Alteration of Ca$^{2+}$ Permeability and Sensitivity to Mg$^{2+}$ and Channel Blockers by a Single Amino Acid Substitution in the N-Methyl-D-aspartate Receptor*

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The N-methyl-D-aspartate (NMDA) receptor plays an important role in glutamate-mediated neuronal plasticity and neurotoxicity in the central nervous system. This receptor is composed of a fundamental subunit (NMDAR1) and its potentiating subunits (NMDAR2A–NMDAR2D). The NMDA receptor is distinct from other glutamate receptor channels because of its high Ca$^{2+}$ permeability and inhibition by selective cationic channel blockers such as Mg$^{2+}$, Zn$^{2+}$, and MK-801. In this study, we investigated the structural features that control Ca$^{2+}$ permeation and channel blockade of the NMDA receptor by in vitro mutagenesis and expression in Xenopus oocytes. We constructed a series of mutations with single amino acid substitutions in the second transmembrane segment of NMDAR1 and examined channel properties of the resultant mutants in combined expression with the NMDAR2A subunit. Substitution of the asparagine with either glutamine or arginine altered both the Ca$^{2+}$ permeability and the sensitivity to blockades by Mg$^{2+}$ and MK-801. These mutations also reduced the inhibitory effects of Zn$^{2+}$ and an antidepressant, desipramine. Based on these results, we concluded that an asparagine ring formed in the central part of the channel-forming second transmembrane segments plays a critical role in determining the Ca$^{2+}$ permeability and the inhibition of open channel blockers.

Glutamate, a major excitatory neurotransmitter, plays an important role in neuronal plasticity and neurotoxicity (Monaghan et al., 1989). The diverse functions of glutamate neurotransmission in the central nervous system are mediated by a variety of glutamate receptors that are classified into two major groups termed ionotropic and metabotropic glutamate receptors (Monaghan et al., 1989). The ionotropic receptors can be subdivided into N-methyl-D-aspartate (NMDA)$^1$ receptors and non-NMDA receptors, both of which contain glutamate-gated, cation-specific ion channels (Monaghan et al., 1989). The metabotropic receptors are coupled to intracellular signal transduction through G proteins (Masu et al., 1991; Tanabe et al., 1992).

The NMDA receptor plays a key role in many functions of glutamate transmission in the central nervous system. This receptor is essential for inducing long-term potentiation, a long-lasting change in neuronal responsiveness that is thought to underlie learning and memory (Collingridge and Bliss, 1987). It also plays a critical role in pathophysiological processes such as epilepsy and acute and delayed ischemic neuronal cell death as well as some neurodegenerative diseases (Meldrum and Garthwaite, 1990). The integral channel of the NMDA receptor is highly permeable to Ca$^{2+}$, and the increased intracellular Ca$^{2+}$ is thought to play an essential role in inducing both glutamate-mediated neuronal plasticity and neurotoxicity (Collingridge and Bliss, 1987; Meldrum and Garthwaite, 1990). The NMDA receptor channel also possesses unique features, including the voltage-dependent inhibition by Mg$^{2+}$, Zn$^{2+}$ inhibition, and channel blockade by selective channel blockers such as MK-801 (Monaghan et al., 1989). The voltage-dependent Mg$^{2+}$ blockade of the NMDA receptor channel is postulated to be crucial for changing synaptic efficacy involved in glutamate-mediated neuronal plasticity (Collingridge and Bliss, 1987).

We recently reported the molecular cloning of an NMDA receptor subunit (NMDAR1) that possesses all properties characteristic of the NMDA receptor-channel complex (Moriyoshi et al., 1991). Subsequent molecular studies identified four additional NMDA receptor subunits (NMDAR2A–NMDAR2D) that potentiate NMDAR1 activity by heteromeric formation (Meguro et al., 1992; Monyer et al., 1992; Kutsuwada et al., 1992; Ishii et al., 1993). Both NMDAR1 and NMDAR2 subunits share structural characteristics with the ligand-gated ion channels and contain four putative transmembrane segments (Moriyoshi et al., 1991; Meguro et al., 1992; Monyer et al., 1992; Kutsuwada et al., 1992; Ishii, et al., 1993). Thus, the NMDA receptor subunits can fit into a transmembrane model of the ligand-gated ion channels in which the second transmembrane segments are involved in lining the channel pore (Unwin, 1989; Betz, 1990). In this investigation, we studied the structural features that control Ca$^{2+}$ permeation and channel blockade of the NMDA receptor by site-directed mutagenesis of the putative channel-forming second transmembrane segment of NMDAR1 and by electrophysiological characterization in Xenopus oocytes. We report here that the asparagine that is conserved in the second transmembrane segments of all NMDA receptor subunits plays a key role in Ca$^{2+}$ permeation and channel blockade of the NMDA receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were purchased from the following sources: Muta-Gene® phagemid in vitro mutagenesis kit from Bio-Rad; Sequenase DNA sequencing kit from U. S. Biochemical Corp.; NMDA, glycine, and desipramine from Sigma; (+)-MK-801 from Research Biochemicals; and T7 RNA polymerase from Stratagene.

Site-directed Mutagenesis—Site-directed mutagenesis was per-
The mutagenized base pair of the corresponding fragment of pN60. Nucleotide changes in the region of NMDAR1 was excised from clone pN60 (Moriyoshi et al., 1991) and subcloned into pUC118. The subsequent digestion was conducted according to the method recommended by the vendor. The mutagenized cDNAs were verified by sequence determination.

Expression and Electrophysiological Measurements in Xenopus Oocytes—RNA was transcribed in vitro in the presence of the capping nucleotide using T7 RNA polymerase (Moriyoshi et al., 1991). cDNA templates were prepared from the pN60 clone (Moriyoshi et al., 1991), the mutagenized pN60 clones (see above), and the pNR2A clone for the NMDAR2A subunit (Ishii et al., 1993) and were used after NotI digestion. Xenopus oocytes were isolated from follicles and injected with in vitro synthesized mRNAs (~1 ng) for the wild-type and mutant NMDAR1 subunits in combination with that (~5 ng) for the NMDAR2A subunit. After incubation in a modified Barth's solution at 18 °C for 2–4 days, oocytes were treated with 2 mg/ml collagenase and placed into a modified Barth's solution (95 mM NaCl, 2 mM KCl, 2 mM CaCl2, and 5 mM HEPES, pH adjusted to 7.5 with NaOH) under conventional two-electrode voltage clamp at −80 mV. Ca2+/Ringer’s solution used for the evaluation of Ca2+ permeation was composed of 10 mM CaCl2, 74 mM N-methylglucamine, 5 mM HEPES, pH adjusted to 7.5 with HCl, and 24 mM sucrose to adjust osmotic balance. Current-voltage curves were constructed by ramping voltage slowly (56 mV/s) from −100 to 40 mV during application of 100 μM NMDA and 10 μM glycine in the presence or absence of Mg2+ (100 μM) or Mg–801 (1 μM) in normal Ringer’s or Ca2+/Ringer’s solution. Leakage current in the absence of agonists was subtracted from currents measured in the presence of agonists. Current responses were recorded and analyzed using pCLAMP (Axon Instruments, Inc.). Dose-response curves for the activation by NMDA and the inhibition by Mg2+ or desipramine were determined by measuring the steady-state currents after serial application of various concentrations of these compounds.

Results

Effects of Single Amino Acid Substitutions of Second Transmembrane Segment of NMDAR1 on Channel Properties—To determine the amino acids responsible for Ca2+ permeability and the actions of channel blockers, we systematically substituted amino acids in the vicinity of the second transmembrane segment of NMDAR1 (Fig. 1). Single amino acid substitutions were introduced into charged and polar residues, which are
expected to interact with cationic ions, and into tryptophan 608, which is conserved in all ionotropic glutamate receptors (Fig. 1). Phenylalanine 609 was also replaced with leucine because the leucine residue at the corresponding position of the nicotinic acetylcholine receptor has been shown to be involved in binding a channel blocker and changing desensitization responses (Revah et al., 1991). Each of the mutant NMDAR1 subunits was named by indicating the amino acid letters in the wild type and the mutant preceding and following the number of the altered residue, respectively. We examined the NMDA receptor channel activity of the resultant mutant proteins in Xenopus oocytes injected with the mRNAs synthesized in vitro from the mutant cDNA templates. In this and all subsequent experiments, the properties of the wild-type and mutant NMDAR1 subunits were analyzed in combined expression with the NMDAR2A subunit. The mRNA-injected oocytes were incubated for 2–4 days, and the current responses after application of 100 μM NMDA and 10 μM glycine were measured in Mg2+-free normal Ringer’s solution and in an Na+/K+-free medium supplemented with 10 mM Ca2+ (Ca2+/Ringer’s solution). In Xenopus oocytes, an increase in intracellular Ca2+ is known to induce two components of Ca2+-activated Cl− conductance (Boton et al., 1989). One is a fast component that requires a high concentration of intracellular Ca2+, and the other is a slow component that is activated by a moderate increase in intracellular Ca2+. The two components of Ca2+-activated Cl− conductance were evoked as a result of Ca2+ permeation through the NMDA receptor channel and were superimposed on the currents evoked by Na+, K+, and Ca2+ fluxes of this receptor channel. Wild-type NMDAR1 thus showed a rapid initial spike (fast component) followed by a steady-state current (slow component) (Fig. 3A). Current magnitudes of both components were larger in Ca2+/Ringer’s solution than in normal Ringer’s solution. N616Q, on the other hand, reduced ion current permeation and channel blockade.

**Effects of N616Q and N616R Mutations on Ca2+ Permeability**—We first determined the dose-response curves for NMDA in the wild type and the N616Q and N616R mutants to ascertain that substitutions at Asn-616 had no effect on the affinity for the agonist. The result of this analysis showed virtually identical dose-response patterns between the wild type and the two mutants in response to application of different concentrations of NMDA (Fig. 2), indicating that the substitutions of Asn-616 do not alter the affinity for the agonist.

The NMDA receptor is more efficiently permeable to Ca2+ than to Na+ and K+ (MacDonald and Nowak, 1990). To test the effects of mutations N616Q and N616R on Ca2+ permeability, we examined NMDA-induced currents in normal Ringer’s solution and in an Na+/K+-free medium supplemented with 10 mM Ca2+ (Ca2+/Ringer’s solution). Effects of mutations N616Q and N616R on Ca2+ permeability were evoked as a result of Ca2+ permeation through the NMDA receptor channel and were superimposed on the currents evoked by Na+, K+, and Ca2+ fluxes of this receptor channel. Wild-type NMDAR1 thus showed a rapid initial spike (fast component) followed by a steady-state current (slow component) (Fig. 3A). Current magnitudes of both components were larger in Ca2+/Ringer’s solution than in normal Ringer’s solution. N616Q, on the other hand, reduced ion current permeation in both normal and Ca2+/Ringer’s solutions and showed a disappearance of the fast component of Ca2+-activated Cl− conductance (Fig. 3A), suggesting that this substitution lowered the Ca2+ permeability. Because the NMDA receptor not only allows Ca2+, Na+, and K+ to permeate, but also induces Cl− flux as a result of Ca2+ permeation, it would be complicated to quantify the reduced levels of Ca2+...
permeation in N616Q on the basis of changes in reversal potentials. However, the following predictions could be made. Because the reversal potential of the wild type (−16 mV) is near an oocyte Cl− equilibrium potential (Dascal, 1987) (Fig. 4A), an outward flux of Cl− would greatly contribute to the total inward current in oocytes expressing wild-type NMDAR1. If Ca2+ permeation is lowered, thus resulting in a decrease in Cl− flux, the reversal potential would shift to a more positive potential in Na+−containing normal Ringer’s solution; and when an Na+-free, high Ca2+ solution containing the same concentration of Cl− (Ca2+/Ringer’s solution) is used as a perfusion medium, the reversal potential would shift to a more negative potential as a result of the major contribution of an outward flux of intracellular K+ across the membrane. The observed changes in reversal potentials of N616Q measured in normal Ringer’s solution (−10 mV) and in Ca2+/Ringer’s solution (−23 mV) were very much consistent with this prediction (see Fig. 4, A and B), indicating that this mutation indeed interferes with Ca2+ permeation. The importance of Asn-616 in the Ca2+ permeability was more convincingly evidenced through the analysis of N616R, in which no obvious response to NMDA was observed in Ca2+/Ringer’s solution (Fig. 3A). Thus, we concluded that Asn-616 is essential for governing Ca2+ permeation in the NMDA receptor.

Effects of N616Q and N616R Mutations on Mg2+ Blockade—We next examined the sensitivity to Mg2+ blockade in N616Q and N616R. In contrast to the wild-type receptor (Figs. 3B and 4A), N616Q showed only a slight blockade by 100 μM Mg2+ under voltage clamp at −80 mV (Fig. 3B) and at hyperpolarized potentials under voltage ramp from −100 to 40 mV (Fig. 4B). Furthermore, no Mg2+ blockade was observed in N616R even under highly hyperpolarized conditions (Figs. 3B and 4C). The reduction or loss of the Mg2+ blockade in these two mutations was further confirmed by dose-response analysis of Mg2+ blockade measured under voltage clamp at −80 mV (Fig. 5A). The concentrations for half-maximal response (IC50) of Mg2+ inhibition shifted from 1 μM in the wild type to 500 μM in N616Q. In N616R, no inhibition was observed by increasing concentrations of Mg2+ up to 1 mM. Thus, Asn-616 serves as a site for Mg2+ blockade in the NMDA receptor channel.

Effects of N616Q and N616R Mutations on Other Blockers—The sensitivity to the MK-801 channel blocker was also changed in mutations N616Q and N616R (Fig. 3C). In the wild type, the application of NMDA evoked a rapid initial spike representing a fast component of Ca2+-activated Cl− conductance in the presence of 1 μM MK-801. However, once the NMDA receptor channel opened, the MK-801 open channel blocker immediately inactivated the channel activity, and this inactivation was not easily recovered by washing and subsequent application of NMDA. In contrast, the MK-801 blockade was markedly reduced in the N616Q mutant, and the NMDA response was recovered more rapidly in this mutant than in the wild type. Furthermore, MK-801 was totally ineffective in blocking the NMDA response of N616R (Figs. 3C and 4C). Thus, Asn-616 also contributes as a blocking site of MK-801 in the NMDA receptor channel.

It has been reported that Zn2+ inhibition is achieved by the actions at two different sites of the NMDA receptor, one acting outside the membrane electrical field and the other directly interfering with the passage of ions inside the channel (Christine and Choi, 1990). We found that 1 μM Zn2+ inhibited the NMDA receptor channel activity in a voltage-dependent manner similar to Mg2+ blockade (data not shown). To address whether Asn-616 is shared in the Zn2+ inhibition, we determined dose-response curves for Zn2+ inhibition under voltage clamp at −80 mV. The NMDA response of the wild type was inhibited in a dose-dependent manner by Zn2+ with an IC50 of 10 μM (Fig. 5B). This value shifted to 100 μM in both N616Q and N616R mutants, indicating that Asn-616 also participates in evoking Zn2+ inhibition. In this case, however, N616R showed an inhibitory pattern similar to that of N616Q, and this was in contrast to the complete loss of the Mg2+ blockade in N616R. This finding may suggest that the high concentrations of Zn2+ act at an additional site for the inhibition of the NMDA receptor.

The above observations suggested that the Asn-616 mutants would be useful to characterize sites of the actions of other NMDA receptor antagonists. Various tricyclic antide-
paragine serves as blocking sites for divalent cations (Mg$^2+$, Zn$^2+$) as well as Mg$^2+$ and Zn$^2+$ for 1 min prior to addition of NMDA, glycine, and the respective divalent cation.

pressants such as desipramine were reported to act at a Zn$^2+$-binding site outside the membrane electrical field (Reynolds and Miller, 1988). However, this conclusion was argued against by indicating the action of desipramine at a binding site of open channel blockers (Sernagor et al., 1989). To examine the action of desipramine, we determined dose-response curves for this antagonist in the wild type and the two mutants (Fig. 5C). The inhibitory effect of desipramine was weakened in the two Asn-616 mutants in the order of mutation. Thus, two types of amide-containing residues (asparagine and glutamine) and a positively charged residue at the central portion of the channel pore are critical in controlling the permeability of divalent cations in the glutamate-gated ion channels.

Our observation that a single channel site contributes to both ion selectivity and blocker binding is consistent with previous reports indicating the interaction between Ca$^{2+}$ and Mg$^{2+}$ as well as between Mg$^{2+}$ and the above cationic compounds within the channel pore (Mayer and Westbrook, 1987; Huetter and Bean, 1988; Sernagor et al., 1989; Christine and Choi, 1990). It was initially proposed through electrophysiological studies that the Mg$^{2+}$-binding site is situated at a very deep site near the intracellular surface (Ascher and Nowak, 1988). This binding site was, however, re-examined, and the argument that the Mg$^{2+}$-binding site is located in the middle portion of the channel pore has now also been made (Johnson and Ascher, 1990). It is feasible that the latter proposed site corresponds to the asparagine ring that determines binding to Mg$^{2+}$ and the other channel blockers discussed in this investigation. Recent molecular characterization of heteromeric assemblies of NMDAR1 and NMDAR2, on the other hand, indicated that different combinations of the NMDAR2 subunits with NMDAR1 confer variability in the sensitivity to Mg$^{2+}$ and channel blockers (Monyer et al., 1992; Kutsuwada et al., 1992; Ishii et al., 1993). All NMDAR2 subunits, however, contain an asparagine residue at equivalent positions and also show a high sequence conservation at the channel-forming second transmembrane segments (see Fig. 1). Thus, it seems unlikely that the above asparagine ring is responsible for differentiating the sensitivity to Mg$^{2+}$ blockade between different heteromeric assemblies. Instead, as pointed out by

**FIG. 5.** Dose-response curves of Mg$^{2+}$ (A), Zn$^{2+}$ (B), and desipramine (C) blockades for wild type (WT) and N616Q and N616R mutants. Amplitudes of steady-state currents induced by 100 μM NMDA and 10 μM glycine in the presence of the indicated concentrations of antagonists were determined; the results are expressed as percent control (no antagonist added) and are the mean ± S.E. of three separate experiments. In A and B, oocytes were pre-exposed to normal Ringer’s solution containing the indicated concentrations of Mg$^{2+}$ or Zn$^{2+}$ for 1 min prior to addition of NMDA, glycine, and the respective divalent cation.

**DISCUSSION**

The NMDA receptor possesses structural characteristics similar to those of other ligand-gated ion channels and can be postulated to contain properties of these mutants in combined expression with permeation in the resultant receptors. Furthermore, this asparagine substitution has no effect on the affinity for agonists, it is very unlikely that this single amino acid substitution causes alternation of a normal heteromeric configuration between the NMDAR1 and NMDAR2 subunits. Instead, as pointed out by indicating the action of desipramine at a binding site of open channel blockers (Sernagor et al., 1989). To examine the action of desipramine, we determined dose-response curves for this antagonist in the wild type and the two mutants (Fig. 5C). The inhibitory effect of desipramine was weakened in the two Asn-616 mutants in the order of mutation. Thus, two types of amide-containing residues (asparagine and glutamine) and a positively charged residue at the central portion of the channel pore are critical in controlling the permeability of divalent cations in the glutamate-gated ion channels.

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Ascher and Nowak (1988), a negatively charged surface potential may exist that controls the accumulation of divalent cations at an extracellular vestibule of the NMDA receptor channel. This negative surface potential may differ among different heteromeric assemblies, and permeation and blockade of cationic ions could in turn be determined by the asparagine ring in the middle portion of the channel pore once these ions reach the channel pore.

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REFERENCES