The NR2B-specific Interactions of Polyamines and Protons with the N-Methyl-D-aspartate Receptor*

(Received for publication, January 28, 1997, and in revised form, July 24, 1997)

Michael J. Gallagher‡, Hui Huang‡, Elfrida R. Grant‡, and David R. Lynch§¶

From the Departments of §Neurology, ¶Pharmacology, and ¶Pediatrics, University of Pennsylvania School of Medicine, Children's Seashore House, Philadelphia, Pennsylvania 19104

Many compounds exhibit NR2B-specific modulation of the N-methyl-D-aspartate receptor, although their mechanism(s) of action are largely unknown. Using chimeric NR2A/NR2B subunits, we have located a region of NR2B (amino acids 138–238) which regulated glycine-independent polyamine stimulation. Mutation of glutamate 201 in this region affected stimulation by polyamines in the order E201D < E201A < E201N < E201R. The relief of proton inhibition of the N-methyl-D-aspartate-induced currents mediated by these mutant receptors correlated with the reduction in glycine-independent polyamine stimulation. Electrophysiological evidence with a triple mutant of NR2A further supports the hypothesis that polyamine stimulation may be linked to the relief of tonic inhibition by protons and demonstrates the crucial role of amino acids 200 and 201 in polyamine stimulation. Polyamines and protons, therefore, share common NR2B determinants.

The N-methyl-D-aspartate (NMDA)1 receptor is a multimeric ligand-gated ion channel that plays a key role in glutamatergic transmission in the central nervous system (1–4). The activated NMDA receptor increases the neuronal membrane permeability to Ca2+ and has been implicated in epilepsy (5), Huntington's disease (6), and the delayed neuronal death following cerebral ischemia (7). NMDA receptor activation requires both glutamate and glycine and is modulated by many channel-blocking agents and noncompetitive inhibitors (8). Dizocilpine (MK-801) (9) and phencyclidine block the channel in the open conformation (10) and have been vital for the pharmacologic characterization of these receptors, although the psychomimetic effects conferred by these agents prohibit their clinical use (4). Agents that modulate NMDA receptors at other sites, including the noncompetitive antagonist ifenprodil (11), the endogenous polyamine spermidine (12), and the ε-site ligand haloperidol (13), may provide better models for novel therapeutic design because they do not produce psychomimetic effects.

The differential assembly of NMDA receptor subunits leads to receptors with distinct pharmacologic properties. The receptor is proposed to exist as multimeric channels composed of five subunits of two types (NR1 and NR2) (14). There are eight forms of NR1 (NR1A–H), derived by alternate splicing (1, 15), and four known NR2 subunits (NR2A–NR2D) (14, 16, 17). The cDNAs for murine NR1 subunits (ζ) and NR2 subunits (ε1–ε4) (18–20) share greater than 99% amino acid homology with their rat counterparts, explaining the observation that coexpression of rat NR1 with murine NR2 subunits yields receptors with properties identical to those of channels made from all rat subunits (21–23). Heterologous expression of NR1 and NR2 subunits in oocytes and cell culture systems has shown that NR1A2A receptors differ pharmacologically from NR1A2B receptors (16–19, 24–27). Modulators that exhibit NR2B-specific interactions include ifenprodil, polyamines, and haloperidol (22, 27, 28).

Polyamines are endogenous compounds in the central nervous system, although their function in the brain is largely unknown (29). These compounds modulate the NMDA receptor by at least four distinct mechanisms, possibly occurring at distinct receptor sites (27, 30, 31). In subsaturating concentrations of the coagonist glycine, polyamines enhance the binding of glycine to NMDA receptors (glycine-dependent stimulation) (32, 33). In saturating glycine concentrations, polyamines stimulate receptor opening (glycine-independent stimulation) at concentrations below 200 μM (28, 34), whereas at higher concentrations, polyamines block NMDA receptors in a voltage-dependent manner (35, 36) and decrease the affinity of the receptor for glutamate (31). Glycine-independent stimulation depends on subunit composition, with receptors containing NR1 splice variants lacking the 5′-insert (such as NR1A) exhibiting stimulation, whereas receptors containing NR1 subunits with the 5′-insert (NR1B) do not exhibit glycine-independent stimulation (21, 30). In addition, glycine-independent stimulation is seen only in receptors containing NR2B and is not exhibited by NR2A-, NR2C-, or NR2D-containing receptors (27, 37). Several specific residues of NR1 affect glycine-independent stimulation. A residue between the M3 and M4 putative transmembrane regions (Asp669) (37) and the NH2-terminal residues Glu-342 and Glu-339 of NR1A (38) have all been implicated in the control of polyamine sensitivity. Mutation of these residues causes a loss or reduction in glycine-independent polyamine stimulation. Specific residues of the NR2B subunit which are involved in glycine-independent polyamine stimulation have not been reported, although we have previously localized the determinants of NR2B-specific polyamine stimulation to the NH2-terminal third of this subunit (21).

The current mediated by NMDA receptors is sensitive to protons in a subunit-specific manner (39). Like polyamines, proton sensitivity is altered by the presence or absence of the 5′-insert. NR1B (5′-insert present)-containing receptors show an EC50 for proton inhibition of pH 6.3, whereas NR1A (lacking insert)-containing receptors have a greater proton sensitivity (EC50 = pH of 7.3) (39). Proton inhibition depends on NR2.
subunit expression, although coexpression of either NR2A or NR2B subunits with NR1A yields receptors with a half-maximal pH inhibition of 7.3; NR2C-containing receptors are insensitive to protons (half-maximal pH = 6.8) (39). The NR1A mutations (Asp-669, Glu-342, and Glu-339), implicated in glycinestimulated polyamine polyanion stimulation (37–39), also affect proton sensitivity, suggesting that these modulatory effects may be linked.

In the present study we have probed the NR2B-specific interaction of the polyamine spermidine and protons with the NMDA receptor to understand further the allosteric modulation of these agents at the molecular level. Chimeric ε2/ε1 subunits were used to localize the NR2B-specific determinants of glycine-independent polyamine stimulation to the NH2-terminal region of NR2B. Mutation of a glutamate residue (Glu-201(ε2)) in this region altered glycine-independent polyamine stimulation. In addition, replacing the three amino acids of ε1 (MQN) with the corresponding ε2 residues (LLE) formed a subunit that partially conferred glycine-independent polyamine stimulation. This mutant showed an increase in proton sensitivity compared with wild type NR1A/ε1 receptors, further suggesting that glycine-independent polyamine stimulation may be linked to proton inhibition. Understanding the mechanism(s) of modulation of the NMDA receptor at the molecular level will provide vital information for the design of agents with higher therapeutic potential for the treatment of ischemia or other neurological diseases.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and Taq DNA polymerase were purchased from either Life Technologies, Inc. or New England Biolabs. Deoxynucleotide triphosphates used in PCR applications were bought from either New England Biolabs (dNTPs) or Boehringer Mannheim (dNTPs). Fetal bovine serum was a product of Life Technologies, Inc. Sodium azide, streptomycin, and trypsin were all products of Life Technologies, Inc. Other reagents were used from standard commercial sources.

Chimeric NR2 Subunit Construction—The constructions of the ε1/ε2 chimeras CH5 and CH6 were described previously (21). The constructions of the chimeric NR2 subunit MK-801 (hydropathy-matched ε1, ε2, and ε1/ε2 chimeras) were described previously (21, 28). Cells were transfected with a 1:1 ratio of NR1A and either chimeric or mutant NR2 subunits by the calcium precipitation method (40, 41). Cells were harvested (Brandel Harvester) onto polyethyleneimine-coated glass slides, fixed in 4% formaldehyde, and subsequently counted with a Beckman (model 5500B) γ-counter.

Electrophysiology—Xenopus oocytes were prepared for injection as described previously (42) and were maintained in ND-96 (96 mM NaCl, 2 mM KCl, 1.8 mM BaCl2, and 10 mM Heps, pH 7.5) supplemented with 0.55 μl/liter pyruvate and 50 mg/ml gentamicin. NR1A, ε2, and E201N cRNA were synthesized in vitro after digestion with either XhoI (NR1) or EcoRI (NR2 types). Recordings were performed 2–4 days after coinjection of 1 ng of NR1A and 5 ng of ε2, ε2, or E201 mutant cRNA. Oocytes were perfused continuously with ND-96 solution (10 ml/min, 22 °C) during two electrode voltage clamp experiments. Oocytes were perfused with ND-96 supplemented with the desired concentration of drug for 1 min before inducing current with 100 μM cold MK-801. Membranes were incubated in saturating glycine (100 μM) and glutamate (10 μM), 300 μM 125I-MK-801, and the desired concentration of either ifenprodil or spermidine for 3 h to allow the ligand to reach equilibrium. The experiments assessing glycine effects, membranes were washed without glycine twice before incubations were commenced with 20 mM Hepes, pH 7.5, 100 μM glutamate, 100 μM spermidine, and the designated concentration of glycine. Membranes were harvested (Brandel Harvester) on to polyethyleneimine-coated glass slides, fixed in 4% formaldehyde, and subsequently counted with a Beckman (model 5500B) γ-counter.

RESULTS

Because the molecular basis for the NR2B-specific effects of polyamines is currently unknown, chimeric NR2A/NR2B subunits could be valuable tools for determining the subunit-specific interactions of these modulators. We designed six chimeric ε1(NR2A)/ε2(NR2B) subunits that, when expressed with NR1A, form intact receptors that allowed the localization of the effects of polyamines to a specific region of the NH2 terminus of NR2B.

Glycine-independent Polyamine Stimulation Localizes to the NH2 Terminus of NR2B—We have previously localized the determinants of glycine-independent polyamine stimulation to the NH2-terminal region between amino acids 198 and 464, using chimeras CH5, CH25, CH5/CH23, and CH23. Figure 1 shows the polyamine stimulation profiles for chimeras CH5 and CH6 (top panel), demonstrating that the peak stimulation of 1A/CH5 receptors is approximately 80% of wild type stimulation, whereas 1A/CH6 showed only 63% wild type levels of stimulation. These and our previous studies suggested that although components downstream from amino acid 464 (perhaps in the
NR2B-specific Modulation of NMDA Receptors

Stimulation—Because the data from the chimera experiments implicated the region between amino acids 198 and 238 for polyamine stimulation, the protein sequences of NR2A were compared with the known NR2B sequences to search for residues in this region which were unique to NR2B. This search revealed a conserved negatively charged amino acid (Glu-201 in ε2 and NR2B) which was an asparagine (Asn-202) in both the NR2A and ε2 sequences (Fig. 2A).

Site-directed mutagenesis demonstrated that glycine-independent polyamine stimulation preferred a negatively charged residue at amino acid 201 (Fig. 2B). Conservation of negative charge at position 201 had a small effect on glycine-independent polyamine stimulation. 1A/E201D receptors exhibit polyamine stimulation levels that are 88% of 1A/ε2 receptors, whereas 1A/E201A receptors only exhibit 63% the level of wild type stimulation (Fig. 2B). Glycine-independent polyamine stimulation was eliminated with substitution of a positively charged amino acid (E201R). Thus, the loss of a negatively charged residue at amino acid 201 reduced polyamine stimulation, whereas substitution of a positively charged residue at this position created receptors that were inhibited by polyamines.

To ensure that the effects noted were truly independent of the glycine concentration, the effects of spermidine were assessed in the presence of multiple glycine concentrations. The amount of polyamine stimulation of wild type ε2-containing receptors was unchanged by varying the glycine concentration (Fig. 3, A and B). Similarly, the glycine dependence curves resulting from radioligand binding assays are identical for 1A/ε2, 1A/ε3, and 1A/E201R receptors. This further confirms that alterations in the effects of glycine cannot explain the differences seen in our study.

Further Characterization of the Effects of E201 Mutants on Polyamine Stimulation—Electrophysiological measurements of the effects of spermidine on the NMDA-induced currents in oocytes injected with NR1 and the E201 mutants were used to characterize further the voltage dependence of the observed changes in polyamine stimulation exhibited by these mutant receptors. It has been described previously that at depolarized potentials (more positive than −70 mV) polyamine stimulation is increased, whereas at more negative potentials voltage-dependent block predominates over potentiation by polyamines (27). The top panel of Fig. 4 shows the polyamine effects on NMDA-induced currents at a holding potential of −40 mV. At this holding potential there is a stimulation of both wild type ε2 receptors, whereas substitution of a positively charged residue at amino acid 201 reduced polyamine stimulation, whereas substitution of a positively charged residue at this position created receptors that were inhibited by polyamines.

Polyamine stimulation increases at more acidic pH conditions (37). We therefore studied the polyamine stimulation of both wild type and mutant receptors at a pH of 6.8 (holding potential = −70 mV) (Fig. 4, lower panel). Both 1A/ε2 and 1A/E201D receptors showed a 2–3-fold increase in glycine-independent polyamine stimulation, whereas a slight increase was seen in E201N- and E201A-containing receptors. Both 1A/E201R and 1A/ε3 receptors were unaffected by the shift in pH. This provides additional evidence that glycine-independent-

Fig. 1. Determinants of glycine-independent polyamine stimulation map to the region near amino acid 198. The chimeras CH5, CH6, CH9, and CH10 were used to localize the determinants of polyamine stimulation. The two panels show the dose-response curves for spermidine modulation of iodo-MK-801 binding. The curves for 1A/CH5 (Δ) and 1A/CH6 (○) receptors (upper panel) are shown with the curves for 1A/ε2 receptors (––) and 1A/ε3 receptors (— —). The bottom panel shows the curves for 1A/CH9 (▲) and 1A/CH10 (○) receptors along with the curves for the wild type combinations 1A/ε2 (––) and 1A/ε3 (— —). 1A/CH5, 1A/CH6, and 1A/CH10 all exhibited stimulation, whereas CH9, which expresses the ε2 sequence between amino acids 198 and 238, showed wild type levels of stimulation, suggesting that the determinants of stimulation localize to this region. The curves shown have been drawn by hand based on the results from 4–10 experiments. In the insets, ε2 is white, and ε3 is black.

channel region) may be important for polyamine stimulation, the region between amino acids 138 and 356 must be important for polyamine effects. Results from two additional chimeras make the localization of the stimulatory region less clear (Fig. 1, bottom panel). 1A/CH9 receptors (containing amino acids 138–238 of ε2) lacked polyamine stimulation and mimicked closely the profile of 1A/ε2 receptors. Surprisingly, the polyamine stimulation of 125I-MK-801 binding mediated by 1A/CH10 receptors (Fig. 1, bottom), which possess even less NR2B sequence (amino acids 198–238), was identical to 1A/ε2 receptors. These results suggest that multiple regions of NR2B are required to form an intact polyamine stimulatory site and that tertiary and quaternary structural components must be involved in spermidine stimulation, although the region between amino acids 198 and 238 is likely to contain important determinants for NR2B-specific polyamine stimulation.

Glu-201 Is Implicated in Glycine-independent Polyamine
experiments showed a slight difference in the IC\textsubscript{50} values for
the binding of \textsuperscript{125}I-MK-801 for the Glu-201 mutant and wild type receptors is shown.

Because glycine-independent polyamine stimulation and proton inhibition may result from the relief of the tonic inhibition of NMDA receptor by protons, the proton inhibition of the Glu-201 mutant receptors was investigated (Fig. 5, panel A). Polyamine stimulation was similar at all glycine concentrations. The effect of glycine on \textsuperscript{125}I-MK-801 binding was also examined for 1A/\textepsilon_4 (○), 1A/\textepsilon_1 (○), and 1A/E201R (○) receptors (panel B). Although glycine had a slight stimulatory effect on 1A/\textepsilon_1 receptors, this was only seen at concentrations around 1 mM or less. At the concentration of glycine used in all other binding assays (100 \textmu M) all mutant and wild type receptors are therefore maximally stimulated by glycine.

Mutation of Glu-201 Alters the pH Dependence of the NMDA Receptor—Because glycine-independent polyamine stimulation may result from the relief of the tonic inhibition of the NMDA receptor by protons (39), the proton inhibition of the Glu-201 mutant receptors was investigated (Fig. 5, A and B). Previous measurements of the pH dependence of heterologous combinations of NR1A with either NR2A or NR2B show a trend toward subunit dependence for proton inhibition (39), whereas our experiments showed a slight difference in the IC\textsubscript{50} values for NR1A/\textepsilon_4 (\textsuperscript{+H} = 100 ± 13 nM, pH = 7.0) and NR1A/\textepsilon_1 (\textsuperscript{+H} = 50 ± 4 nM, pH = 7.3) receptors. Like the results for spermidine, receptors with a negatively charged residue at position 201 (E201D) exhibited no change in the pH dependence (IC\textsubscript{50} for E201D = 50 ± 6 nM, pH = 7.3), whereas the mutation E201N (the residue found in \textepsilon_0) demonstrated a pH dependence identical to that of 1A/\textepsilon_1 receptors. The pH dependence of NR1A/E201A receptors was shifted to the left (IC\textsubscript{50} = 160 ± 20 nM, pH = 6.8), whereas the greatest change was again seen with the E201R substitution (IC\textsubscript{50} = 300 ± 40 nM, pH = 6.5). These results suggest that polyamine stimulation may share a mechanism with proton inhibition and support the hypothesis that glycine-independent polyamine stimulation occurs by relief of the tonic inhibition of NMDA receptor by protons.

Mutation of Glu-200 Has No Effect on Either Polyamine Stimulation or Proton Inhibition—A conserved glutamate residue in NR2B subunits found adjacent to Glu-201 at position 200 (19) was also mutated to determine the necessity of this residue for NR2B-specific modulation. Truncation of the glutamate side chain by the substitution of an alanine at this position, E200A, had no effect on glycine-independent polyamine stimulation in \textsuperscript{125}I-MK-801 binding assays (Fig. 6A, upper panel), whereas spermidine stimulated NMDA-induced currents 61\% (± 9\%) with this receptor, slightly less than that seen with 1A/\textepsilon_1 receptors (holding potential = −40 mV, pH 7.5, data not shown). In the oocyte expression system, 1A/E200A receptors demonstrated a pH dependence identical to that of wild type 1A/\textepsilon_1 receptors (Fig. 6A, bottom panel). Therefore, the residue Glu-200 is not required for either glycine-independent polyamine stimulation or proton-dependent inhibition.

The Double Mutant E200Q,E201N—The glutamate residues at positions 200 and 201 were replaced with the corresponding residues of \textepsilon_2 (Gln-200, Asn-201) (mutant E200Q,E201N) to determine whether a negatively charged amino acid at position Glu-201 is necessary for 2B-specific polyamine or proton mod-
NR2B-specific Modulation of NMDA Receptors

A Triple Mutation in ε2 Imparts Partial Polyamine Stimulation—To assess further the involvement of Glu-201 in NR2B-specific modulation, we changed the three residues of ε2 (Leu-199, Glu-200, Glu-201) to see if we could create an ε2 subunit that possesses ε2-like properties. When coexpressed with NR1, the resulting triple mutant (M200L,N201E,Q202E) exhibited about 60% polyamine stimulation (125% of base line) (Fig. 7A, top panel) compared with wild type 1A/ε2 receptors, further implicating these residues in NR2B-specific spermidine interaction. The pH dependence of NR1A/M200L,N201E,Q202E receptors (Fig. 7A, bottom panel) was also shifted to the right, being even more sensitive to proton inhibition than 1A/ε2 receptors. ([H+] = 32 mM, pH 7.5). This provides additional evidence that glycine-independent polyamine stimulation is linked to proton inhibition.

To confirm the effects of spermidine and pH on the M200L,N201E,Q202E mutant, I-V curves were performed (Fig. 7B). The NR1A/ε1 receptor demonstrated voltage-dependent block by spermidine with no stimulation at any voltage, whereas NR1A/ε2 was stimulated by spermidine with a superimposed voltage-dependent block being seen as flattening of the curve at more negative holding potentials and at higher spermidine concentrations. These results resemble previously reported curves for these receptors when the effects of spermidine were tested (27). The NR1A/M200L,N201E,Q202E mutant was stimulated by spermidine at all voltages, although not to the same extent as 1A/ε2 combinations. Voltage-dependent block was also noted at higher spermidine concentrations. No voltage-dependent effects of pH were noted on the I-V curve for the M200L,N201E,Q202 mutant (Fig. 7B) or in other subunit combinations (data not shown).

DISCUSSION

In the present study, chimeric ε1/ε2 receptors facilitated the further localization of the NR2B-specific determinants of glycine-independent stimulation on the NH2 terminus of NR2B. We have shown previously that the NR2B-specific determinants of ifenprodil and polyamine interaction localize to the NH2-terminal third of NR2B (21) by using the chimeras 1A/CH8 and 1A/CH25. Our detailed mapping suggests that multiple regions of NR2B may play a role in polyamine stimulation, with full stimulation requiring distinct tertiary structural elements from both the NH2-terminal and other regions of the subunit. In this and our previous study (21), we have localized the determinants of glycine-independent polyamine stimulation to the region around amino acid 198, with the chimera containing a minimal component of NR2B (only 40 amino acids of ε2 (198–238)) exhibiting wild type levels of polyamine stimulation. The region between amino acids 138 and 238 is highly conserved between the NR2A and NR2B protein sequences (14, 16–20). Searching this region for residues that were uniquely conserved in NR2B type receptors but not in NR2A type revealed a single acidic residue, Glu-201 (ε2), which was the nonconserved residue asparagine in NR2A and ε1. Because polyanymes are highly basic compounds, their potential interaction with acidic residues could be postulated.

Mutation of Glu-201 revealed the importance of this residue as an NR2B-specific determinant of polyamine interaction with the NMDA receptor. Substitution of Glu-201 (ε2) with the other negatively charged residue aspartate yielded receptors with glycine-independent polyamine stimulation identical to that of wild type receptors. Truncation of the side chain of Glu-201 by substitution of alanine (E201A) exhibited the mildest reduction in polyamine stimulation (60% reduction), which suggests that there is a steric constraint at this site for efficacious binding of modulators. Substitution with asparagine, the residue found in ε2 and NR2A, yielded receptors virtually identical to NR2A type receptors with respect to polyamine stimulation, whereas substitution to the positively charged arginine abolished glycine-independent polyamine stimulation. The arginine at this position could produce these effects by either a steric hindrance or by exerting an undesirable electrostatic repulsion with a nearby residue in the receptor complex, or it could act by...
repelling the positively charged spermidine molecule from the receptor complex.

The residue Glu-201 may play many possible roles in the NR2B-specific modulation by polyamines. Because polyamine interactions are linked to the binding of the coagonist glycine, mutations at residue Glu-201 could alter the glycine affinity of the receptor. This is unlikely because all of the Glu-201 mutants when coexpressed with NR1A exhibit comparable levels of $^{125}$I-MK-801 binding and peak NMDA-induced current, which require the open channel conformation (43, 44) and thus an intact glycine site. In addition, the residues that affect glycine affinity (Ser-669, Tyr-666, Phe-390, Tyr-392 etc.) are found exclusively on the NR1 subunit, in a distal region of the polypeptide sequence from the homologous region near Glu-201.

**Fig. 5.** Proton dependence of the Glu-201 mutants. The proton dependence of the Glu-201 mutant receptors was dependent on amino acid substitution. Panel A, the representative electrophysiologic traces for 1A/E201A (upper left), 1A/E201D (upper right), 1A/E201N (lower left), and 1A/E201R (lower right) upon the 1-min application of 100 μM NMDA, 100 μM glycine at the desired pH followed by a wash in ND-96 at pH 7.5 (which causes a perfusion artifact as the proton block is removed with glutamate and glycine still bound). This was repeated throughout the pH titration for each receptor concentration. Oocytes were equilibrated at the desired pH before application of NMDA and glycine. Panel B, the pH dependence of NMDA-induced current curves for 1A/e1 (○), 1A/e2 (●), 1A/E201A (△), 1A/E201D (○), 1A/E201N (●), and 1A/E201R (△) are shown. The proton dependence of 1A/E201D was identical to 1A/e2, whereas 1A/E201A and 1A/E201N receptors shared similar proton dependence with 1A/e1. The curve for 1A/E201R showed the most dramatic reduction on proton dependence, being shifted an entire pH unit to the left from 1A/e2 receptors. EC$_{50}$ values were calculated using the following equation: response = (maximum − minimum)/(1 + ([H$^+$]/EC$_{50}$)$^n$) + minimum. The curves shown were drawn by hand based on cumulation of the data and resulted from four to six experiments (for the mean ± S.E., see "Results").
Furthermore, the glycine dependence of the 1A/E201R receptor is identical to the 1A/e2 receptor. It is therefore unlikely that mutations at Glu-201 altered the glycine affinity of our expressed receptors.

Mutations of Glu-201 likely affect the actions of polyamines by a unique mechanism. The direct interaction of the positively charged spermidine with Glu-201 is unlikely because the double mutant E200Q,E201N, lacking a negatively charged amino acid, exhibited wild type levels of glycine-independent polyamine stimulation. It is possible that instead of a “stimulatory” sequence being present on the NR2B subunit, there may be a structural component of the NR2A subunit which interferes with polyamine stimulation. Perhaps the substitution of Glu-201 to arginine creates a structural change that inhibits efficacious polyamine stimulation, much the same way as an inhibitory region of NR2A and permit stimulation. In this alternative, polyamines could bind directly to the NR1 subunit, and this binding is regulated by the NR2 subunits, either directly or through allosteric modulation at the channel pore. Thus, the study of the mechanisms of polyamine stimulation may eventually provide information on the molecular interactions between NR1 and NR2 subunits.

Glycine-independent polyamine stimulation and pH-dependent effects of the Glu-201 mutants closely correlated in our study. Possibly, spermidine is unable to relieve the proton inhibition for receptors such as 1A/E201R because the proton inhibition of this receptor has already been reduced by the mutation. The results of the E200Q,E201N mutant also correlated glycine-independent polyamine stimulation with proton inhibition, both exhibiting wild type levels. The region near Glu-201 is likely to be very near to, or form an integral part of, the proton sensor of the NMDA receptor. The proton sensor has been proposed to be on the NR1 subunit because homomeric receptors exhibit proton sensitivity and because experiments with the splice variants of NR1 which contain the 5'-insert (such as NR1B) have shown that this insert relieves the tonic inhibition by the receptor and glycine-independent polyamine

**FIG. 6.** Mutants E200A and E200Q,E201N show the role of the residue at position 200. Panel A, the mutation of Glu-200 to alanine (E200A) had no effect on glycine-independent polyamine stimulation, being indistinguishable from e2-containing receptors in the MK-801 binding assay (upper panel). There was also no change in pH dependence of 1A/E200A receptors (lower panel), suggesting that Glu-200 is not an important residue for either polyamine stimulation or proton dependence. In both panels the mutant receptor 1A/E200A (•) is shown along with the curves for 1Ae1 (○) and 1Ae2 (●) receptors. Data shown are the result of four or five repetitions. Panel B, the double mutant E200Q,E201N, when expressed with 1A, yielded receptors that exhibited no change in either polyamine or proton modulatory effects. Both glycine-independent polyamine stimulation, as measured by the 125I-MK-801 displacement assay (top panel), and the proton dependence (bottom panel) of this mutant were identical to 1Ae2 receptors. The mutant receptor 1A/E200Q,E201N (●) is shown along with the curves for 1Ae1 (○) and 1Ae2 (●) receptors. Data shown were obtained and analyzed as described in the legends to Figs. 1 and 4.
stimulation (39). Another mutation in the proposed extracellular segment between the putative membrane-spanning regions M2 and M3 in NR1 also affects both pH dependence and polyamine stimulation in a way analogous to that of the 5′-insert (37). If one compares the homologous regions of NR1 and NR2 NH2 termini, the region surrounding Glu-201 is very homolo-

**Fig. 7.** The triple mutant of e1 (M200L,N201E,Q202E) affects spermidine and proton sensitivity. Panel A, the triple mutant M200L,N201E,Q202E (e1) exhibited glycine-independent polyamine stimulation in our MK-801 binding assay (top panel) and in our electrophysiologic assay (data not shown). The proton dependence of 1A/M200L,N201E,Q202E receptors (bottom panel) was (determined as described in the legend to Fig. 4) increased in this mutation, demonstrating an additional correlation between spermidine potentiation and the relief of proton inhibition. The spermidine and the pH curves for 1A/M200L,N201E,Q202E (A) are shown along with the curves for 1Ae1 (——) and 1Ae2 (— — —) receptors (n = 4). Panel B, I-V curves were performed for the NR1/e2 (upper left panel), NR1/e1 (upper right panel), and NR1/M200L,N201E, Q202E (lower right panel) receptors in the presence (●) or absence (□) of 1 mM spermidine. Spermidine stimulated NR1/e2 and NR1/M200L,N201E,Q202E receptors but not 1Ae1, 1Ae2, and 1Ae1 receptors. All receptors exhibited voltage-dependent block at hyperpolarizing potentials. Data are shown as means from three to five curves with data normalized to the current value at −70 mV for each oocyte. The I-V curves for 1A/M200L,N201E,Q202E at pH values 8.5 (●), 7.5 (○), and 6.0 (□) are also shown (lower right panel). These results demonstrate the increased sensitivity of the 1A/M200L,N201E,Q202E mutant to proton inhibition. Data shown are the mean of three separate curves for each pH and are displayed in nA.
...lead to a better understanding of the events that polyamines with NMDA receptors at a molecular level may cause. The mutation exhibited both glycine-independent polyamine stimulation and an enhanced sensitivity to protons. This adds further the role of the region around Glu-201 in both proton sensitivity and polyamine interaction.

Acknowledgments—We give special thanks to Dr. Brian Bacekai and Dr. Michael Robinson for help in reviewing and revising this manuscript. We also give special thanks to Dr. Karen Wilcox for helpful discussions on the results of our electrophysiological experiments.

REFERENCES

1. Nakanichi, S. (1992) Science 258, 597–603
2. McBay, C. J., and Mayer, M. L. (1994) Physiol. Rev. 74, 723–760
3. Lynch, D. R., Gallagher, M. J., Lenz, S. J., Anegawa, N. J., and Grant, E. R. (1996) Pharmacology of Recombinant NMDA Receptors: The Ionotropic Glutamate Receptors (Monaghan, D., and Wenthold, R., eds) pp. 325–347, Humana Press, Totowa, NJ
4. Lipton, S. A. (1993) Trends Neurosci. 16, 527–532
5. Parsons, G. C., Quack, G., Bressink, I., Baran, L., Przegalinski, E., Kostowski, W., Krasicki, P., Hartmann, S., and Danysz, W. (1995) Neuropharmacology 34, 1239–1258
6. Reynolds, G. P., Pearson, S. J., and Hutson, P. H. (1994) J. Neurosci. 14, 46–49
7. Choi, D. W., and Rothman, S. M. (1996) Annu. Rev. Neurosci. 19, 171–182
8. Monaghan, D. T., Bridges, R. J., and Cotman, C. W. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 365–402
9. Haeutter, J. E., and Bean, B. P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1307–1311
10. Fagg, G. E. (1987) Neurosci. Lett. 76, 221–227
11. Carter, B., Benavides, J., Legendre, P., Vincent, J. D., Noel, F., Thuret, F., Legoix, K. G., Archilla, S., Zivkovic, B., Mackenzie, E. T., Rousseau, J., Scatton, B., and Wick, A. (1988) J. Pharmacol. Exp. Ther. 247, 1222–1232
12. Ransohoff, R. W., and Stee, N. L. (1988) J. Neurochem. 51, 830–836
13. Fletcher, E. J., and MacDonald, J. F. (1993) Eur. J. Pharmacol. 235, 291–295
14. Moriyoashi, K., Masu, M., Ishii, T., Shimogori, R., Mizuno, N., and Nakashiba, S. (1991) Nature 354, 31–37
15. Durand, G. M., Gregori, P., Zheng, X., Bennett, M. V., Uhl, G. R., and Zukin, R. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9359–9363
16. Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lameli, H., Burnashev, N., Sakmann, B., and Seeburg, P. H. (1992) Science 256, 1217–1221
17. Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shimogori, R., Mizuno, N., Masu, M., and Nakashiba, S. (1993) J. Biol. Chem. 268, 2836–2843
18. Meguro, H., Morii, H., Araki, K., Kushiyama, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., and Mishina, M. (1992) Nature 357, 70–74
19. Kutsuwada, T., Kashiwabuchi, N., Morii, H., Sakimura, K., Kushiyama, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M., and Mishina, M. (1992) Nature 358, 36–41
20. Ikeda, K., Nagasawa, M., Morii, H., Araki, K., Sakimura, K., Watanabe, M., Inoue, Y., and Mishina, M. (1993) FEBS Lett. 313, 34–38
21. Gallagher, M. J., Huang, H., Pritchett, D. B., and Lynch, D. R. (1996) J. Biol. Chem. 271, 9603–9611
22. Lynch, D. R., and Gallagher, M. J. (1996) J. Pharmacol. Exp. Ther. 279, 154–161
23. Chazot, P. L., Coleman, S. K., Cik, M., and Stephenson, F. A. (1994) J. Biol. Chem. 269, 24403–24409
24. Brose, N., Gasic, G. P., Vetter, D. E., Sullivan, J. M., and Heinemann, S. F. (1995) J. Biol. Chem. 270, 23963–23971
25. Sakurai, S. Y., Penney, J. B., and Young, A. B. (1993) J. Neurochem. 60, 1344–1353
26. Williams, K. (1993) Mol. Pharmacol. 44, 851–859
27. Williams, K., Zappia, A. M., Pritchett, D. B., Shen, Y. M., and Molinoff, P. B. (1994) Mol. Pharmacol. 45, 803–809
28. Lynch, D. R., Lawrence, J. J., Lenz, S., Anegawa, N. J., Dichter, M., and Pritchett, D. B. (1995) J. Neurochem. 64, 1462–1468
29. Johnson, T. D. (1996) Trends Pharmacol. Sci. 17, 22–27
30. Durand, G. M., Bennett, M. V., and Zakin, R. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6731–6735
31. Williams, K., Kashikawa, K., Fukuchi, J. I., and Igarashi, K. (1995) Mol. Pharmacol. 48, 1087–1098
32. Sacca, A. I., and Johnson, K. M. (1989) Mol. Pharmacol. 36, 836–839
33. Benveniste, M., and Mayer, M. L. (1993) J. Physiol. 464, 131–163
34. Williams, K., Dawson, V. L., Romano, C., Dichter, M. A., and Molinoff, P. B. (1990) Neuron 5, 199–208
35. Rock, D. M., and Macdonald, R. L. (1992) Mol. Pharmacol. 41, 83–88
36. Rod, R. L., and Macdonald, R. L. (1992) Mol. Pharmacol. 41, 157–164
37. Kashikawa, K., Fukuchi, J., Chao, J., Igarashi, K., and Williams, K. (1996) Mol. Pharmacol. 49, 1131–1141
38. Williams, K. (1995) in Polyamines: Regulation and Molecular Interaction (Casero, R. A., ed) pp. 129–170, R. G. Landes Company, Austin, TX
39. Trayanos, S. F., Hartley, M., and Heinemann, S. F. (1995) Science 268, 873–876
40. Chen, C., and Okuyama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
41. Lynch, D. R., Anegawa, N. J., Verdroomd, T., and Pritchett, D. B. (1994) Mol. Pharmacol. 45, 540–545
42. Zhong, J., Russell, S. L., Pritchett, D. B., Molinoff, P. B., and Williams, K. (1994) Mol. Pharmacol. 45, 846–853
43. Ferrer-Montiel, A. V., Sun, W., and Montal, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8021–8025
44. Koeg, Y., Harting, R., and Sokolovsky, M. (1988) Biochemistry 27, 843–848
45. Kuryatov, A., Laube, B., Liao, H., and Kuhse, J. (1994) Neuron 12, 1291–1300
46. Wafford, K. A., Kathoria, M., Bain, C. J., Marshall, G., Le Bourdelles, B., Kemp, J. A., and Whiting, P. J. (1995) Mol. Pharmacol. 47, 374–380
47. Zheng, X., Zhang, L., Durand, G. M., Bennett, M. V., and Zakin, R. S. (1994) Neuron 12, 811–818
48. Fage, D., Voltoz, C., Scatton, B., and Carter, C. (1992) J. Neurochem. 58, 2170–2175
49. Paschen, W. (1992) Cerebrovasc. Brain Metabol. Rev. 4, 59–88
50. Harman, R. J., and Shaw, G. H. (1981) Br. J. Pharmacol. 73, 165–174
51. Pouletti, P., Neyton, J., and Ascher, P. (1995) Neuron 15, 1109–1120