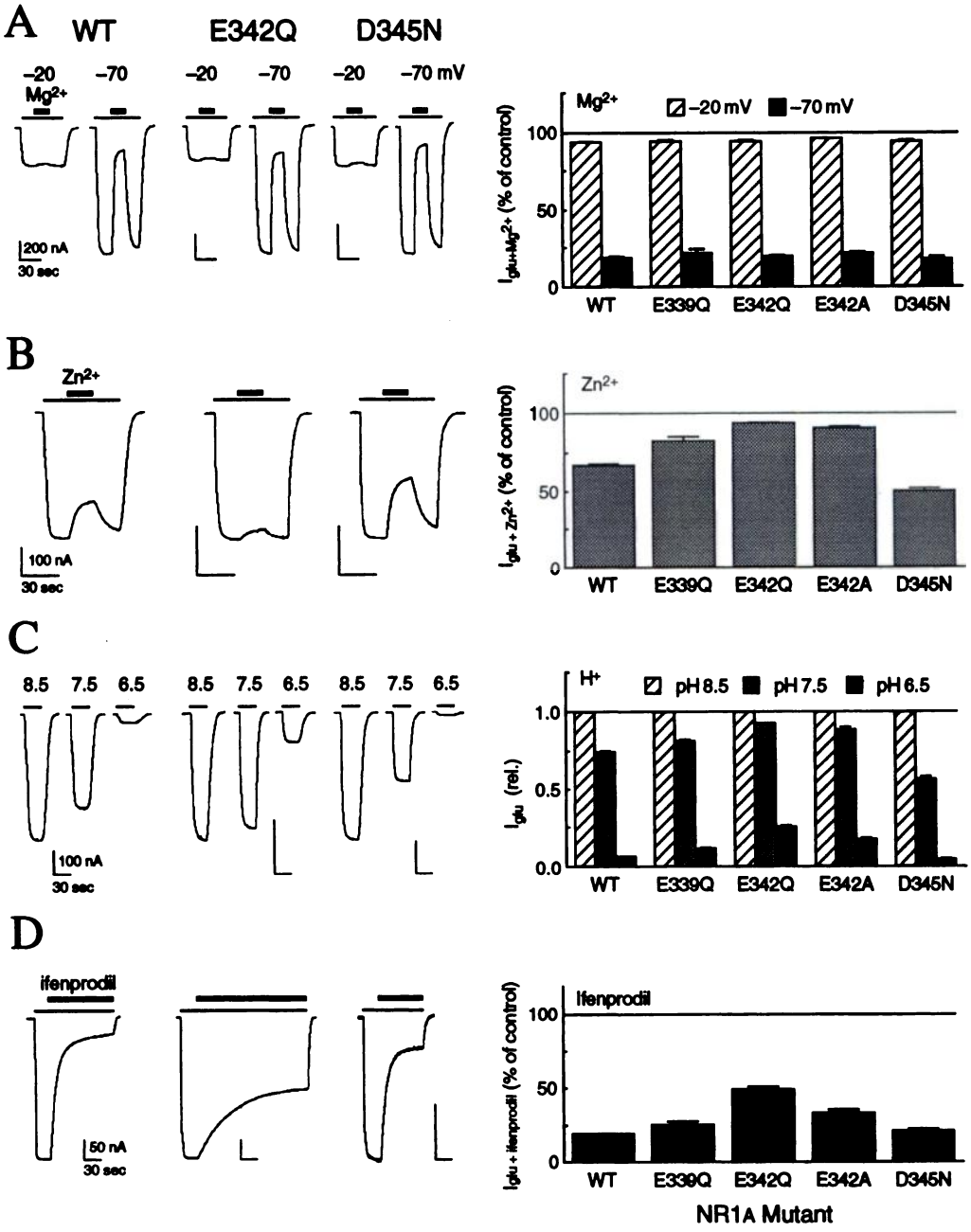

Fig. 6. Effects of modulators at NR1A/NR2B receptors. The effects of 100 μM Mg^{2+} (A), 1 μM Zn^{2+} (B), pH (C), and 1 μM ifenprodil (D) on responses to glutamate (10 μM; with 10 μM glycine) were measured at NR1A/NR2B receptors containing wild-type (*WT*) and mutant NR1A subunits. The effects of Mg^{2+} were determined using oocytes voltage-clamped at −20 mV and −100 mV. In B, C, and D, oocytes were voltage-clamped at −20 mV. Data in A, B, and D are expressed as a percentage of the control response to glutamate. Data in C are expressed as a fraction of the glutamate-induced current measured at pH 8.5 (I_{glu} at pH 8.5 = 1.0). Values are mean ± standard error from 6 (A, B, and C) or 5–11 (D) oocytes for each subunit combination. The effects of 100 μM spermine on responses to 10 μM glutamate and glycine at −20 mV were also measured at each subunit combination on the same oocytes (A, B, and C) or in oocytes from the same batch (D) (data not shown), and results with spermine were identical to those illustrated in Fig. 3.

Mutations at NR2A(E352Q) and NR2B(E353Q) had no effect on sensitivity to spermine, pH, and ifenprodil compared with the respective wild-type subunits (Fig. 8).

Discussion

A number of studies have documented homology in the amino acid sequences of glutamate receptors with bacterial periplasmic amino acid binding proteins (2, 9, 17–19). The amino acid binding sites of glutamate receptors may be formed by pockets that involve structural motifs similar to those of bacterial amino acid binding proteins (9, 17, 19). With this in mind, we set out to look for regions of NR1 that may be involved in forming binding sites for polyamines by comparing the sequence of NR1 with a number of other proteins that bind polyamines, in particular, the PotD protein from *E. coli*. A region of NR1A that shows homology with part of the PotD protein was located. The amino acids in NR1A that we have identified in this study as being important for spermine stimulation (E339 and E342) are just proximal to the region that shows homology with PotD. Three acidic amino acids, one of which is proximal to an RKL motif, have been found to form part of a polyamine binding site in PotD.[1] If E342 forms part of a spermine binding site, it is possible that the homologous regions of NR1A and PotD form similar secondary structures that are important for positioning amino acids around this region to form a polyamine binding site. It is also possible that amino acid residues in other parts of the NR1 subunit or in NR2 subunits contribute to the spermine binding site. In this regard, the glycine binding site of the NMDA receptor appears to be formed by residues in the distal portion of the large amino-terminal domain and in the loop between transmembrane segments 3 and 4 (9, 10).

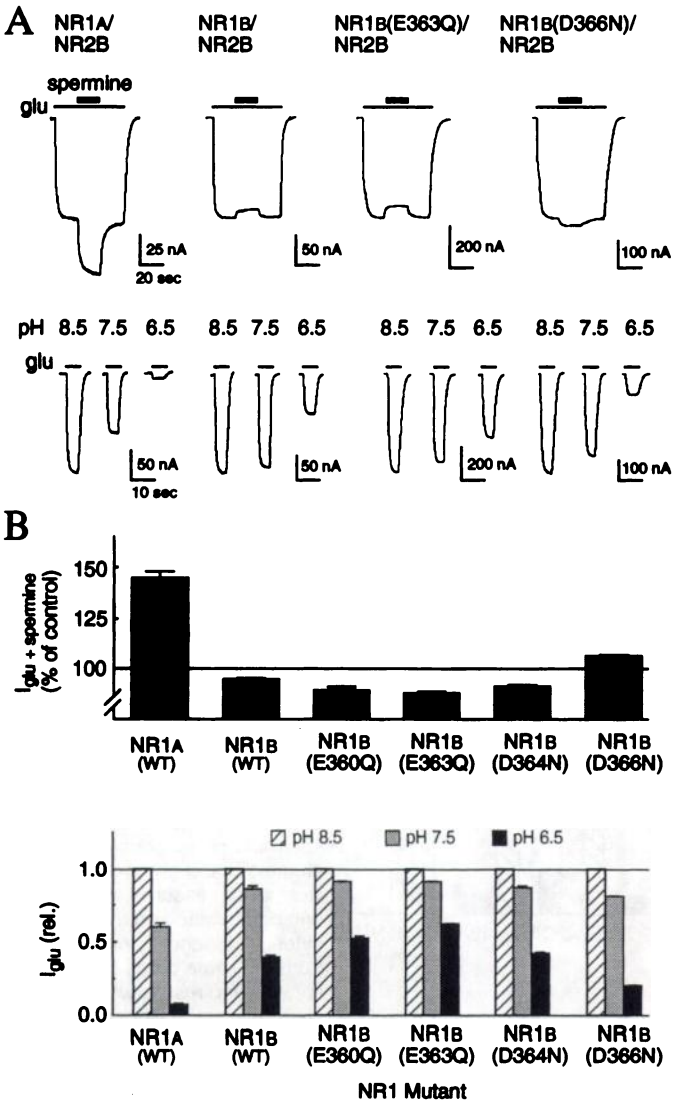

Fig. 7. Properties of receptors containing mutant NR1B subunits. The effects of spermine and pH were studied at wild-type (*WT*) NR1A/NR2B receptors and at NR1B/NR2B receptors containing wild-type and mutant NR1B subunits. The NR1B variant contains a 21-amino acid amino-terminal insert; thus, mutations E360Q, E363Q, D364N, and D366N in NR1B correspond to mutations E339Q, E342Q, D343N, and D345N, respectively, in NR1A. A, Representative traces showing effects of 100 μM spermine on responses to glutamate (10 μM; with 10 μM glycine) (*top row*) and responses to glutamate measured at pH 8.5, 7.5, and 6.5 (*bottom row*) in oocytes voltage-clamped at −20 mV. All horizontal scale bars are 20 sec for experiments with spermine and 10 sec for pH experiments. B, Data (mean ± standard error, five oocytes) for each subunit combination were measured using the protocols shown in A. Currents measured in the presence of spermine are expressed as a percentage of the control response to glutamate. For pH experiments, data are expressed as a fraction of the glutamate-induced current measured at pH 8.5 (I_{glu} at pH 8.5 = 1.0). Control responses to glutamate at pH 7.5 were 201 ± 64 nA (*WT NR1A*), 344 ± 74 nA (*WT NR1B*), 437 ± 50 nA (*E360Q*), 384 ± 63 nA (*E363Q*), 351 ± 33 nA (*D364N*), and 426 ± 81 nA (*D366N*).

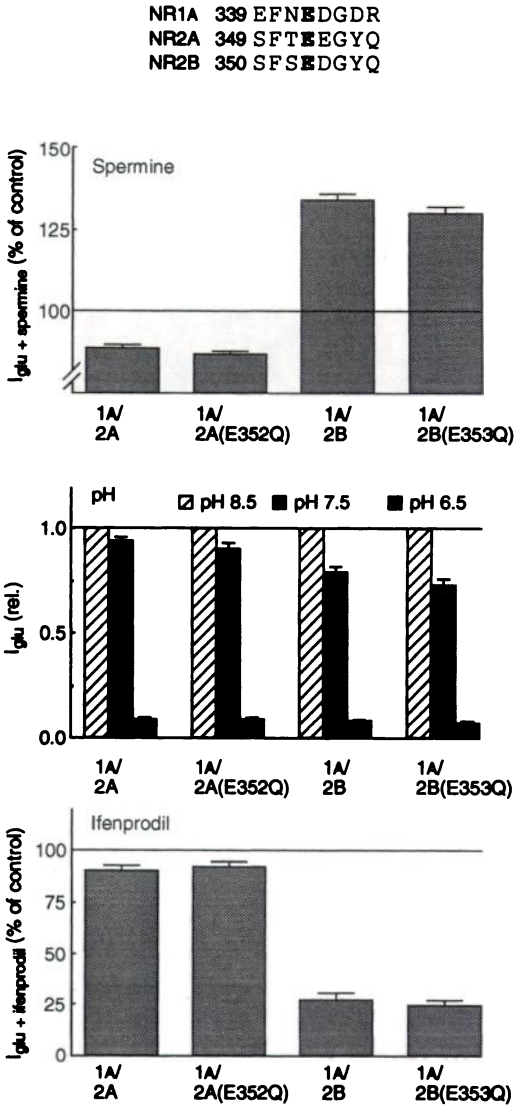

Fig. 8. Properties of receptors containing mutant NR2 subunits. The effects of spermine (100 μM), pH, and ifenprodil (1 μM) were determined at NR1A/NR2 receptors containing wild-type NR2A and NR2B and NR2A(E352Q) or NR2B(E353Q) subunits. The effects of modulators on responses to glutamate (10 μM; with 10 μM glycine) were measured in oocytes voltage-clamped at −20 mV. Data for spermine and ifenprodil are expressed as a percentage of the control response to glutamate. For pH experiments, data are expressed as a fraction of the glutamate-induced current measured at pH 8.5 (I_{glu} at pH 8.5 = 1.0). Values are mean ± standard error from 10 (spermine) or 5 (pH, ifenprodil) oocytes for each subunit combination. *Top*, amino acid sequences of NR2A and NR2B containing the mutated residues, together with the corresponding sequence of NR1A. *Bold*, residues E342 (NR1A), E352 (NR2A), and E353 (NR2B).

Spermine is a tetraamine containing two primary and two secondary amino groups. Potentiation of NMDA receptors is also seen with the triamine spermidine but not with diamines of similar chain length such as 1,7-diaminoheptane or 1,10-diaminodecane (34–37). The binding site responsible for glycine-independent spermine stimulation is therefore likely to contain a minimum of three amine interaction points (36). If E342 forms part of this polyamine binding site, the carboxyl moiety on this amino acid could act as a hydrogen bond acceptor for one of the amino groups present in spermine and spermidine. This hypothesis is supported by the observation that spermine stimulation was reduced with E342Q and E342A, where the carboxyl groups are lost, but was increased slightly with E342D, where the carboxyl group is retained but the chain length is decreased. Because spermine is a highly flexible molecule, it may still interact easily at a site where the chain length of one of the acceptor acidic groups is altered.

Results from a number of previous studies have suggested that the glycine-independent and -dependent forms of stimulation by spermine involve two separate polyamine binding sites. In ligand binding studies, polyamines increase the binding of radiolabeled channel blockers such as MK-801 when measured in the presence of saturating concentrations of glycine (presumed to be equivalent to glycine-independent stimulation) and increase the binding of [^{3}H]glycine (presumed to be equivalent to glycine-dependent stimulation). These two effects have a different concentration dependence and a different pharmacological profile when studied with polyamine analogues (38, 39). At native NMDA receptors on cultured neurons studied by patch-clamp recording, the two forms of spermine stimulation appeared to be mechanistically distinct (37). Furthermore, the two forms of stimulation have a different subunit dependency in terms of both the type of NR2 subunit and the type of NR1 splice variant present in recombinant NMDA receptors (4, 5). The results of the present study provide direct evidence that glycine-independent and glycine-dependent stimulation by spermine involve two separate polyamine binding sites. Mutations at E339 and E342 that reduced or abolished glycine-independent stimulation had no effect on glycine-dependent stimulation. Furthermore, these mutations did not alter voltage-dependent block by spermine, consistent with the proposal that voltage-dependent block involves a separate, presumably third, polyamine binding site that may be located in or near the ion channel pore of the NMDA receptor (5, 37, 40).

In addition to influencing sensitivity to spermine, mutations at E342 and D345 in NR1A altered sensitivity to ifenprodil and pH. The magnitude of glycine-independent spermine stimulation has been shown to be dependent on extracellular pH, and it has been proposed that spermine functions to relieve tonic proton inhibition at NR1A/NR2B receptors (16). Thus, it is possible that the D345N mutation alters spermine sensitivity secondary to changes in pH sensitivity because this mutation has a pronounced effect on pH sensitivity. In contrast, mutations at E342 have only modest effects on inhibition by protons, suggesting that changes in pH sensitivity at E342 mutants cannot account entirely for changes in sensitivity to spermine.

The neuroprotective agent ifenprodil has been suggested to act as an antagonist at a stimulatory polyamine site on NMDA receptors (41), although this interaction does not appear to be strictly competitive (42). However, ifenprodil and polyamines have a similar subunit-dependent profile at NMDA receptors; only receptors containing the NR2B subunit have a high affinity for ifenprodil and are sensitive to glycine-independent spermine stimulation (7, 26). Polyamines and ifenprodil may share overlapping binding sites or their effects may be influenced by similar structural features in NMDA receptors. Mutations at E342 that reduce stimulation by spermine were also found to reduce inhibition by ifenprodil at NR1A/NR2B receptors. Thus, E342 may contribute to or be located in a region near to a binding site for ifenprodil. The cluster of acidic residues around E342 (i.e., E339, E342, D343, and D345) may form part of a spermine binding site (E342) and/or a "hot spot" for binding, modulation, or charge recognition by organic and inorganic cations, including Zn^{2+} and protons. Alternatively, this region may be a common downstream effector site for a variety of modulators that alter channel gating by related mechanisms.

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