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An Aspartate Residue in the Extracellular Loop of the *N*-Methyl-D-aspartate Receptor Controls Sensitivity to Spermine and Protons

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SUMMARY

To study the role of acidic residues in modulation of NMDA receptors by spermine, we used site-directed mutagenesis of receptor subunits and voltage-clamp recording in *Xenopus* oocytes. Sixteen glutamate and aspartate residues, located in the first two thirds of the putative extracellular loop of the NR1A subunit, were individually mutated. This region of NR1A shows homology with bacterial amino acid binding proteins, a bacterial polyamine binding protein, and a bacterial spermidine acetyltransferase. Mutation of D669 to asparagine (D669N), alanine (D669A), or glutamate (D669E) abolished the "glycine-independent" form of spermine stimulation in heteromeric NR1A/NR2B receptors. These mutations also markedly reduced inhibition by ifenprodil and by protons at NR1A/NR2B receptors. Mutations at the equivalent position (D690) in NR1B, which contains the insert encoded by exon 5, reduced the pH sensitivity of NR1B/NR2B receptors. Thus, the effects of mutations at D669 are not prevented by the presence of exon 5, and the influence of exon 5 is not prevented by mutations at D669 (D690 in NR1B). Mutations at NR1A (D669) had little or no effect on the potencies of glutamate and glycine and did not alter voltage-dependent block by Mg^{2+} or the "glycine-dependent" form of spermine stimulation. Surprisingly, the D669N and D669A mutations, but not the D669E mutation, reduced voltage-dependent block by spermine at NR1A/NR2 receptors. Mutations in NR2B at a position (D668) equivalent to D669 did not alter spermine stimulation or sensitivity to pH and ifenprodil. However, mutations D668N and D668A but not D668E in NR2B reduced voltage-dependent block by spermine. Screening of the negative charges at NR1A(D669) and NR2B (D668) may be involved in voltage-dependent block by spermine. D669 in NR1A could form part of a binding site for polyamines and ifenprodil and/or part of the proton sensor of the NMDA receptor. Alternatively, this residue may be critical for coupling of modulators such as spermine, protons, and ifenprodil to channel gating.

NMDA receptors are sensitive to modulation by a variety of endogenous ligands, including glycine, Mg^{2+}, polyamines, and protons. Site-directed mutagenesis and heterologous expression of receptor subunits provide approaches to study the molecular basis for modulation of NMDA receptors and to locate residues that may contribute to binding sites for modulators and antagonists on the receptor. Through these approaches, residues that are important for modulation by glycine, Mg^{2+}, redox reagents, protons, and polyamines have been identified (1–10).

There are two families of NMDA receptor subunits: NR1, which is a single gene product expressed as eight alternatively spliced mRNAs, and NR2A, NR2B, NR2C, and NR2D, which are distinct gene products (11, 12). Some GluR subunits, including NR1, have homology with bacterial amino acid binding proteins, and regions of the receptor subunits may have a tertiary structure that forms an amino acid binding pocket similar to that of bacterial periplasmic binding proteins (5, 11, 13, 14). The properties of many modulators are dependent on the type of NR1 splice variant and the type of NR2 subunit present in heteromeric NR1/NR2 receptors.

Spermine has four macroscopic effects on NMDA receptors, all of which are dependent on the subunit composition of the receptor (15–17). These effects may involve three discrete polyamine binding sites (Fig. 1A). The "glycine-independent" form of spermine stimulation (Fig. 1A, *1*) is seen with saturating concentrations of glycine and occurs at receptors containing NR1 variants, such as NR1A, that lack a 21-amino acid insert encoded by exon 5. This form of stimulation is seen at homomeric NR1A receptors and at heteromeric NR1A/

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; ESAT, *E. coli* spermidine acetyltransferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GluR, glutamate receptor.

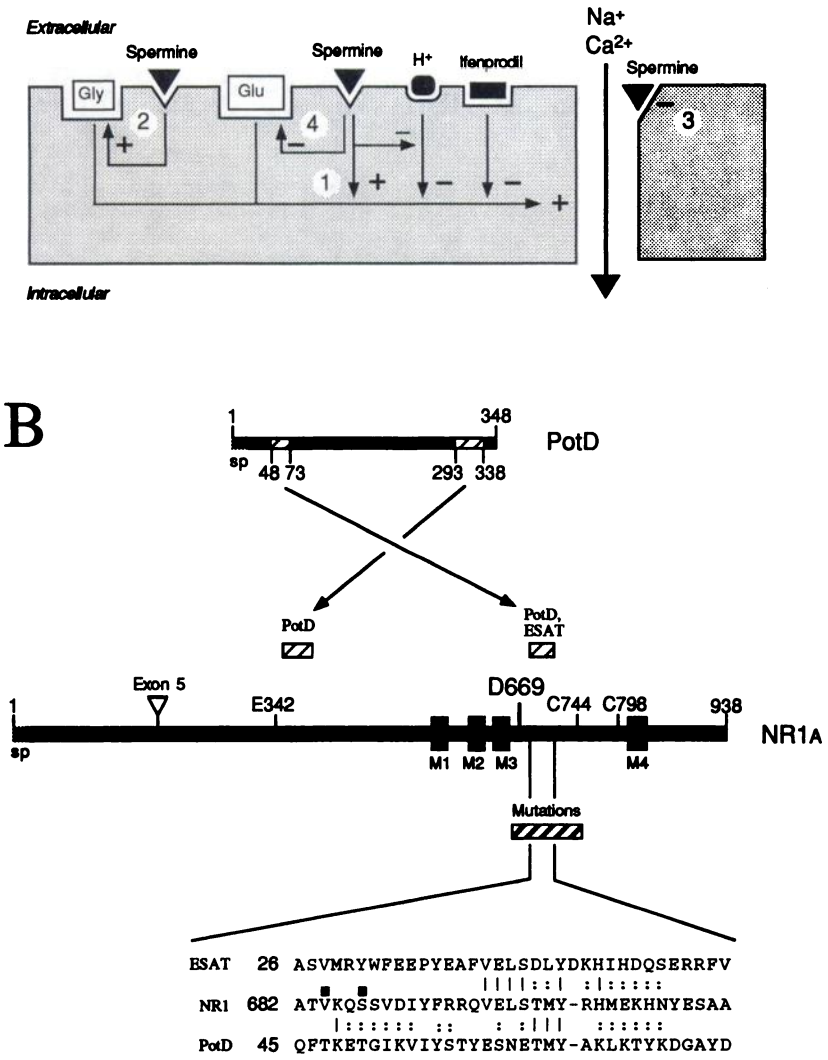

Fig. 1. A, Schematic model of the NMDA receptor to illustrate the effects of spermine, protons, and ifenprodil. Spermine has four macroscopic effects at NMDA receptors: 1) glycine-independent stimulation, which is seen in the presence of saturating concentrations of glycine; 2) glycine-dependent stimulation, which involves an increase in the affinity for glycine; 3) voltage-dependent block, which is more pronounced at hyperpolarized membrane potentials; and 4) a decrease in agonist affinity. Part of the mechanism underlying glycine-independent stimulation may involve relief of inhibition by protons. B, Amino acid similarity in NR1, ESAT, and PotD. *Top*, open reading frame of PotD (18); *hatched boxes*, regions of PotD that show homology with NR1A; *bottom*, the NR1A subunit, indicating the positions of D669, of other residues that influence sensitivity to spermine and protons (E342, C744, and C798), and of the 21-amino acid insert encoded by exon 5 that is absent in NR1A but is present in NR1B. *Filled vertical boxes*, (M1–4), positions of the originally proposed four transmembrane domains (11). *sp*, signal peptide. *Hatched boxes above NR1A*, regions that show homology with PotD (351–393) (Ref. 10) and with ESAT and PotD (685–710); *heavy shaded box below NR1A*, region (D658 to E751) where mutations were made. In the sequence comparisons, identical residues are indicated by lines and favored substitutions by colons; *black squares*, positions of residues (V684 and S687) that are important for modulation by glycine (5). Amino acids are numbered from the initiator methionine in NR1A.

NR2B receptors but not at receptors containing NR2A, NR2C, or NR2D. Because stimulatory effects of spermine are seen at homomeric NR1A receptors as well as at heteromeric NR1A/NR2B receptors, the polyamine binding site(s) may be located on the NR1 subunit. As a strategy to search for regions or amino acid residues in the NMDA receptor that are involved in modulation by spermine, we compared the amino acid sequence of NR1 with sequences of polyamine binding proteins including PotD, a periplasmic polyamine binding protein from *Escherichia coli* (18, 19), and ESAT, a bacterial spermidine acetyltransferase (20). Through this approach, we previously identified an acidic residue (E342), located just upstream of a region of NR1 that shows homology with PotD, which is critical for the glycine-independent form of spermine stimulation (10). Another region of NR1 (residues 685–719) that shows homology with PotD and with ESAT (a spermidine acetyltransferase) has been located (Fig. 1B).

We report the effects of mutations at acidic residues in the first two thirds (D658 to E751) of the loop between M3 and M4 in NR1A (see Fig. 1B). By analogy with subunits of other types of GluRs, subunits of NMDA receptors may contain only three transmembrane domains (M1, M3, and M4), with the originally proposed M2 making a hairpin loop that contributes to the ion channel pore (21–24). Thus, the region between M3 and M4 may be an extracellular loop, as proposed for subunits of non-NMDA receptors (21–23). We set out to look for residues that may contribute to spermine binding sites and chose to focus on the loop between M3 and M4 because it shows some homology (residues 685 to 710) with PotD and with ESAT (Fig. 1B). This region is upstream of two cysteine residues (C744 and C798; see Fig. 1B) that have been reported to control sensitivity to polyamines, protons, and redox reagents (6). We mutated acidic (glutamate and aspartate) residues because acidic residues have been found to contribute to polyamine binding sites in other proteins, including PotD (24a) and casein kinase 2 (25), and because an acidic residue (E342) in the amino-terminal domain of NR1A was previously shown to be critical for spermine stimulation at NMDA receptors (10). In the present work, we found that an aspartate residue (D669) in the M3–M4 loop of NR1A controls sensitivity to spermine and to pH.

Materials and Methods

Sequence comparisons and site-directed mutagenesis. Analysis of the sequence homology of NR1A, PotD, and ESAT was carried out according to the methods of Needleman and Wunsch (26) with the program DNASIS (Hitachi Software Engineering Co. Ltd.). Homology among NR1A, PotD, and ESAT is shown in Fig. 1B. The effects of mutations at acidic residues in and around the only other region of NR1A (residues 351–393) that shows homology with PotD have been reported previously (10). No significant homology was found between PotD or ESAT and NR2A or NR2B.

NR1A mutants were prepared as described previously (10), using a 2.6-kb *SphI/SalI* fragment of plasmid pN60 (11) inserted into the same sites of M13 mp18 (27). Similarly, to prepare NR2B mutants, a 2.1-kb *BamHI/SphI* fragment of pBSNR2B was inserted into the same sites of M13 mp19. Site-directed mutagenesis was carried out according to the method of Kunkel *et al.* (28) or the method of Sayers *et al.* (29) with the Sculptor *in vitro* mutagenesis system (Amersham International). Mutated DNA fragments were isolated from the replicative form of M13 and religated into the same sites of pN60 and pBSNR2B. Mutations were confirmed by DNA sequencing (30) using the M13 phage system (27). To prepare NR1B mutants, a 1.6-kb *BglII* fragment of each NR1A mutant was ligated with a 5.7-kb *BglII* fragment of pBSNR1B to yield the appropriate NR1B mutant (10). For example, the fragment from NR1A(D669N), when ligated into pBSNR1B, produces NR1B(D690N) because NR1B contains a 21-amino acid insert encoded by exon 5. The orientation of the inserts was verified by digestion with *XhoI*. Plasmids were propagated in *E. coli* DH5α and isolated using standard procedures.

Amino acids are numbered from the initiator methionine in NR1 and NR2 subunits (11, 31, 32). Mutations are referred to after the subunit by the original amino acid, its number, and the mutated amino acid. Thus, NR1A(D669N) contains an asparagine (N) at position 669 in place of the aspartate (D) found in the wild-type NR1A subunit.

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 (33–35). Oocytes were injected with NR1A or NR1B plus NR2A or NR2B cRNAs in a ratio of 1:5 (0.5–2 ng of NR1 plus 2.5–10 ng of NR2). For homomeric NR1 receptors, oocytes were injected with 10 ng of NR1A cRNA.

Macroscopic currents were recorded with a two-electrode voltage-clamp as described previously (33, 35). 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 (33, 36). In most experiments, oocytes were injected with K^+-BAPTA (100 nl; 40 mM, pH 7.4) on the day of recording (33).

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 NR1A and NR1B clones (11, 31) were a gift from Dr. S. Nakanishi (Department of Immunology, Kyoto University, Japan). The NR2A and NR2B clones (37) were a gift from Dr. P. H. Seeburg (Center for Molecular Biology, University of Heidelberg, Germany).

Results

Expression of NR1A mutants. To look for residues that influence sensitivity to polyamines, we first made glutamate-to-glutamine and aspartate-to-asparagine mutations at the 16 asparate and glutamate residues between D658 and E751 in NR1A. When coexpressed with NR2B, all of the mutants except D732N produced large macroscopic currents. Currents with NR1A (D732N)/NR2B receptors were only 5–20 nA in oocytes voltage-clamped at −70 mV (20 oocytes; two independent clones), so the properties of this mutant were not studied further. The D669N mutant abolished spermine stimulation (see below), and we therefore studied other mutations (D669A and D669E) at this position. Initially, we found that receptors containing D669N (but not D669A or D669E) produced currents that were 50–70% smaller than responses at wild-type receptors in the same batches of oocytes. For the detailed characterization of these clones, oocytes were injected with 0.5–1 ng of wild-type, D669A, and D669E cRNAs (plus 2.5–5 ng of NR2 cRNA) and 2 ng of D669N cRNA (plus 10 ng of NR2 cRNA). Currents induced by 10 μM glutamate (with 10 μM glycine) at NR1A/NR2B receptors in oocytes voltage-clamped at −20 mV were 282 ± 28 nA (wild-type, 62 oocytes), 158 ± 21 nA (D669N, 52 oocytes), 400 ± 93 nA (D669A, 31 oocytes), and 513 ± 100 nA (D669E, 35 oocytes). The D669N but not the D669E or D669A mutations increased the apparent affinity for glycine. At NR1A/NR2B receptors, EC_{50} values for glycine were 0.6 ± 0.4 μM (wild-type, eight oocytes), 0.02 ± 0.01 μM (D669N, nine oocytes), 0.30 ± 0.03 μM (D669A, five oocytes), and 0.7 ± 0.2 μM (D669E, five oocytes). The D669 mutations did not change sensitivity to glutamate. EC_{50} values for glutamate were 2.4 ± 0.4 μM (wild-type, six oocytes), 0.9 ± 0.1 μM (D669N, eight oocytes), 3.9 ± 0.8 μM (D669A, four oocytes), and 2.5 ± 0.3 μM (D669E, four oocytes).

Sensitivity to stimulation by spermine. All four macroscopic effects of spermine are seen at NR1A/NR2B receptors (16, 35). By manipulating the concentrations of glutamate and glycine and the holding potential, these effects can be studied in relative isolation at NR1A/NR2B receptors. In oocytes voltage-clamped at −20 mV to minimize voltage-dependent block by spermine and with 10 μM glutamate and glycine to activate the receptors, the glycine-independent form of spermine stimulation can be measured (Fig. 1A, *1*). We studied this effect at wild-type and mutant NR1A/NR2B receptors (Fig. 2). With some mutations (e.g., D658N, E662Q, E707Q), spermine produced a larger stimulation than at wild-type receptors. Stimulation by spermine was abolished with D669N and with other mutations at this position (D669A and D669E) (Fig. 2). Glycine-independent stimulation was also measured at homomeric NR1A receptors. Spermine potentiated responses to glutamate at wild-type NR1A receptors (133 ± 4% of control, eight oocytes) but had no effect at NR1A(D669N) receptors (99 ± 2% of control, eight oocytes).

We measured concentration-response relationships for spermine at NR1A/NR2B receptors in oocytes voltage-clamped at −20 mV. Spermine, at concentrations of 3–300 μM, potentiated responses at wild-type receptors and an additional inhibitory component was seen at concentrations of >300 μM. At receptors containing NR1A(D669) mutants, no stimulation was seen at concentrations of spermine up to 3 mM (data not shown).

The second form of spermine stimulation, "glycine-dependent" stimulation (Fig. 1A, *2*), involves an increase in the affinity of the receptor for glycine and is seen with subsaturating concentrations of glycine. Thus, when effects of spermine are studied at NR1A/NR2B receptors activated by 0.1 μM glycine (with 10 μM glutamate), both the glycine-dependent and glycine-independent forms of stimulation are measured. A comparison of the effects of spermine measured with 10 μM

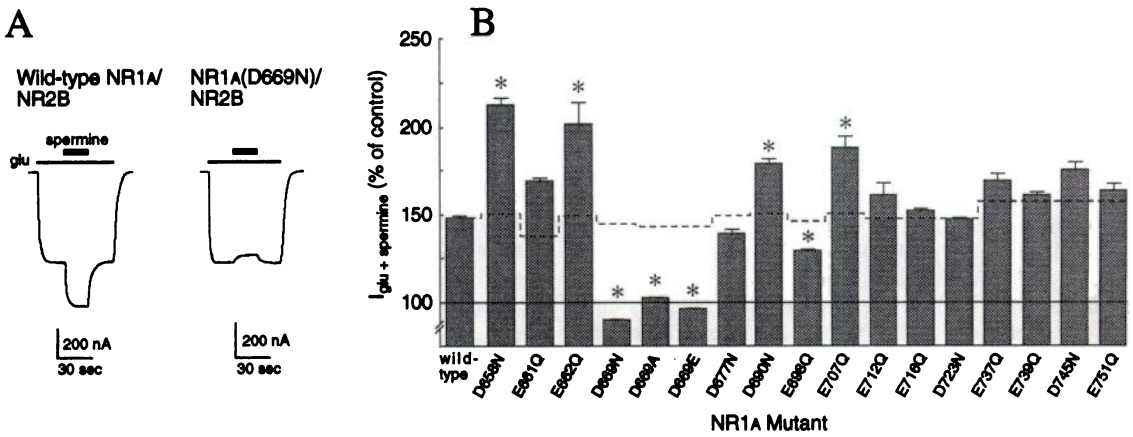

Fig. 2. Glycine-independent stimulation at NR1A/NR2B receptors. A, Effects of 100 μM spermine on responses to glutamate (10 μM; with 10 μM glycine) in oocytes expressing wild-type NR1A/NR2B receptors and NR1A(D669N)/NR2B receptors and voltage-clamped at −20 mV. B, The effects of spermine were measured in oocytes expressing NR1A/NR2B receptors with wild-type and mutant NR1A subunits and voltage-clamped at −20 mV. Currents measured in the presence of spermine are expressed as a percentage of the control response to glutamate. Values are mean ± standard error for 4–15 oocytes for each mutant and 28 oocytes for wild-type NR1A. Effects at wild-type receptors were measured in all batches of oocytes. *Broken line*, effects at wild-type receptors in the same experiments as the corresponding mutants. *, $p < 0.05$ compared with wild-type receptors studied in the same batch of oocytes (Student's unpaired *t* test).

glycine and 0.1 μM glycine provides an index of glycine-dependent stimulation (Fig. 3A). Glycine-dependent stimulation by spermine was seen with all of the NR1A mutants (Fig. 3B). Mutations D669A and D669E did not affect glycine-dependent stimulation. Stimulation was somewhat reduced with D669N. However, this was probably due to the increase in affinity for glycine seen with this mutant (see above). Thus, mutations at D669, which abolish glycine-independent

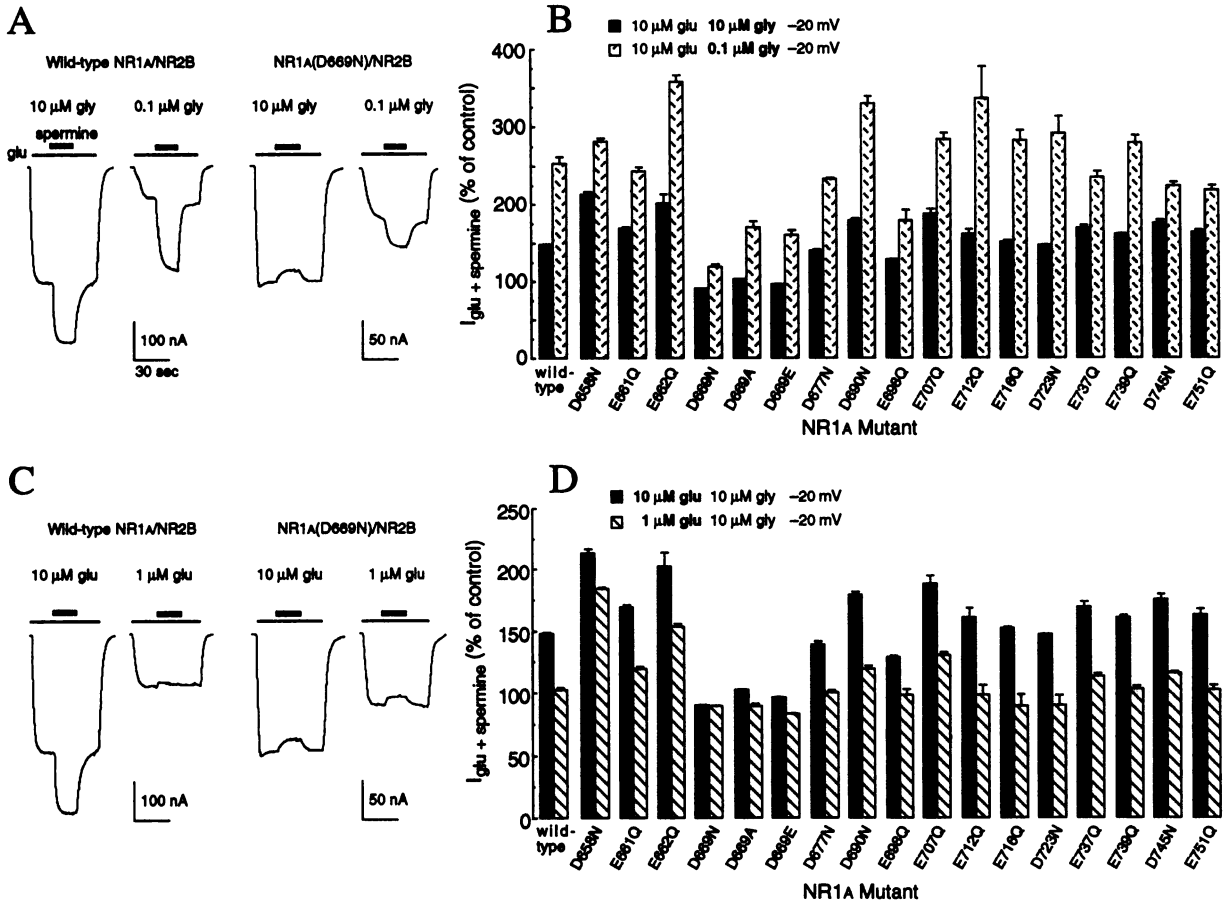

Fig. 3. Glycine-dependent and glutamate-dependent effects of spermine. A and B, Glycine-dependent stimulation was assessed by measuring the effects of 100 μM spermine on responses to glutamate (10 μM) with 10 μM glycine and 0.1 μM glycine in oocytes expressing NR1A/NR2B receptors and voltage-clamped at −20 mV. C and D, The effects of 100 μM spermine on responses to 1 μM and 10 μM glutamate (all with 10 μM glycine) were measured in oocytes expressing NR1A/NR2B receptors and voltage-clamped at −20 mV. Data in B and D are mean ± standard error from 4–15 oocytes for each mutant and 28 oocytes for wild-type NR1A.

stimulation (Fig. 2), do not affect glycine-dependent stimulation by spermine (Fig. 3B).

The effects of spermine are dependent on the concentration of glutamate used to activate NR1A/NR2B receptors. Spermine potentiates responses to 10 μM glutamate (in the presence of 10 μM glycine) but has little or no effect on responses to 1 μM glutamate (Fig. 3C, *traces for wild-type NR1A/NR2B*). This is because spermine reduces the affinity of NR1A/NR2B receptors for glutamate (Fig. 1A, *4*), an effect that decreases macroscopic currents at subsaturating concentrations of glutamate, balancing the stimulation and resulting in no net effect of spermine (35). If the decrease in agonist affinity caused by spermine persists in mutants such as D669N, one would expect to see a 30–50% reduction in macroscopic currents by spermine when 1 μM glutamate is used to activate the receptors. However, spermine had virtually no effect on responses to either 10 μM glutamate or 1 μM glutamate at receptors containing D669N, D669A, or D669E (Fig. 3, C and D). This suggests that mutations at D669 eliminate the effect of spermine on agonist affinity, consistent with the proposal (35) that this effect is mediated at the same site or involves the same mechanism as glycine-independent stimulation (Fig. 1A, *1* and *4*).

Sensitivity to inhibition by spermine. Another effect of spermine is voltage-dependent inhibition (Fig. 1A, *3*), which is more pronounced at hyperpolarized than at depolarized membrane potentials. To evaluate this effect, we compared the effects of spermine at NR1A/NR2B receptors in oocytes voltage-clamped at −20 mV and −100 mV (Fig. 4). Glycine-independent stimulation occurs at both holding potentials, but voltage-dependent block, which is pronounced at −100 mV, is negligible or absent at −20 mV. As previously reported (16, 38), the block of NR1A/NR2B receptors at −100 mV developed slowly, reaching a steady state after 1.5–3 min (Fig. 4A, *traces for wild-type receptors*). Mutations at D669 had complex effects on voltage-dependent block at NR1A/NR2B receptors, depending on the amino acid substituted for D669. With D669N, voltage-dependent block by spermine was reduced (Fig. 4, A and B). With D669E, the steady state block was larger than at wild-type receptors, as one would expect if stimulation was abolished, but voltage-dependent block was unaltered (Fig. 4A). With D669A, the effect on voltage-dependent block was intermediate between that at D669N and D669E (Fig. 4B). Thus, surprisingly, mutations at D669 alter voltage-dependent block by spermine at NR1A/NR2B receptors. Because glycine-independent stimulation by spermine occurs at NR1A/NR2B receptors, voltage-dependent block cannot be studied in isolation at these receptors. To measure voltage-dependent block in the absence of stimulation, we studied NR1A subunits coexpressed with NR2A because NR1A/NR2A receptors show block but not stimulation by spermine (16). Similar to the profile seen at NR1A/NR2B receptors, inhibition by spermine at NR1A/NR2A receptors was reduced by D669N and D669A but unaltered by D669E (Fig. 5). Thus, the effect of mutations at D669 on voltage-dependent block by spermine is independent of the NR2 subunit. Mutations at other acidic residues in this region of NR1A did not alter voltage-dependent block by spermine (Fig. 4B).

Sensitivity to Mg^{2+}. Some mutations at D669 altered voltage-dependent block by spermine. To determine the specificity of this effect, we also studied voltage-dependent block by extracellular Mg^{2+} at NR1A/NR2B receptors. Effects of 100 μM Mg^{2+} on responses to glutamate were measured in oocytes voltage-clamped at −20 and −70 mV. In contrast to effects on inhibition by spermine, mutations at D669 had no

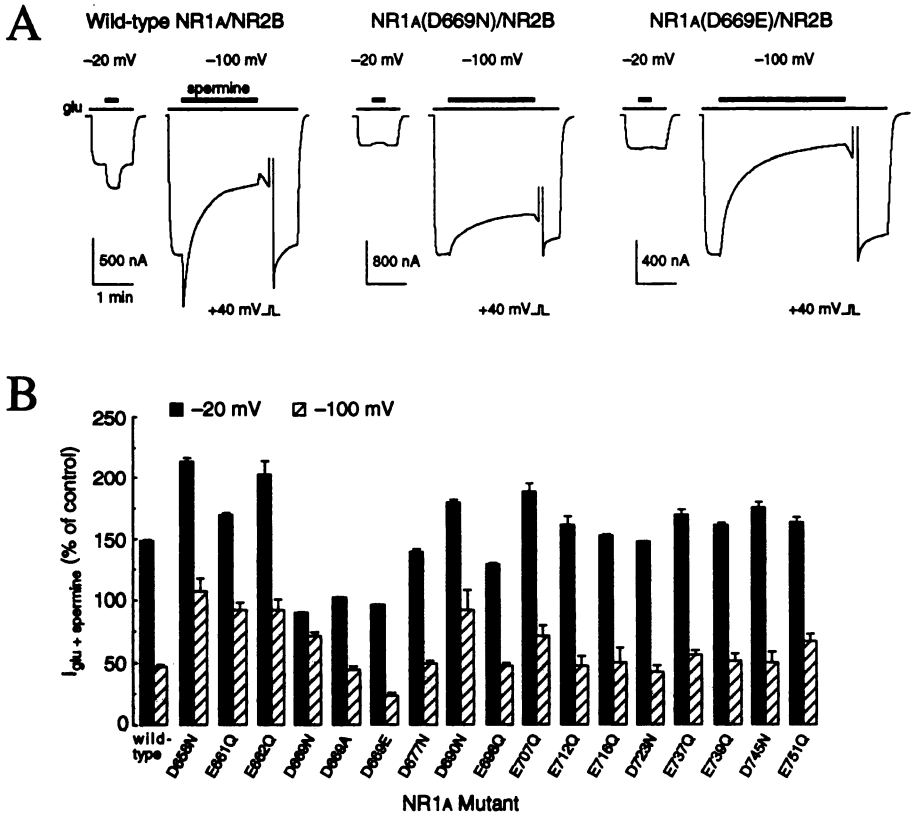

Fig. 4. Voltage-dependent block by spermine at NR1A/NR2B receptors. A, The effects of 100 μM spermine on responses to glutamate (10 μM; with 10 μM glycine) were measured in oocytes expressing NR1A/NR2B, NR1A(D669N)/NR2B, and NR1A(D669E)/NR2B receptors and voltage-clamped at −20 and −100 mV. During recovery from block by spermine at −100 mV, cells were depolarized to +40 mV for 5 sec to speed recovery. All horizontal scale bars are 1 min. B, Steady state effects of spermine were measured in oocytes expressing NR1A/NR2B receptors with wild-type or mutant NR1A subunits and voltage-clamped at −20 or −100 mV. Currents measured in the presence of spermine are expressed as a percentage of the control response to glutamate at each holding potential. Values are mean ± standard error for 4–14 oocytes for each mutant and 29 oocytes for wild-type NR1A.

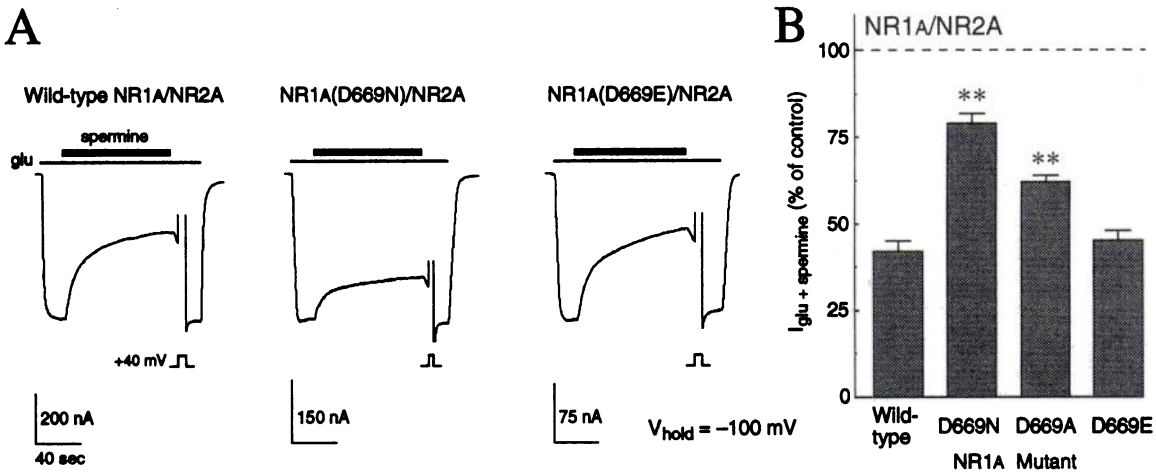

Fig. 5. Voltage-dependent block by spermine at NR1A/NR2A receptors. A, Effects of 100 μM spermine on responses to glutamate (10 μM; with 10 μM glycine) in oocytes expressing NR1A/NR2A, NR1A(D669N)/NR2A, and NR1A(D669E)/NR2A receptors and voltage-clamped at −100 mV. After washout of spermine, oocytes were depolarized to +40 mV for 3–6 sec to speed recovery. All horizontal scale bars are 40 sec. B, Effects of spermine were measured in oocytes expressing NR1A/NR2A receptors containing wild-type and mutant NR1A subunits using the protocols shown in A. Currents measured in the presence of spermine are expressed as a percentage of the control response to glutamate. Values are mean ± standard error for 6–9 oocytes for each subunit combination. **, $p < 0.01$; one-way analysis of variance with post-hoc Dunnett's test.

effect on inhibition by Mg^{2+} (7–12 oocytes for each D669 mutant; data not shown).

Sensitivity to ifenprodil. Ifenprodil is an atypical antagonist at NMDA receptors that has been suggested to act at a stimulatory polyamine site (39), although inhibition by ifenprodil is seen in the absence of extracellular polyamines, implying that ifenprodil does not act simply as a competitive antagonist to block spermine stimulation (33, 40, 41). Ifenprodil has a high affinity only at heteromeric receptors containing NR2B (33, 38), which are also sensitive to glycine-independent stimulation by spermine (16, 38). Thus, polyamines and ifenprodil may share overlapping binding sites or be influenced by similar structural features in NMDA receptors. With this in mind, we studied inhibition by ifenprodil at NR1A/NR2B receptors containing wild-type and mutant NR1A subunits (Fig. 6). Mutations D669N, D669A, and D669E reduced inhibition by ifenprodil. Other asparate-to-asparagine and glutamate-to-glutamine mutations in this region of NR1A had no effect on sensitivity to ifenprodil (Fig. 6).

Sensitivity to pH. Protons inhibit the activity of NMDA receptors. Sensitivity to protons, like sensitivity to spermine, is influenced by the presence of the 21-amino acid insert encoded by exon 5 (8, 15). Variants, like NR1A, that lack the insert are more sensitive to inhibition by protons than are variants that contain the insert. Glycine-independent stimulation by spermine is altered by changes in extracellular pH, and this form of stimulation may involve relief of tonic inhibition by protons when responses to spermine are measured at pH 7.5 (8). Because of the reported interactions between spermine and protons, we carried out experiments to determine whether mutations at D669 alter pH sensitivity at NR1A/NR2B receptors (Fig. 7 and Table 1). At wild-type NR1A/NR2B receptors, inhibition was observed between pH 8.5 and 6.5. At NR1A(D669N)/NR2B receptors, the maximum response to glutamate was observed at pH 7.5 rather than at pH 8.5, and inhibition by protons was greatly reduced (Fig. 7). Similarly, inhibition by protons was reduced in receptors containing D669A and D669E (Fig. 7 and Table 1).

We also examined the pH sensitivity of heteromeric receptors expressed from NR1A(D669) mutants together with NR2A (rather than NR2B). As with NR1A/NR2B receptors, mutations at D669 reduced the sensitivity of NR1A/NR2A receptors to protons (Table 1). However, the pattern seen at

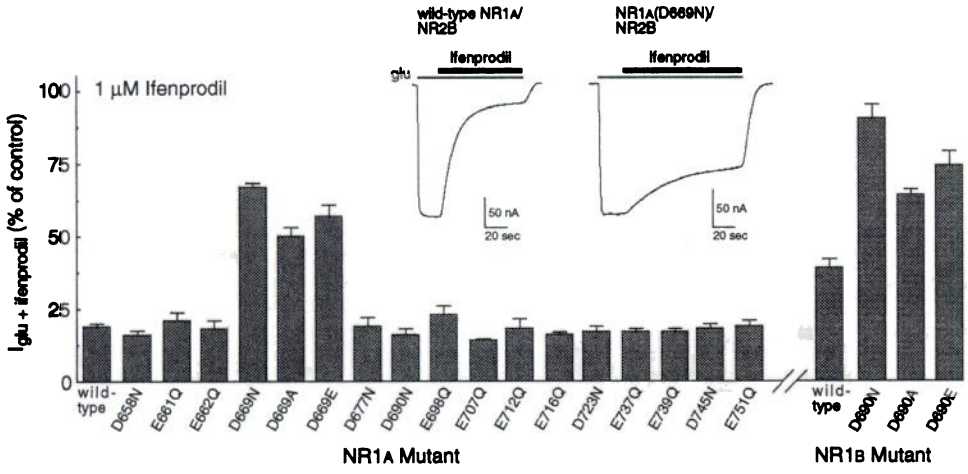

Fig. 6. Effects of ifenprodil at NR1A/NR2B and NR1B/NR2B receptors. The effects of 1 μM ifenprodil on responses to glutamate (10 μM; with 10 μM glycine) were measured in oocytes expressing NMDA receptors with wild-type and mutant NR1A and NR1B subunits and voltage-clamped at −20 mV. Currents measured in the presence of ifenprodil are expressed as a percentage of the control response to glutamate. Values are mean ± standard error for 4–14 oocytes for each mutant and 25 oocytes for wild-type NR1A. *Inset*, effects of ifenprodil at wild-type NR1A/NR2B receptors and NR1A (D669N)/NR2B receptors.

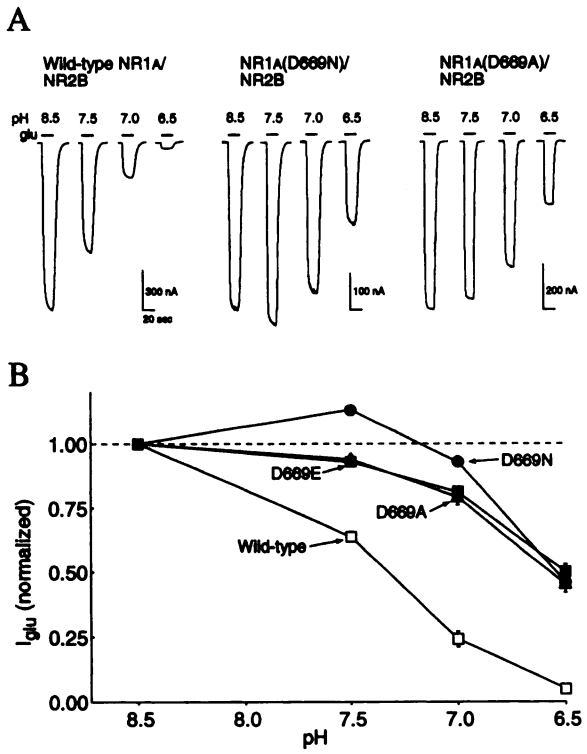

Fig. 7. Proton sensitivity of NR1A/NR2B receptors. A, Currents induced by glutamate (10 μM; with 10 μM glycine) were measured at pH 8.5–6.5 in oocytes expressing wild-type NR1A/NR2B receptors, NR1A(D669N)/NR2B, and NR1A(D669A)/NR2B receptors. Oocytes were perfused with buffer at the appropriate pH for 20 sec before and after application of glutamate and were perfused at pH 7.5 between applications at different pH. For each subunit combination, the same profile was seen regardless of the order of application of pH changes. B, Sensitivity to pH was measured at NR1A/NR2B receptors containing wild-type and mutant NR1A subunits using the protocols shown in A. Data are normalized to the glutamate-induced current measured at pH 8.5 (glutamate-induced current at pH 8.5 = 1.0). Values are mean ± standard error for 5–12 oocytes for each subunit combination. Currents at pH 8.5 were 872 ± 141 nA (wild-type, 10 oocytes), 287 ± 56 nA (D669N, 12 oocytes), 1860 ± 106 nA (D669E, five oocytes), and 1165 ± 157 nA (D669A, five oocytes).

TABLE 1

Effects of mutations on pH sensitivity

Glutamate-induced currents were measured at pH 8.5–6.5. IC_{50} values were calculated by fitting untransformed data (i.e., glutamate current versus pH) to the logistic function: $I = I_{max}/1 + ([pH/IC_{50}])^{n_H}$, in which I is the glutamate current, I_{max} is the theoretical maximum current, IC_{50} is the pH producing a 50% inhibition of macroscopic currents, and n_H is the Hill slope. For all analyses except NR1A(D669N)/NR2B, data measured at pH 8.5–6.5 were analyzed. For NR1A(D669N)/NR2B receptors, at which a maximum response was seen at pH 7.5 rather than at pH 8.5, data measured at pH 7.5–6.5 were analyzed. These analyses assume that protons cause a complete inhibition of macroscopic currents at all receptor types. Values are mean ± standard error.

Subunit combination	IC_{50}	No. of oocytes
	pH	
Wild-type NR1A/NR2B	7.27 ± 0.03	20
NR1A(D669N)/NR2B	6.64 ± 0.03	12
NR1A(D669A)/NR2B	6.57 ± 0.04	5
NR1A(D669E)/NR2B	6.49 ± 0.04	5
NR1A(D658N)/NR2B	7.43 ± 0.02	5
NR1A(E662Q)/NR2B	7.46 ± 0.02	5
NR1A(D690N)/NR2B	7.45 ± 0.01	5
NR1A(E707Q)/NR2B	7.45 ± 0.02	5
NR1A/NR2B(D668N)	7.29 ± 0.02	6
NR1A/NR2B(D668A)	7.18 ± 0.02	6
NR1A/NR2B(D668E)	7.14 ± 0.04	6
Wild-type NR1A/NR2A	7.27 ± 0.03	8
NR1A(D669N)/NR2A	6.84 ± 0.02	8
NR1A(D669A)/NR2A	7.07 ± 0.06	5
NR1A(D669E)/NR2A	6.91 ± 0.03	5

NR1A/NR2A receptors was different from that seen at NR1A/NR2B receptors, with effects on pH sensitivity showing a rank order of D669N > D669E > D669A (Table 1).

In addition to D669 mutants, where spermine stimulation is abolished, we measured the pH sensitivity of NR1A mutants at which glycine-independent spermine stimulation was increased. At D658N, E662Q, D690N, and E707Q, the pH IC_{50} values were shifted to a slightly more alkaline pH than at wild-type NR1A/NR2B receptors (Table 1). Thus, mutations D658N, E662Q, D690N, and E707Q in NR1A produce a small increase in proton sensitivity, leading to a greater proton inhibition at pH 7.5. These effects, which are opposite the effects of mutations at D669 (Table 1), may account for the increase in spermine stimulation seen at the D658N, E662Q, D690N, and E707Q mutants studied at pH 7.5 (Fig. 2).

To determine whether a change in pH sensitivity can account for the lack of spermine stimulation at receptors containing D669 mutants, we studied the effects of spermine at NR1A/NR2B receptors at pH 7.5 and 6.5 (Table 2 and Fig. 8). At wild-type NR1A/NR2B receptors, spermine produced a larger potentiation at pH 6.5 than at pH 7.5. At receptors containing D669 mutants, there was no potentiation by 100 μM spermine at pH 7.5, but some potentiation (18–51%) at pH 6.5 (Table 2). NR1A/NR2B receptors containing the NR1A(D669) mutants are inhibited ~50% by extracellular protons at pH 6.5, whereas a similar degree of inhibition is seen at pH 7.5 in wild-type NR1A/NR2B receptors (Fig. 7 and Table 1). Thus, if the stimulatory effect of spermine is due to a relief of tonic proton inhibition, one would expect to see a similar degree of potentiation by spermine at D669 mutants measured at pH 6.5 as at wild-type receptors measured at pH 7.5. Potentiation by spermine at wild-type NR1A/NR2B receptors at pH 7.5 was similar to that at D669 mutants measured at pH 6.5 (Table 2 and Fig. 8). Thus, a change in pH sensitivity may contribute in large part to the loss of spermine stimulation in receptors containing NR1A(D669) mutants. In these experiments, we also determined spermine sensitivity of wild-type NR1A/NR2A receptors, which do not show glycine-independent spermine stimulation at pH 7.5 (16). At NR1A/NR2A receptors, there was no stimulation by spermine at pH 7.5 or 6.5 (Table 2). Thus, the influence of the NR2A subunit on spermine sensitivity is not related to a change in pH sensitivity of heteromeric receptors containing NR2A.

Effects of mutations in the NR1B subunit. The NR1B variant of NR1 is identical to NR1A in its carboxyl-terminal splicing pattern but contains a 21-amino acid insert, encoded by exon 5, in the amino-terminal domain (31, 42). This insert prevents glycine-independent stimulation by spermine and reduces the sensitivity of NMDA receptors to protons. In light of this, we looked at mutations in NR1B at a position (D690) equivalent to D669 in NR1A. Because of the differential effects seen with asparagine, alanine, and glutamate mutations at D669 in NR1A, we tested all three mutations at D690 in NR1B.

The properties of NR1B subunits were determined after coexpression with NR2B. Wild-type and mutant NR1B/NR2B

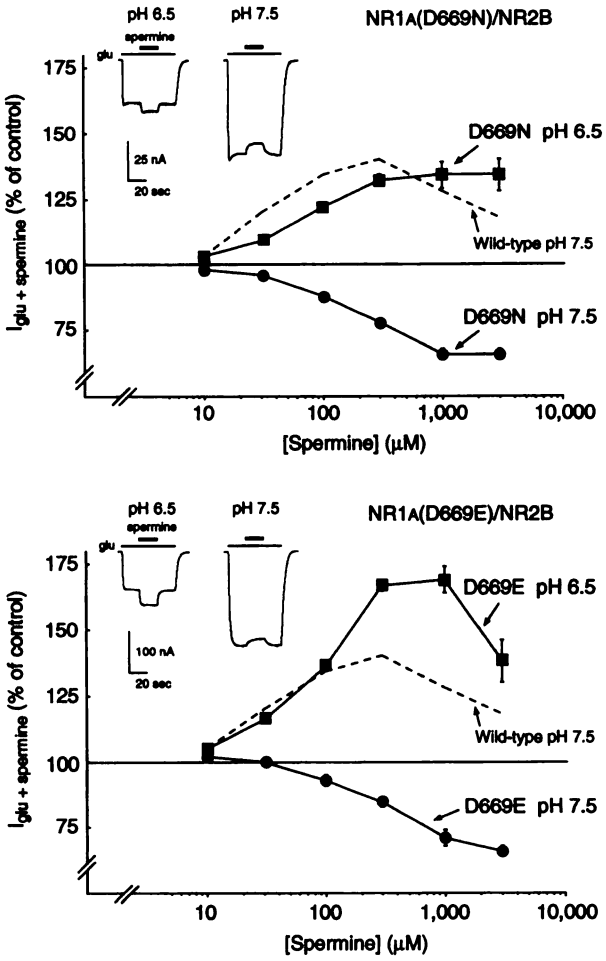

Fig. 8. Dependence of spermine stimulation on extracellular pH. Effects of various concentrations of spermine on responses to glutamate (10 μM; with 10 μM glycine) were determined at NR1A/NR2B receptors with NR1A(D669N) and NR1A(D669E) at pH 6.5 or pH 7.5. Oocytes were voltage-clamped at −20 mV. Data are the steady state response to glutamate plus spermine expressed as a percentage of the control response to glutamate measured at each pH. Values are mean ± standard error for four to six oocytes for each subunit combination. *Insets*, effects of 100 μM spermine measured at pH 6.5 and 7.5 for each receptor type.

TABLE 2

Effects of pH on stimulation by spermine

Responses to glutamate (10 μM; with 10 μM glycine) were measured in the absence and presence of 100 μM spermine at pH 7.5 and 6.5 in oocytes voltage-clamped at −20 mV. Responses measured in the presence of spermine are expressed as a percentage of the control response to glutamate at each pH. Values are mean ± standard error.

Subunit combination	$I_{glu+spermine}$		No. of oocytes
	pH 7.5	pH 6.5	
	% of control		
Wild-type NR1A/NR2B	143 ± 1	293 ± 7	8
NR1A(D669N)/NR2B	88 ± 1	118 ± 8	8
NR1A(D669A)/NR2B	104 ± 1	151 ± 3	8
NR1A(D669E)/NR2B	92 ± 1	146 ± 3	8
Wild-type NR1A/NR2A	85 ± 5	92 ± 3	5

receptors were insensitive to stimulation by spermine (data not shown). Inhibition by ifenprodil was somewhat smaller at wild-type NR1B/NR2B receptors that at wild-type NR1A/NR2B receptors, but this inhibition was reduced by NR1B (D690) mutants, similar to effects seen with the corresponding NR1A(D669) mutants (Fig. 6). Thus, the inclusion of exon 5 in NR1 produces a small reduction in the sensitivity to ifenprodil but does not alter the effects of D669 mutations on sensitivity to ifenprodil (Fig. 6).

As reported by other investigators (8), receptors containing wild-type NR1B were less sensitive to inhibition by protons than were receptors containing NR1A (Fig. 9). The pH sensitivity of NR1B(D690) mutants was greatly reduced compared with wild-type NR1B, and the maximum response to glutamate was seen at pH 7.5–7.0 rather than at pH 8.5 (Fig. 9). The proton sensitivity of NR1B(D690) mutants was also reduced compared with the respective NR1A(D669) mutants; an example is shown for NR1B(D690A) versus NR1A(D669A) (Fig. 9B, *inset*). Thus, the effects of D669/D690 mutations and the inclusion of exon 5 are additive: the inclusion of exon 5 does not prevent the change in pH sensitivity caused by D669 mutants, and *vice versa*.

Effects of mutations in the NR2B subunit. The NR2 subunits contain aspartate residues at positions equivalent

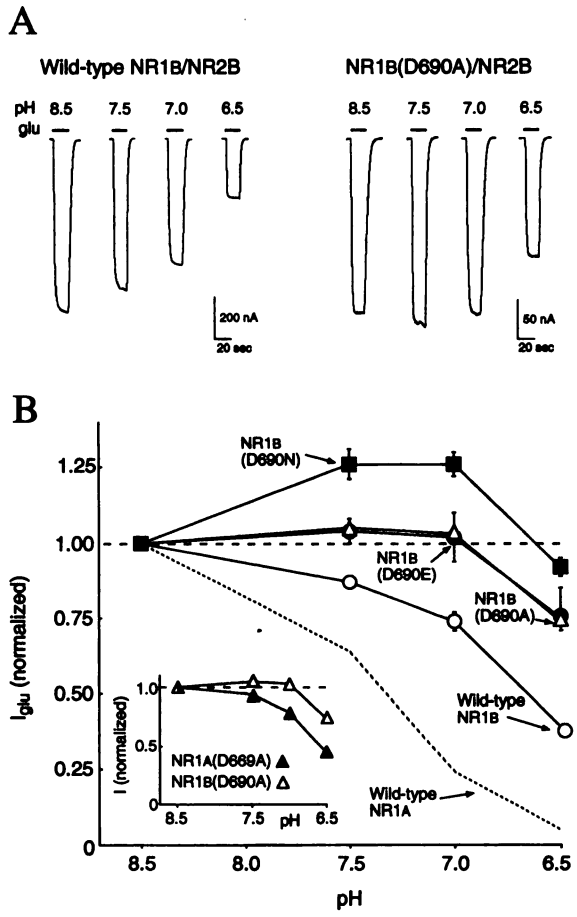

Fig. 9. Proton sensitivity of NR1B/NR2B receptors. A, Currents induced by glutamate (10 μM; with 10 μM glycine) were measured at pH 8.5–6.5 in oocytes expressing wild-type NR1B/NR2B receptors and NR1B(D690A)/NR2B receptors. B, Sensitivity to pH was measured at NR1B/NR2B receptors containing wild-type and mutant NR1B subunits using the protocols shown in A. Data are normalized to the glutamate-induced current measured at pH 8.5 (glutamate-induced current at pH 8.5 = 1.0). Data for wild-type NR1A/NR2B receptors are replotted from Fig. 7. Values are mean ± standard error for three to six oocytes for each subunit combination. *Inset*, data for NR1B(D690A)/NR2B receptors are plotted with data for the corresponding NR1A mutant, NR1A(D669A)/NR2B, which are replotted from Fig. 7.

to D669 in NR1A. To determine whether the corresponding aspartate residue in an NR2 subunit influences sensitivity to spermine, pH, and ifenprodil, we studied mutations at position D668 in NR2B (Fig. 10A). Wild-type and mutant NR2B subunits were studied after coexpression with wild-type NR1A. Currents induced by glutamate (10 μM; with 10 μM glycine) at −20 mV were 367 ± 66 nA (wild-type, 17 oocytes), 59 ± 9 nA (D668N, 15 oocytes), 186 ± 48 nA (D668A, 14 oocytes), and 338 ± 73 nA (D668E, 14 oocytes). Mutations at NR2B (D668) had no effect on glycine-independent stimulation by spermine measured at −20 mV (Fig. 10B) or on sensitivity to ifenprodil (Fig. 10C) or pH (Table 1). However, mutations D668N and D668A (but not D668E) reduced voltage-dependent block by spermine measured at −100 mV (Fig. 10B). This is similar to effects seen with D669N and D669A mutations in NR1A (Figs. 4 and 5). Thus, the negative charges at D668 in NR2B and D669 in NR1A may both be important for voltage-dependent block by spermine.

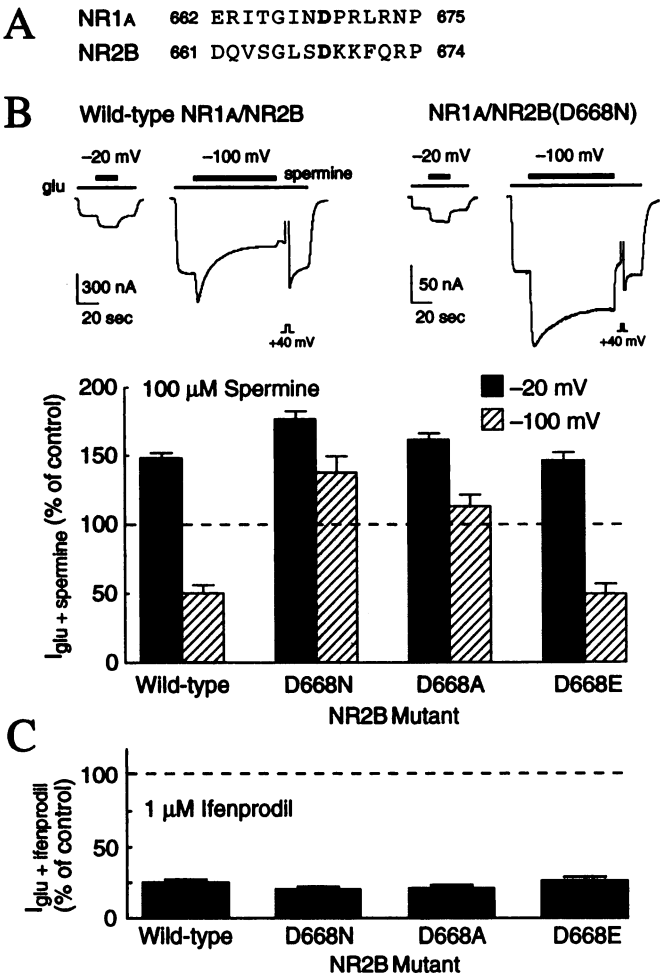

Fig. 10. Properties of mutant NR2B subunits. A, Comparison of the sequences of NR1A and NR2B in the regions containing residues that were mutated (D669 in NR1A and D668 in NR2B). B, Effects of spermine on responses to glutamate (10 μM; with 10 μM glycine) were measured in oocytes expressing NR1A/NR2B receptors containing wild-type and mutant NR2B subunits and voltage-clamped at −20 and −100 mV. Currents measured in the presence of spermine are expressed as a percentage of the control response to glutamate. C, Effects of ifenprodil were measured in oocytes expressing NR1A/NR2B receptors containing wild-type and mutant NR2B subunits and voltage-clamped at −20 mV. Currents measured in the presence of ifenprodil are expressed as a percentage of the control response to glutamate. Values in B and C are mean ± standard error for 5–11 oocytes for each subunit combination.

Discussion

Results of previous studies have shown that the effects of polyamines and protons at NMDA receptors are influenced by the presence of exon 5 in the NR1 subunit, by cysteine residues in the presumed extracellular loop between M3 and M4, and by a glutamate residue (E342) in the center of the large amino-terminal domain (see Fig. 1B). In the present work, we found that an aspartate residue (D669) in the loop between M3 and M4 also controls sensitivity to spermine and pH. The initial strategy for the studies described in the current and a previous report (10) was based on homology of NR1A and PotD and was designed to look for residues that may contribute to spermine binding sites on NR1A. The region of NR1A (685–710) that shows homology with PotD overlaps with a region of NR1A that is homologous to bacterial periplasmic amino acid binding proteins, including the glutamine binding protein and the lysine/arginine/ornithine binding protein (5, 11). PotD shows only limited amino acid sequence identity with other bacterial periplasmic binding proteins, but X-ray crystallographic analysis has shown that PotD has a major fold and a three-dimensional structure similar to those of the bacterial maltodextrin binding protein, the sulfate binding protein, and the lysine/arginine/ornithine binding protein (24a). Thus, the similarity in the M3–M4 loop of NR1 with PotD may reflect structural features that are common to NR1 and to a number of bacterial periplasmic binding proteins (5, 14). Alternatively, the amino acid similarities in NR1, PotD, and ESAT may reflect the existence of core regions or residues in these diverse proteins that form similar tertiary structures that are important for binding of polyamines. Residue D669 may contribute to a polyamine binding site or be critical for coupling of polyamine binding to channel gating of NMDA receptors.

D669 in NR1A is located upstream of residues in the M3–M4 loop that are important for glycine binding (5) and upstream of two cysteine residues that control sensitivity to redox reagents, pH, and spermine (6) (see Fig. 1B). The region containing D669 corresponds to a region in GluR 3 and GluR 6 subunits of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors that controls agonist binding properties and may form part of the glutamate binding site (14). Mutations at D669 in NR1A had only small effects on the potency of glutamate and glycine and, presumably, did not disrupt the agonist binding sites.

Three mutations were tested at D669. Spermine stimulation was abolished by a mutation that removes the negative charge (D669N), by a mutation that retains the negative charge but alters chain length (D669E), and by a mutation that removes the side chain (and the negative charge) at this position (D669A). These effects are different than mutations at an acidic residue (E342) in the amino-terminal domain of NR1A that controls spermine sensitivity. Mutations E342Q and E342A but not E342D abolished spermine stimulation (10). This may suggest that E342 and D669 have different roles in modulation of NMDA receptors by spermine or that differences in chain length that retain an acidic residue (E342D versus D669E) are better tolerated at E342 than at D669. Mutations at E342 do not affect voltage-dependent

block by spermine (10), whereas some mutations at D669 reduce voltage-dependent block by spermine. Mutations at E342 have only modest effects on pH sensitivity (10), whereas mutations at D669 greatly reduce pH sensitivity of NR1A/NR2B receptors. Thus, mutations at D669 are functionally different from those at E342. If E342 and D669 form part of a common spermine binding pocket, then mutations at D669 may disrupt other binding sites or gating properties (e.g., proton sensitivity, voltage-dependent block) that are close to or influenced by D669 but not close to E342.

Mutations at D669 that abolished the glycine-independent form of stimulation (Fig. 1A, *1*) did not affect glycine-dependent stimulation by spermine (Fig. 1A, *2*). This is consistent with results of previous studies in which the two forms of stimulation seemed to be mechanistically distinct and/or to involve separate spermine binding sites (16, 43, 44). It is notable that glycine-dependent stimulation by spermine is not affected by mutations at D669 or E342 (10) or by the insert encoded by exon 5 (15), suggesting that glycine-dependent stimulation may involve a region of the NMDA receptor entirely different from that responsible for glycine-independent stimulation.

A surprising finding from the present study was that some mutations at D669 reduced voltage-dependent block by spermine. Until now, this form of inhibition was thought to involve a separate site and mechanism of action compared with stimulation by spermine (16, 44–46). Thus, voltage-dependent inhibition and voltage-independent stimulation by polyamines may share some structural determinants on NMDA receptors, but this cannot be determined unequivocally because spermine stimulation is pH dependent and mutations at D669 also alter pH sensitivity of NMDA receptors. In the current study, mutations that neutralized the negative charge at D669 (D669N or D669A) reduced inhibition by spermine. This effect was not seen with a mutation that retains a negative charge (D669E), suggesting that the presence of a negative charge at D669 is important for block by spermine. The mechanisms responsible for voltage-dependent block by spermine at native NMDA receptors have been suggested to involve both a fast-channel block, like that seen with Mg^{2+}, and screening of surface charge (44–48). The observation that D669 mutants did not alter voltage-dependent block by Mg^{2+} is consistent with the proposal that block by spermine involves a site on the receptor that is distinct from the Mg^{2+} binding site, possibly outside the ion channel pore, and may involve a charge-screening effect rather than fast open-channel block (46, 48). Alternatively, block by the tetraamine spermine may involve more interaction points, including D669, than block by Mg^{2+}. D669 is located in a loop outside the putative membrane-spanning domains of NR1. Thus, residue D669 and, presumably, other regions of the M3–M4 loop may contribute to the mouth of the ion channel pore, and screening of D669 by spermine could in part account for the inhibitory effect of spermine. Mutations at D668 in NR2B also reduced voltage-dependent block by spermine. Thus, NR2B(D668) may have a role similar to that of NR1A(D669) and may also contribute to the mouth of the ion channel pore or at least to the site responsible for voltage-dependent block by spermine.

Inhibition by ifenprodil was reduced by mutations at D669 in NR1A. Ifenprodil has been suggested to act at a stimulatory polyamine site (39), but its interaction does not seem to be strictly competitive (17, 33, 40, 41). The observation that D669 mutations, like some mutations at E342 (10), alter sensitivity to ifenprodil suggests that this residue may be part of a common "effector" linking spermine and ifenprodil to channel gating or that D669 may be close to or form part of an ifenprodil binding site. Because ifenprodil is neuroprotective and does not share the motor and cognitive side effects associated with traditional NMDA receptor antagonists (49), an understanding of the effects of this antagonist at a molecular level may be useful for the rational design of neuroprotective agents.

Mutations at D669 in NR1A had a pronounced effect on pH sensitivity of NR1A/NR2B receptors and, to a lesser extent, of NR1A/NR2A receptors. The change in pH sensitivity may account, at least in part, for the change in sensitivity to spermine stimulation. The results are consistent with the proposal (8) that spermine can function to relieve tonic proton inhibition at NMDA receptors (see Fig. 1A). Residue D669 could form part of the proton sensor on NR1A, although this does not exclude a possible direct contribution of D669 to a spermine binding site on NR1. The inclusion of exon 5 in the NR1 subunit reduces sensitivity to protons (8). This effect can be accounted for by a single positively charged amino acid (K211 in NR1B) in the 21-amino acid insert encoded by exon 5 (8). It has been suggested that this insert may function as a "spermine-like" constitutive modulator of the NMDA receptor and may directly shield or alter access to the proton sensor (8). In the current study, we found that mutations at NR1B(D690) (equivalent to D669 in NR1A) did not prevent the change in pH sensitivity seen with exon 5 and, similarly, that the inclusion of exon 5 did not prevent the shift in pH sensitivity caused by mutations at D669. Mutations at D669 seem to influence receptor properties independent of the effects of exon 5. Thus, if D669 forms part of the proton sensor on NR1, it presumably is not shielded by the exon 5 insert. Alternatively, D669 may not contribute directly to the proton sensor but may be important for coupling of pH (and spermine and ifenprodil) to channel gating, as has been reported for cysteine residues in the M3–M4 loop (6).

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