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Activation of *N*-Methyl-D-Aspartate Receptors by Glycine: Role of an Aspartate Residue in the M3-M4 Loop of the NR1 Subunit

KEITH WILLIAMS, JAMES CHAO, KEIKO KASHIWAGI, TAKASHI MASUKO, and KAZUEI IGARASHI

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084 (K.W., J.C.), and Faculty of Pharmaceutical Sciences, Chiba University, Inage-Ku, Chiba 263, Japan (K.K., T.M., K.I.)

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SUMMARY

Glutamate and glycine are coagonists that act at distinct sites to activate *N*-methyl-D-aspartate (NMDA) receptors. In the NR1 subunit of the NMDA receptor, mutation of D732 to glutamate (D732E), asparagine (D732N), alanine (D732A), or glycine (D732G) reduced the potency of glycine by >4000-fold, but these mutations had no effect on sensitivity to glutamate. Mutations at NR1(D732) also changed sensitivity to the glycine-site agonists D-serine and D-alanine, reducing the potencies and, in some cases, the efficacies of these compounds. Thus, D-serine was a full agonist at the glycine site of receptors containing NR1(D732N) and NR1(D732A), a partial agonist at receptors containing NR1(D732G), and a competitive antagonist at receptors containing NR1(D732E). Mutations at NR1(D732) had no effect or produced an increase in sensitivity to the glycine-site antagonists 6,7-dichloroquinoxaline-2,3-dione and 5,7-dichlorokynurenic acid. These mutations did not affect the reversal potential, voltage-dependent block by extracellular Mg^{2+}, block by ifenprodil, or stimulation by spermine at NR1/NR2B receptors. NR2 subunits containing mutations at NR2A(D731) and NR2B(D732), which correspond to NR1(D732), did not produce functional receptors when coexpressed with NR1. Residue D732 in NR1 may be close to a glycine binding site on the NMDA receptor and may directly affect the properties of this site or be critical for coupling of glycine binding to channel activation.

NMDA receptors are involved in the induction of various forms of synaptic plasticity in the central nervous system and in neurotoxicity that occurs after ischemia (1, 2). Glycine is an obligatory coagonist for activation of NMDA receptors and binds to a site that is distinct from the glutamate recognition site (3, 4). In addition to the obvious physiological importance of the glycine binding site on the NMDA receptor, this site represents a potential therapeutic target for neuroprotective agents (5).

The cloning of cDNAs encoding subunits of NMDA receptors (6–8) allows analyses of these receptors at a molecular level, including studies of the site and the mechanism of action of glycine. Two families of NMDA receptor subunits have been cloned: NR1, which is a single gene product expressed as eight alternatively spliced mRNAs, and NR2A–NR2D, which are distinct gene products (6–8). The NR1 subunit can form functional homomeric receptors that produce small responses (6), whereas heteromeric NR1/NR2 receptors gate much larger macroscopic currents and have distinct functional and pharmacological properties, including differences in sensitivity to glycine (9–12). Because homomeric NR1 receptors are sensitive to glycine (6, 13), a glycine binding site is presumably located on the NR1 subunit or can be formed by the interaction of two or more NR1 subunits in a homomeric complex. In two studies, amino acids in the NR1 subunit were identified that influence sensitivity to glycine (14, 15). These include aromatic (F408, Y410, and F484) and charged (D481 and K483) residues in the distal part of the amino-terminal domain and two residues (V684 and S687) in the loop between M3 and M4 of NR1 (14, 15) (Fig. 1A). Both

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; 5,7-DCK, 5,7-dichlorokynurenic acid; DCQX, 6,7-dichloroquinoxaline-2,3-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I-V, current-voltage.

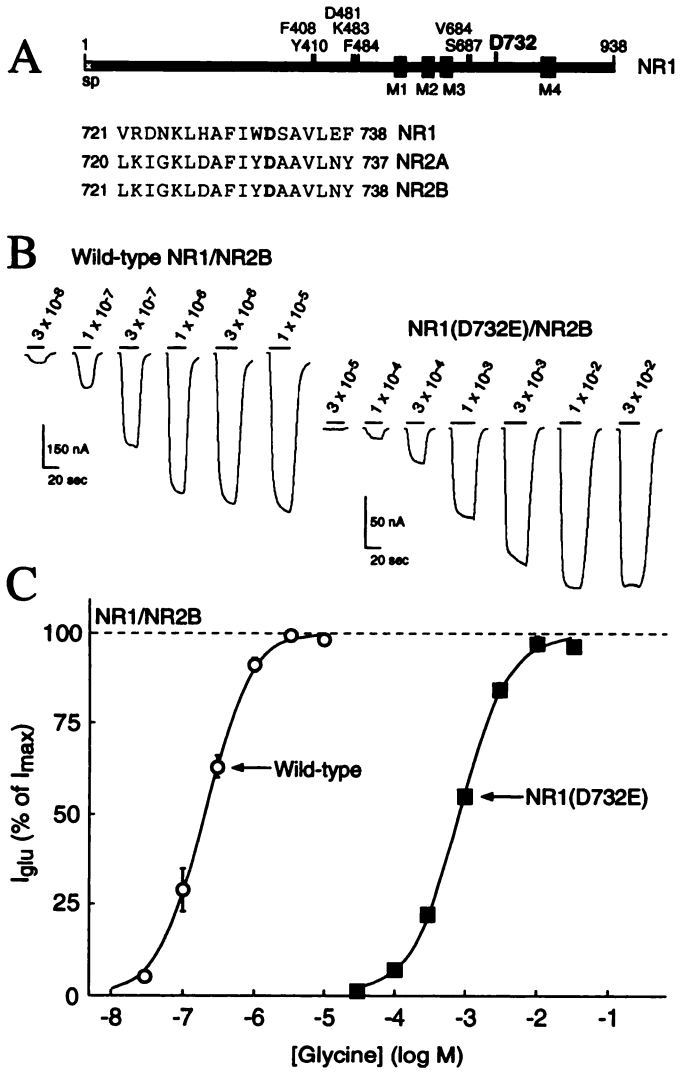

Fig. 1. Effects of NR1(D732) mutations on glycine sensitivity. A, Schematic of the NR1 subunit cDNA showing the position of D732 and of residues in the amino-terminal domain and M3-M4 loop that influence sensitivity to glycine. Amino acids are numbered from the initiator methionine. *sp*, signal peptide. The sequences of NR1, NR2A, and NR2B around D732 are shown below the schematic. B, Responses induced by various concentrations of glycine (3×10^{-8} to 3×10^{-2} M) with glutamate (10 μM for wild-type; 300 μM for D732E) at NR1/NR2B and NR1(D732E)/NR2B receptors in oocytes voltage-clamped at −70 mV. C, Concentration-response curves for glycine at NR1/NR2B and NR1(D732E)/NR2B receptors. *glu*, glutamate. Values are mean ± standard error from six to nine oocytes for each subunit combination. Where error bars are not shown, errors are within the size of the symbol.

of these domains are presumed to be extracellular, and a glycine binding site may be formed in a pocket involving residues from the amino-terminal domain and from the M3-M4 loop (14). Mutations at an aspartate residue, D481, in the amino-terminal domain reduce the potency of glycine and of glycine-site antagonists such as L-689,560 (14, 15). Thus, D481 may interact directly with glycine via hydrogen bonding with the amino group of glycine and the corresponding amino groups in glycine-site antagonists. The role of the residues (V684 and S687) in the M3-M4 loop is less clear because mutations at V684 and S687 (numbered V666 and S669 in Ref. 14) reduce the potency of glycine but not of glycine-site antagonists (14).

In the course of studies in which site-directed mutagenesis was used to locate amino acids that may contribute to binding sites for polyamines (16, 17), we found that an aspartate-to-asparagine mutation at NR1(D732N) almost abolished the response to 10 μM glutamate and glycine at NR1/NR2B receptors, but the mechanism underlying this effect was not determined (17). We now report that mutations at NR1(D732) reduce the potency of glycine by 4,000–40,000-fold without altering sensitivity to glutamate, block by extracellular Mg^{2+}, or a number of other properties of NMDA receptors.

Experimental Procedures

Site-directed mutagenesis. NR1 mutants were prepared as described previously (16, 17), using a 2.6-kb *SphI/SalI* fragment of plasmid pN60 (6) inserted into the same sites of M13mp18 (18). NR2A and NR2B mutants were prepared using a 2.2-kb *BamHI/XmaI* fragment of pBSNR2A and a 2.4-kb *SphI/EcoRI* fragment of pBSNR2B (9) inserted into the same sites of M13mp18. Mutagenesis was carried out according to the method of Kunkel *et al.* (19) or the method of Sayers *et al.* (20) with the Sculptor *in vitro* mutagenesis system (Amersham International, Buckinghamshire, UK). The oligonucleotides for preparation of the NR1 mutants (untranslated strands) were 5′-GCA CGG CCG ACT CCC AGA TAA for NR1(D732E), 5′-ACG GCC GAG TTC CAG ATA AAG for NR1(D732N), 5′-CAC GGC CGA GGC CCA GAT AAA for NR1(D732A), 5′-CAC GGC CGA GCC CCA GAT AAA for NR1(D732G), 5′-AGC TTG TTG TTC CGC ACA GCC for NR1(D723N), and 5′-GCC TCA AAC TGC AGC ACG GCC for NR1(E737Q). The oligonucleotides for preparation of NR2A and NR2B mutants (translated strands) were 5′-TTC ATC TAT GCC GCA GCC GTC for NR2A(D731A), 5′-TTT CAT CTA TGA GGC AGC CGT CT for NR2A(D731E), 5′-TCA TCT ATG AGG CAG CTG TGC for NR2B(D732E), and 5′-TTC ATC TAT GCT GCA GCT GTG for NR2B(D732A). Mutated DNA fragments were isolated from the replicative form of M13 and re-ligated into the corresponding sites of pN60, pBSNR2A, or pBSNR2B. Mutations were confirmed by DNA sequencing (21) using the M13 phage system. For the NR1 mutants, nucleotides 1855–2256 (corresponding to amino acids 619–752) were sequenced. For NR2 mutants, nucleotides 2170–2502 (NR2A; amino acids 724–834) and 2029–2493 (NR2B; amino acids 677–831) were sequenced. The sequences were as expected for all mutations. In the mutant and wild-type NR2B clones, nucleotide 2305 was found to be thymidine (T) rather than guanosine (G) as reported in the published sequence (9). Thus, amino acid 769 in NR2B is tyrosine (Y), encoded by TAC, rather than aspartate (D), encoded by GAC.

Mutations are referred to after the subunit by the original amino acid, its number, and the mutated amino acid. Thus, NR1(D732E) contains a glutamate (E) at position 732 of NR1 in place of the aspartate (D) found in the wild-type clone. Amino acids are numbered from the initiator methionine in NR1 and NR2 subunits (6, 12). This numbering system is identical to that used by Moriyoshi *et al.* (6) and Wafford *et al.* (15) but differs from the system used by Kuryatov *et al.* (14), in which amino acids are numbered starting from the first arginine in the mature peptide (after the 18-amino acid signal peptide). Thus, residues F408, Y410, D481, K483, F484, V684, and S687 shown in Fig. 1A correspond to residues F390, Y392, D463, K465, F466, V666, and S669, respectively, in Kuryatov *et al.* (14). Residue D732 corresponds to residue D714 in the numbering system used by Kuryatov *et al.* (14). The authors reported effects of mutations at "W703A" and "D704A," although the amino acids at positions 703 and 704 (corresponding to positions 721 and 722 using the numbering system shown in Fig. 1A) are valine (V) and arginine (R), respectively. The mutations at W703A and D704A (14) presumably correspond to positions W731 and D732 according to the system shown in Fig. 1. The D-to-A mutation at D704 in NR1 (presumed to

be equivalent to D732) was reported not to alter sensitivity to glycine (14). We have no explanation for the apparent discrepancy between the D704A mutation reported by Kuryatov *et al.* (14) and the results with D732 mutations in the current report.

Expression in oocytes and voltage-clamp recording. The preparation of cRNAs and the preparation, injection, and maintenance of oocytes were carried out as described previously (22–24). Oocytes were injected with NR1 plus NR2 cRNAs in a ratio of 1:5 (1–2 ng of NR1 plus 5–10 ng of NR2). Macroscopic currents were recorded with a two-electrode voltage-clamp as described previously (22, 24). 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) that contained $BaCl_2$ rather than $CaCl_2$ to minimize Ca^{2+}-activated Cl^- currents (24, 25). In most experiments, oocytes were injected with K^+-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (100 nl; 40 mM, pH 7.4) on the day of recording (24).

To obtain the EC_{50} values for agonists, data were fit to the following logistic function: $I = I_{max}/1 + ([agonist]/EC_{50})^{nH}$, where I is the glutamate-induced current, I_{max} is the maximum response, and n_H is the Hill slope. To obtain the IC_{50} values for antagonists, data were fit to the following equation: $I = 100/1 + ([antagonist]/IC_{50})^{nH}$, where I is the response to glutamate measured in the presence of antagonist and is expressed as a percentage of the control response to glutamate (control = 100%). K_i values for antagonists $\{K_i = IC_{50}/1 + ([glycine]/EC_{50})\}$ were determined from the IC_{50} value for each antagonist using the EC_{50} values for glycine shown in Table 1 for each receptor type (26). All compounds except DCQX were dissolved in saline solution. In experiments with DCQX, a stock solution of 10 mM DCQX was prepared in dimethylsulfoxide, and all test solutions (with or without DCQX) contained dimethylsulfoxide at a final concentration of 1% to circumvent any effects of dimethylsulfoxide on NMDA responses. I-V curves were constructed by using linear voltage ramps (−100 to +40 mV; 12 sec). Leakage currents, measured by ramps before and after test ramps, were digitally subtracted. Reversal potentials were calculated by linear regression of the data from −10 mV to +10 mV.

Materials. The wild-type NR1 clone (pN60) used in this study was the NR1A variant (6, 27) (a gift from Dr. S. Nakanishi, Kyoto University, Kyoto, Japan). The NR2A and NR2B clones (9) were gifts from Dr. P. H. Seeburg (University of Heidelberg, Heidelberg, Germany), and the mouse NR2D (ε4) clone (28) was a gift from Dr. M. Mishina (University of Tokyo, Tokyo, Japan). Glutamate, glycine, D-serine, and D-alanine were purchased from Sigma Chemical (St. Louis, MO). Spermine tetrahydrochloride was from Aldrich Chemical (Milwaukee, WI). DCQX was purchased from Research Biochemicals (Natick, MA). 5,7-DCK was a gift from Marion Merrell Dow Research Institute, Marion Merrell Dow (Cincinnati, OH). Ifenprodil was a gift from Synthélabo Recherche (Bagneux, France).

Results

Sensitivity to agonists. Currents induced by 10 μM glutamate plus 10 μM glycine at NR1(D732N)/NR2B receptors were very small compared with the responses at wild-type receptors (17). We reasoned that this may be due to a change in the sensitivity of the mutant receptors to glutamate or glycine. In preliminary experiments, we found that larger currents were induced at NR1(D732N)/NR2B receptors with concentrations of 100 μM to 30 mM glycine (together with 10 μM glutamate). However, the concentration-response curve for glycine was n-shaped, and the response to 10 μM glutamate was depressed at concentrations of 10–30 mM glycine (data not shown). A similar effect was seen at wild-type NR1/NR2B receptors: the response to 10 μM glutamate was decreased at glycine concentrations of >10 mM (compared with responses to 10 μM or 3 mM glycine). The inhibitory effect of high concentrations of glycine seems to be due to an antagonist effect of glycine at the glutamate binding site because the inhibition can be overcome by increasing the concentration of glutamate (data not shown). Therefore, concentration-response curves for glycine at receptors containing the NR1(D732) mutants were carried out in the presence of 300 μM or 1 mM glutamate, whereas experiments with wild-type receptors were done with 10 μM glutamate. Concentration-response curves for glutamate were carried out with 3 mM glycine for both mutant and wild-type subunits. Using these paradigms, the EC_{50} value for glutamate at wild-type NR1/NR2B receptors (4.0 μM) studied with 3 mM glycine was similar to the EC_{50} value previously seen for glutamate (2.1 μM) at NR1/NR2B receptors studied with 10 μM glycine (22).

We tested four mutations (D-to-E, D-to-N, D-to-A, and D-to-G) at NR1(D732). These mutations reduced the potency of glycine by 4,000–40,000-fold when the mutant NR1 subunits were coexpressed with NR2B (Fig. 1 and Table 1). The sizes of the macroscopic currents produced by the wild-type and mutant receptors were similar (Fig. 1B and Table 1). To determine whether the effects of mutations at NR1(D732) were dependent on the type of NR2 subunit coexpressed with

TABLE 1

Properties of NR1 mutants

The potency of glycine was measured at various NR1/NR2 receptors in oocytes voltage-clamped at −70 mV. I is the response to saturating concentrations of glutamate and glycine or the response to glutamate with the highest concentration of glycine that was tested (30 mM).

Subunit combination	Glycine		I	No. of oocytes
	EC_{50}	n_H		
	μM		nA	
Wild-type NR1/NR2B	0.20 ± 0.02	1.5 ± 0.1	330 ± 130	6
NR1(D732E)/NR2B	849 ± 57	1.3 ± 0.1	561 ± 202	9
NR1(D732N)/NR2B	2892 ± 135	0.9 ± 0.1	521 ± 142	9
NR1(D732A)/NR2B	7284 ± 523	1.1 ± 0.1	192 ± 80	5
NR1(D732G)/NR2B	1736 ± 236	1.1 ± 0.1	160 ± 35	5
NR1(D723N)/NR2B	0.19 ± 0.02	1.2 ± 0.1	554 ± 155	5
NR1(E737Q)/NR2B	0.28 ± 0.11	1.0 ± 0.1	430 ± 186	5
Wild-type NR1/NR2A	1.0 ± 0.1	1.7 ± 0.1	378 ± 66	7
NR1(D732E)/NR2A	1796 ± 114	1.4 ± 0.1	201 ± 33	5
Wild-type NR1/NR2D	0.10 ± 0.01	1.8 ± 0.1	128 ± 15	7
NR1(D732E)/NR2D	620 ± 10	1.5 ± 0.03	266 ± 50	6

NR1, we measured glycine sensitivity at NR1/NR2A and NR1/NR2D receptors containing wild-type and NR1(D732E) subunits. Similar to the effects seen at receptors containing NR2B, the potency of glycine was greatly reduced at NR1(D732E)/NR2A and NR1(D732E)/NR2D compared with the wild-type receptors (Table 1). Mutations at two other acidic residues, D723 and E737, which are close to D732 (Fig. 1A), had no effect on sensitivity to glycine (Table 1).

To determine whether NR1(D732) mutations alter permeability or voltage dependence of NMDA receptors, we measured I-V relationships and the reversal potential at wild-type and NR1(D732E)/NR2B receptors (Fig. 2). The NR1(D732E) mutation had no effect on the shape of the I-V curve (Fig. 2) or the reversal potential, which was $+1.1 \pm 0.8$ mV at wild-type NR1/NR2B receptors (five oocytes) and -2.3 ± 0.9 mV at NR1(D732E)/NR2B receptors (five oocytes; $p > 0.2$, Student's t test).

The sensitivity of NR1 mutants to two other glycine-site agonists, D-serine and D-alanine, was determined. Similar to the effects seen with glycine, the potencies of D-serine and D-alanine were reduced by ≥10,000-fold at receptors containing NR1(D732N), NR1(D732A), and NR1(D732G) (Fig. 3A and Table 2). Furthermore, the D732E and D732G mutations reduced the efficacies of D-serine and D-alanine. Efficacies at NR1(D732G) were reduced by ~50% (Fig. 3A and Table 2). Receptors containing NR1(D732E) were insensitive to stimulation by D-serine and D-alanine, having only very small responses to 30 mM D-serine and D-alanine compared with 30 mM glycine in the same oocytes. However, D-serine and D-alanine were able to inhibit the response to glycine at NR1(D732E)/NR2B receptors (Fig. 3B). The mean K_i values (−standard error, +standard error) were 0.88 mM (0.86, 0.91 mM) for D-serine (seven oocytes) and 1.88 mM (1.66, 2.13 mM) for D-alanine (seven oocytes). To determine whether D-serine acts as a competitive antagonist at the glycine binding site of NR1(D732E)/NR2B receptors, glycine concentration-response curves were measured in the absence and presence of 3 mM D-serine (Fig. 4). Inhibition of glycine responses by D-serine was surmountable, and D-serine caused a parallel shift in the glycine curve (Fig. 4). Thus, D-serine is a competitive antagonist at the glycine site of receptors containing NR1(D732E).

Sensitivity to glycine-site antagonists. To determine whether mutations at NR1(D732) alter sensitivity to glycine-site antagonists, we measured concentration-inhibition curves for the antagonists 5,7-DCK and DCQX (Fig. 5 and Table 3). The NR1(D732E) mutation had no effect or produced an increase in sensitivity to 5,7-DCK and DCQX (Fig. 5 and Table 3). NR1(D732G) produced a small (3-fold) decrease in sensitivity to 5,7-DCK, whereas NR1(D732N) and NR1(D732A) increased the sensitivity to 5,7-DCK and DCQX by ~10-fold (Table 3). Thus, the effects of NR1(D732) mutations on sensitivity to glycine-site antagonists are markedly different from their effects on sensitivity to agonists; although the mutations reduce agonist sensitivity by >4000-fold, they have no effect or produce an increase in sensitivity to antagonists.

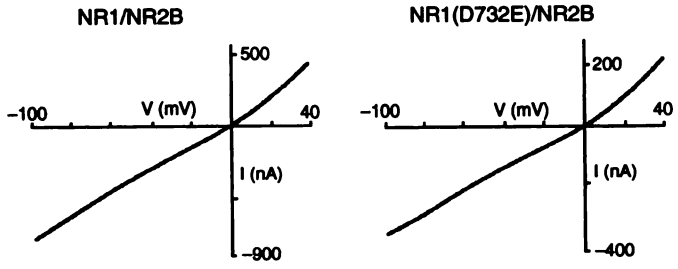

Fig. 2. I-V relationships at NR1/NR2B and NR1(D732E)/NR2B receptors. I-V curves were measured by voltage ramps (−100 mV to +40 mV; 12 sec) at receptors activated by 10 μM glutamate plus 10 μM glycine (wild-type) or 300 μM glutamate plus 30 mM glycine (D732E). Leak currents have been subtracted.

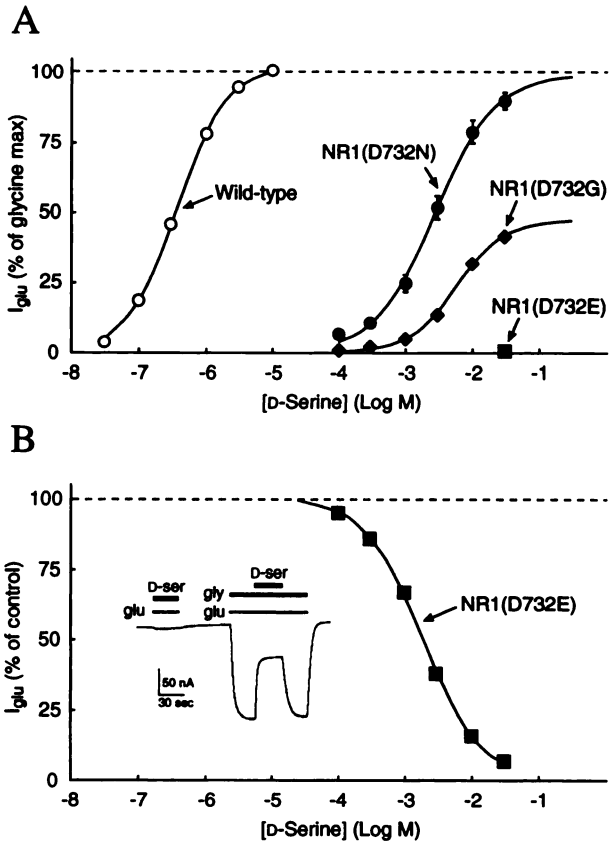

Fig. 3. Effects of D-serine at NR1/NR2B receptors. A, Concentration-response curves for D-serine at wild-type, NR1(D732N)/NR2B, and NR1(D732G)/NR2B receptors were measured in the presence of 10 μM glutamate (*glu*) (wild-type) or 300 μM glutamate (D732 mutants) in oocytes voltage-clamped at −70 mV. Data are expressed as a percentage of the maximum response to glycine at each receptor type (*broken line*) measured in the same oocytes. D-Serine (30 mM) (with 300 μM glutamate) does not activate receptors containing NR1(D732E). B, Concentration-inhibition curves for D-serine were measured in the presence of 300 μM glutamate and 1 mM glycine in oocytes expressing NR1(D732E)/NR2B receptors. Data are expressed as a percentage of the control response to glutamate plus glycine (*broken line*). *Inset*, effects of D-serine (*D-ser*; 3 mM) on responses to glutamate (*glu*; 300 μM) and glycine (*gly*; 1 mM) at NR1(D732E)/NR2B. Data in A and B are mean ± standard error from six or seven oocytes for each subunit combination. Where error bars are not shown, the errors are within the size of the symbol.

Sensitivity to glutamate. Mutations at NR1(D732) had no effect on sensitivity to glutamate at NR1/NR2B receptors (Fig. 6A). The EC_{50} values for glutamate were 4.0 ± 0.7 μM (wild-type; five oocytes), 6.4 ± 0.4 μM (D732E; five oocytes), 3.9 ± 0.3 μM (D732N; five oocytes), 2.3 ± 0.1 μM (D732A; five oocytes), and 7.8 ± 1.4 μM (D732G; five oocytes). Thus, mutations at D732 affect activation of NMDA receptors by glycine but not activation by glutamate (Fig. 1 and Fig. 6A).

TABLE 2

Sensitivity to D-serine and D-alanine

EC_{50} values for D-serine and D-alanine were determined from concentration-response curves measured in the presence of 10 μM glutamate (wild-type) or 300 μM glutamate (NR1 mutants). Oocytes were voltage-clamped at −70 mV.

Subunit combination	D-Serine		No. of oocytes	D-Alanine		No. of oocytes
	EC_{50}	Efficacy[a]		EC_{50}	Efficacy[a]	
	μM			μM		
Wild-type NR1/NR2B	0.36 ± 0.01	1.03 ± 0.01	6	0.69 ± 0.03	0.94 ± 0.02	6
NR1(D732E)/NR2B	[b]	0.01 ± 0.003	8	[b]	0.02 ± 0.01	8
NR1(D732N)/NR2B	3,038 ± 400	1.01 ± 0.02	6	>10,000	n.d.	6
NR1(D732A)/NR2B	6,324 ± 260	1.36 ± 0.05	5	>10,000	n.d.	6
NR1(D732G)/NR2B	5,897 ± 269	0.47 ± 0.01	6	7,512 ± 424	0.46 ± 0.01	5

[a] The efficacies of D-serine and D-alanine are expressed relative to the maximum response to glycine measured in the same oocytes. For wild-type receptors, the maximum response was determined with 10 μM glycine; and for the D732 mutants, the response was determined, with 30 mM glycine. Because 30 mM glycine is subsaturating at NR1(D732N) and NR1(D732A) receptors, responses to 30 mM glycine were corrected (based on glycine concentration-response curves) to obtain a theoretical maximum response at these receptors.

[b] D-Serine and D-alanine are almost inactive at NR1(D732E)/NR2B receptors. Responses to 30 mM D-serine at NR1(D732E)/NR2B receptors were 5 ± 1 nA, whereas responses to 30 mM glycine in the same oocytes were 317 ± 69 nA; responses to 30 mM D-alanine were 15 ± 4 nA, whereas responses to 30 mM glycine in the same oocytes were 505 ± 127 nA (all with 300 μM glutamate; mean ± standard error for eight oocytes).

n.d. = not determined.

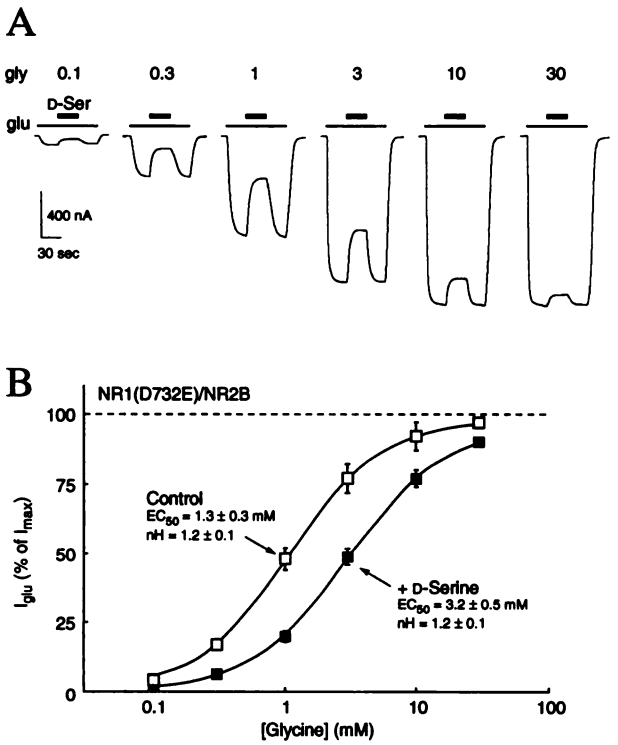

Fig. 4. D-Serine is a competitive antagonist at the glycine site of NR1(D732E)/NR2B receptors. A, Effects of D-serine (3 mM) on responses to glycine (*gly*) (0.1–30 mM; all with 1 mM glutamate; *glu*) were measured in an oocyte expressing NR1(D732E)/NR2B receptors and voltage-clamped at −70 mV. B, Concentration-response curves for glycine (all with 1 mM glutamate) were measured in the absence and presence of D-serine in oocytes voltage-clamped at −70 mV according to the protocols for A. Data, expressed as a percentage of the maximum response to glycine in each oocyte, are mean ± standard error from six oocytes. Where error bars are not shown, the errors are within the size of the symbol.

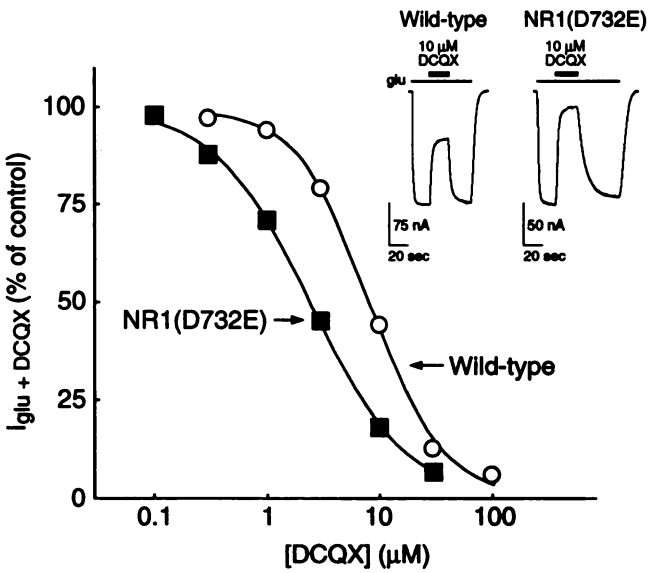

Fig. 5. Effects of DCQX at NR1/NR2B receptors. Concentration-inhibition curves for DCQX were measured at wild-type NR1/NR2B and NR1(D732E)/NR2B receptors in the presence of 10 μM glutamate (*glu*) and 3 μM glycine (wild-type) or 300 μM glutamate and 10 mM glycine (D732E) in oocytes voltage-clamped at −70 mV. Data (mean ± standard error from six oocytes) are expressed as a percentage of the control response to glutamate plus glycine. Where error bars are not shown, the errors are within the size of the symbol.

Sensitivity to other modulators. To further define the specificity of mutations at NR1(D732), we studied the effects of a number of other modulators and antagonists, including protons, Mg^{2+}, spermine, and ifenprodil, at NR1/NR2B receptors. Other residues in the M3-M4 loop (D669, C744, and C798) have previously been shown to influence sensitivity to pH, spermine, and ifenprodil (17, 29). The pH sensitivity of NR1/NR2B receptors containing NR1(D732E) or NR1(D732G) mutants was not altered, whereas receptors containing NR1(D732N) or NR1(D732A) showed an increased pH sensitivity with a greater proton inhibition at alkaline pH compared with wild-type receptors. IC_{50} values for pH were 7.4 ± 0.04 (wild-type; three oocytes), 7.3 ± 0.03 (D732E; four oocytes), 7.8 ± 0.04 (D732N; four oocytes), 8.3 ± 0.17 (D732A; four oocytes), and 7.3 ± 0.03 (D732G; four oocytes). Voltage-dependent block by extracellular Mg^{2+} was assessed by measuring the effects of 100 μM Mg^{2+} in oocytes voltage-clamped at −20 and −70 mV. The mutations had no effect on sensitivity to Mg^{2+} (Fig. 6B). Inhibition by the atypical antagonist ifenprodil was not altered by NR1(D732) mutants (Fig. 6C). Spermine has multiple effects on NR1/NR2B receptors, including "glycine-independent" stimula-

TABLE 3

Sensitivity of NR1/NR2B receptors to glycine-site antagonists

The concentrations of glycine used were 3–10 μM (with 10 μM glutamate; wild-type) and 10–30 mM (with 300 μM glutamate; D732 mutants). K_i values were calculated as the geometric mean of the pK_i in each experiment and are presented as the mean (−standard error, +standard error).

Subunit combination	5,7-DCK		DCQX	
	K_i	No. of oocytes	K_i	No. of oocytes
	nM		nM	
Wild-type NR1/NR2B	95 (92, 98)	5	505 (480, 532)	6
NR1(D732E)/NR2B	122 (95, 156)	5	191 (175, 210)	6
NR1(D732N)/NR2B	10 (9, 12)	4	36 (34, 38)	6
NR1(D732A)/NR2B	10 (9, 11)	5	59 (52, 66)	5
NR1(D732G)/NR2B	327 (292, 367)	5	688 (654, 724)	5

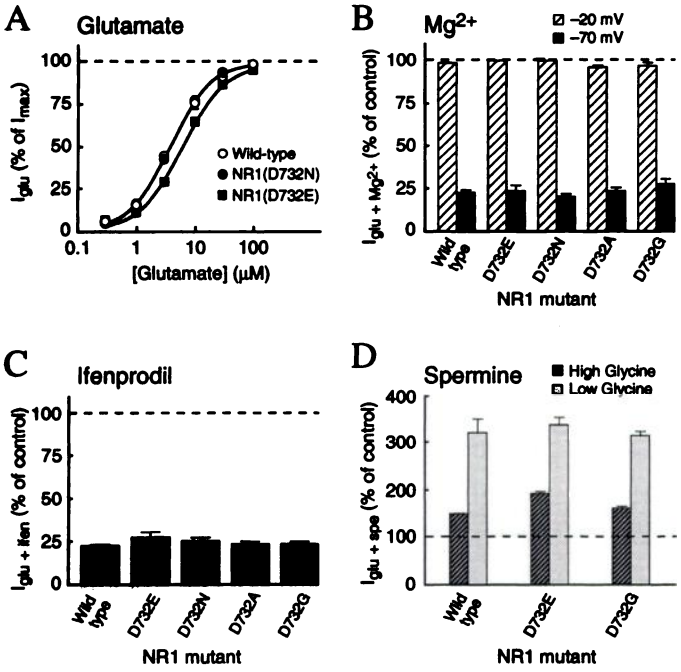

Fig. 6. Modulation and properties of NR1/NR2B receptors. The effects of glutamate (*glu*), Mg^{2+}, ifenprodil (*ifen*), and spermine (*spe*) were determined at NR1/NR2B receptors containing wild-type and mutant NR1 subunits. A, Concentration-response curves for glutamate were measured in the presence of 3 mM glycine for all subunits. B, The effects of 100 μM Mg^{2+} on responses to 10 μM glutamate plus 10 μM glycine (wild-type) or 300 μM glutamate plus 10 mM glycine (D732 mutants) were measured in oocytes voltage-clamped at −20 and −70 mV. Data are expressed as a percentage of the control response at each holding potential. C, The effects of 1 μM ifenprodil on responses to 10 μM glutamate plus 10 μM glycine (wild-type) or 300 μM glutamate plus 30 mM glycine (D732 mutants) were measured in oocytes voltage-clamped at −20 mV. Data are expressed as a percentage of the control response in each oocyte. D, The effects of 100 μM spermine were determined in the presence of 10 μM glutamate (wild-type) or 300 μM glutamate (D732 mutants) in the presence of high (10 μM, wild-type; 30 mM, D732 mutants) and low (0.03 μM wild-type; 0.3 mM, D732 mutants) concentrations of glycine in oocytes voltage-clamped at −20 mV. Data are expressed as a percentage of the control response at each concentration of glycine.

tion, which is seen with saturating concentrations of glycine, and "glycine-dependent" stimulation, which involves an increase in the affinity for glycine and is seen with subsaturating concentrations of glycine (30). Because the degree of spermine stimulation is dependent on extracellular pH (31), we studied the effects of spermine only at NR1 mutants (D732E and D732G) that do not show changes in pH sensitivity. The effects of spermine were measured on responses to saturating or near-saturating concentrations of glycine (10 μM for wild-type; 30 mM for D732 mutants) and low concentrations of glycine (0.03 μM for wild-type; 0.3 mM for D732 mutants). Both the glycine-independent and -dependent components of spermine stimulation were seen in receptors containing NR1(D732) mutants (Fig. 6D). Thus, mutations at D732 do not alter sensitivity to spermine.

Mutations in NR2A and NR2B. The NR2 subunits also contain aspartate residues at positions equivalent to NR1(D732) (shown for NR2A and NR2B in Fig. 1A). To determine whether the corresponding residues in NR2A and NR2B influence sensitivity to glycine, we tested D-to-E and D-to-A mutations at NR2A(D731) and at NR2B(D732). Oocytes injected with wild-type NR1 together with NR2A(D731A) (two independent clones), NR2A(D731E), NR2B(D732A), or NR2B(D732E) and voltage-clamped at −70 mV showed no response or very small responses (2–10 nA) to 10 mM glutamate plus 10–30 mM glycine (8 to 33 oocytes for each mutant). Oocytes expressing homomeric NR1 receptors also gave small responses (2–10 nA) to 10 mM glutamate and glycine. Responses at wild-type NR1/NR2 receptors in the same batches of oocytes were 200-2000 nA. Thus, mutations at NR2A(D731) and NR2B(D732) may render NMDA receptors nonfunctional or reduce the assembly or expression of NR1/NR2 receptors.

Discussion

We characterized the effects of mutations at an aspartate residue in the extracellular loop of NR1. Any of four mutations (D-to-E, D-to-N, D-to-A, or D-to-G) at D732 decreased the sensitivity of NMDA receptors to glycine by >4000-fold without altering sensitivity to glutamate. Mutations that neutralize the negative charge at D732 (D-to-A, D-to-G, or D-to-N) have effects similar to a mutation that retains the negative charge but increases the length of the side chain (D-to-E). One possible interpretation of these results is that D732 contributes directly to a binding site for glycine. In this case, the carboxyl group of D732 could interact with the amino group of glycine, similar to the interaction proposed for residue D481 (14, 15). However, mutations at D732 did not reduce sensitivity to the glycine-site antagonists DCQX and 5,7-DCK. These antagonists contain secondary amines that are thought to interact with the same pharmacophore group in the receptor as does the primary amine in glycine (5, 15). This suggests that D732 may not be involved directly in the binding of glycine and glycine-site antagonists. D732 is in the presumed extracellular loop of NR1 and is downstream of other residues (V684 and S687) that control sensitivity to

glycine and that may form part of a glycine binding pocket (14). Although there is no information about the tertiary structure of the M3-M4 loop of NMDA receptor subunits, it is possible that D732 is located near the glycine binding site. If D732 does not interact directly with glycine, this residue may be critical for coupling of glycine binding to channel opening.

Mutations at D732 had complex effects on sensitivity to the glycine-site agonists D-serine and D-alanine, altering both the potency and efficacy of these compounds. The NR1(D732E) mutation abolished stimulation by D-serine and D-alanine, and these amino acids act as antagonists at NR1(D732E)/NR2B receptors. These effects are reminiscent of effects seen with mutations at residues in the amino-terminal domain of the nicotinic acetylcholine receptor (32) and at residue R271 in the α1 subunit of the strychnine-sensitive glycine receptor (33). In the acetylcholine receptor, mutations at Y190 and D200 reduce sensitivity to acetylcholine and convert partial agonists into competitive antagonists (32). These residues are near the acetylcholine binding site and may be important for coupling of ligand binding to channel opening (32). In the α1 subunit of the glycine receptor, mutations that neutralize R271 greatly reduce sensitivity to glycine and convert the agonists β-alanine and taurine into competitive antagonists (33–35). Residue R271 is in the extracellular loop between M2 and M3 of the glycine receptor, and it has been proposed that this residue does not participate directly in binding of glycine but is important for coupling of agonist binding to channel opening (33, 34). Although NMDA receptors are not directly activated by glycine (they require cobinding of glutamate), it is possible that residue NR1(D732) in the NMDA receptor has a role analogous to that of R271 in the strychnine-sensitive glycine receptor.

Results of previous studies have identified residues in the M3-M4 loop (D669, C744, and C798) that are involved in modulation of channel opening by redox reagents, spermine, ifenprodil, and protons (17, 29). In the current study, we found that mutations at NR1(D732) had no effect on sensitivity to ifenprodil and spermine and did not alter the ability of spermine to increase the affinity for glycine ("glycine-dependent" stimulation). However, mutations D732A and D732N (but not D732E and D732G) increased proton sensitivity. Thus, in addition to altering sensitivity to glycine, mutations at NR1(D732) may have other subtle effects on NMDA receptor/channel properties depending on the amino acid that replaces D732.

Glycine is an obligatory coagonist at NMDA receptors, but the molecular mechanisms that underlie the activation of NMDA receptors by glycine are unknown. Glycine may facilitate transitions of the ion channel to an open state after binding of glutamate, and part of the mechanism of action of glycine involves a decrease in desensitization of NMDA receptors (36). Desensitization is not seen at recombinant NMDA receptors expressed in oocytes, but this may be because the solution exchange time during application of agonists is relatively slow, which could mask desensitization. Thus, it is possible that mutations at D732 alter desensitization of NMDA receptors or alter the effects of glycine on desensitization. The ability to drastically reduce glycine sensitivity by making mutations at D732 may be useful in dissecting the molecular basis for activation and desensitization of NMDA receptors using fast solution exchange and single-channel recording. Furthermore, because mutations at D732 alter the efficacy of some glycine-site agonists, these mutations may also be useful in probing the molecular basis for partial agonism at the glycine site.

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Send reprint requests to: Dr. Keith Williams, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084.