Ethanol Inhibition of \(N\)-Methyl-\(d\)-aspartate Receptors Is Reduced by Site-directed Mutagenesis of a Transmembrane Domain Phenylalanine Residue

Received for publication, March 29, 2001, and in revised form, August 6, 2001
Published, JBC Papers in Press, September 25, 2001, DOI 10.1074/jbc.M102800200

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\(N\)-Methyl-\(d\)-aspartate (NMDA) receptors (NRs) are ionotropic receptors activated by glutamate and the co-agonist glycine. Ethanol inhibits NMDA receptor function, although its site of action is undefined. We hypothesized that ethanol acts at specific amino acids contained within the transmembrane (TM) domains of the receptor. In this study, NR1 and NR2A subunits were altered by mutagenesis and tested for sensitivity to ethanol. Three NR1 mutants (W636A, F817A, and L819A) and one NR2A mutant (F637A) failed to generate functional receptors. Pre-TM1 (I546A, L551A, F554A, and F558A), TM1 (W563A), and TM2 (W611A) NR1 mutations did not affect ethanol sensitivity of heteromeric receptors. In contrast, altering a TM3 phenylalanine to alanine (F639A) reduced the ethanol inhibition of NMDA receptors expressed in oocytes and human embryonic kidney 293 cells. Mutation of the nearby methionine (M641) to alanine did not affect ethanol sensitivity, whereas changing Phe629 to tryptophan slightly enhanced ethanol inhibition. NR1(F639A) did not alter the agonist potency of glutamate but did produce a leftward shift in the glycine concentration response for receptors containing NR2A and NR2B subunits. NR1(F639A) also reduced the potency of the competitive glycine antagonist 5,7-dichlorokynurenic acid and increased the efficacy of the glycine partial agonist 3-amino-1-hydroxy-2-pyrrrolidinone (\(\pm\)-HA-966). These results suggest that ethanol may interact with amino acids contained in the TM3 domain of NMDA subunits that are involved in transducing agonist binding to channel opening.

\(N\)-Methyl-\(d\)-aspartate (NMDA)\(^1\) receptors are calcium-permeable ion channels expressed by neurons and require both glutamate and glycine for activation. Combinations of NMDA receptor 1 (NR1) and NR2 subunits yield receptors with different biophysical and pharmacological properties such as differences in desensitization and sensitivity to agonists and antagonists. NMDA receptors play an important role in neuronal development and are required for some forms of synaptic plasticity such as associative long-term potentiation that may underlie some forms of learning and memory (1). NMDA receptors are also involved in the excitotoxic effects of glutamate that accompany traumatic brain injury and stroke-induced ischemia.

Ethanol inhibits native NMDA receptor function in vitro and in vivo (2–7). Chronic exposure of neurons to ethanol results in up-regulation of NMDA receptor function and enhanced glutamate-mediated excitotoxicity (8–10). NMDA antagonists block the seizures associated with ethanol withdrawal (11, 12), and human alcoholics report ethanol-like subjective effects after administration of ketamine, a noncompetitive anesthetic that inhibits NMDA channel function (13). Despite the wealth of knowledge indicating that the NMDA receptor is an important target for ethanol in the brain, there is no consensus as to how ethanol inhibits receptor function. Ethanol behaves as a non-competitive and voltage-independent antagonist of the receptor, and attempts to correlate its inhibitory actions with any of the known modulatory sites on the receptor have been largely negative (14–16). In single-channel studies, the inhibitory effects of ethanol were best accounted for by decreases in the mean open time and frequency of channel opening, effects consistent with an allosteric reduction in agonist-induced channel gating (17).

Studies with recombinant NMDA receptors have shown that receptors containing NR1/2A or NR1/2B subunits are generally more sensitive to ethanol inhibition than NR1/2C or NR1/2D receptors (16, 18, 19). In addition, ethanol inhibition of NMDA-induced currents in oocytes expressing NR1, NR2A, and NR2C subunits was less than that observed with NR1 and NR2A receptors, suggesting that subunit composition significantly influences overall ethanol sensitivity (19). Recent studies from this laboratory have also shown that ethanol inhibition of NR1/2A receptors expressed in human embryonic kidney 293 (HEK293) cells is reduced by Fyn tyrosine kinase-mediated phosphorylation of the NR2A subunit as well as by conditions that block calcium-dependent inactivation of NR1/2A receptors (20, 21). However, these manipulations only partially reduce ethanol inhibition of receptor function and C-terminal truncated NMDA subunits retain substantial sensitivity to inhibition by ethanol (21–23). Overall, these data suggest that although C-terminal modifications may influence the ethanol sensitivity of the NMDA receptor, it is unlikely that these intracellular domains represent the major site of action for ethanol.

Results from recent mutagenesis studies with alcohol- and anesthetic-sensitive \(\gamma\)-aminobutyric acid A (GABA\(_A\)) and glycine receptors have shown that mutation of a serine residue in the second transmembrane (TM) domain or an alanine residue in the TM3 greatly affected the potentiation of GABA\(_A\) and glycine receptors. NMDA receptors are calcium-permeable ion channels expressed by neurons and require both glutamate and glycine for activation. Combinations of NMDA receptor 1 (NR1) and NR2 subunits yield receptors with different biophysical and pharmacological properties such as differences in desensitization and sensitivity to agonists and antagonists. NMDA receptors play an important role in neuronal development and are required for some forms of synaptic plasticity such as associative long-term potentiation that may underlie some forms of learning and memory (1). NMDA receptors are also involved in the excitotoxic effects of glutamate that accompany traumatic brain injury and stroke-induced ischemia.

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glycine channel function by ethanol and volatile anesthetics (24). The magnitude of this effect was correlated with the molecular volume of the substituted amino acid, with larger amino acids producing inhibition by ethanol and volatile anesthetics and smaller amino acids producing enhanced potentiation. These results suggested that specific amino acids in these subunits may define an alcohol- and anesthetic-sensitive site (25). Although the sequence identity and structural homology between GABA_A and glycine receptors and glutamate receptors is extremely low, we hypothesized that NMDA receptors also possess an ethanol-sensitive site that is defined by specific amino acids contained in one or more of the TM domains of the receptor. We reasoned that these amino acids would be relatively large and conserved between various NMDA subunits and not face the pore of the ion channel.

In this study, a series of amino acids fitting these criteria were altered by site-directed mutagenesis, and the resulting mutant receptors were tested for their ethanol sensitivity. The results demonstrate that substitution of a single phenylalanine residue in the TM3 domain markedly reduces ethanol inhibition of NMDA receptor currents and that this effect is influenced by amino acid volume. A preliminary report of these findings has been presented in abstract form (26).

**EXPERIMENTAL PROCEDURES**

**Molecular Biology and Mutagenesis**—The rat NR1 and NR2 cDNA clones were kindly provided by Dr. S. Nakashima, Dr. P. H. Seeburg, and Dr. D. Lynch. These cDNAs were subcloned into cytomegalovirus-containing vectors (pcDNA3, Invitrogen; or pGFP-N3, CLONTECH) as directed by Dr. D. Lynch. These cDNAs were subcloned into cytomegalovirus-con

| NR1 | 546 |
|-----|-----|
| NR2A | TPRSTLDSAPQLPPFDST |
| NR2B | NWTVSIAAPAQPAS |
| NR2C | NWTVSIAAPAQPAS |
| NR2D | NWTVSIAAPAQPAS |

**TM1**

| NR1 | 562 |
|-----|-----|
| NR2A | LWLLVLGLSVHVVANVLYL |
| NR2B | WWMVFMVLLVSAVVFV |
| NR2C | WWMVFMVLLVSAVVFV |
| NR2D | WWMVFMVLLVSAVVFV |

**TM2**

| NR1 | 631 |
|-----|-----|
| NR2A | ILGMVQAFGMIFIVASYTANL |
| NR2B | IMYSVWAFVAFISTRYANL |
| NR2C | IMYSVWAFVAFISTRYANL |
| NR2D | IMYSVWAFVAFISTRYANL |

**TM3**

| NR1 | 611 |
|-----|-----|
| NR2A | MAGFYVLMAGVAMLSSLTFFIE |
| NR2B | MAGFYVLMAGVAMLSSLTFFIE |
| NR2C | MAGFYVLMAGVAMLSSLTFFIE |
| NR2D | MAGFYVLMAGVAMLSSLTFFIE |

**TM4**

| NR1 | 611 |
|-----|-----|
| NR2A | 813 |
| NR2B | 813 |
| NR2C | 813 |
| NR2D | 813 |

**FIG. 1. Sequence alignment of transmembrane domain regions of NMDA receptor subunits.** NR1 subunit residues shown in boldface were mutated to alanine in the present study and tested for ethanol sensitivity. Asterisks indicate residues in the NR1 subunit that are hypothesized to be pore-facing based on results from cysteine scanning mutagenesis (29, 30).
binding site and to prevent glycine-dependent desensitization. Na2EDTA (0.01 mM) was added to all external solutions to eliminate residual zinc found in experimental solutions. HEK293 cells showing green fluorescent protein fluorescence were selected for recording and were held at -50 mV. Series resistance was routinely compensated by 70–80%. A multibarrel fast perfusion system (SF77A; Warner Instruments, Hamden, CT) with a switching time of ~8 ms was used to perfuse the cells with extracellular control solution. NMDA receptor-mediated currents were activated by switching from the control solution to one containing glutamate (200 μM) and glycine (50 μM). Currents were filtered at 5 kHz, low pass-filtered at 0.2 kHz, digitized using an Instutech analog-to-digital interface, and collected on a Macintosh computer running the Pulse Control voltage clamp software under the Igor Pro graphics platform (Wavemetrics).

**RESULTS**

Fig. 1 shows the sequences of the TM domains of the NR1 and NR2 NMDA receptor subunits. Asterisks indicate residues shown in the NR1 subunit that have been previously assigned as pore-facing by cysteine scanning mutagenesis (29, 30). Residues in the NR1 subunit shown in **boldface** were mutated to alanine in the present study and tested for ethanol sensitivity. Most of these mutant receptor subtypes gave rise to large NMDA-mediated currents when expressed in *Xenopus* oocytes (Table I). However, some of the NR1 mutants (W636A, F817A, I546A, L551A, F554A, or F558A) plus the NR2A subunit were all inhibited to the same extent by 25–100 mM ethanol (Fig. 2A). The range of ethanol sensitivity among these mutants was not different from that determined for wild-type NR1/2A receptors. Similarly, selected mutations made in either the TM1 (W563A) or TM2 (L819A) domain of the NR1 receptor resulted in functional and ethanol-sensitive NMDA receptors that showed normal sensitivity to ethanol (Fig. 3, A and B).

TABLE I
Mean current amplitudes of wild-type and mutant NMDA receptors expressed in *Xenopus* oocytes

| Receptor subtype | Mean current amplitude (nA) | No. tested |
|------------------|---------------------------|-----------|
| RI/2A            | 1258 ± 158                | 75        |
| RI/F546A/2A      | 598 ± 120                 | 12        |
| RI/L551A/2A      | 688 ± 192                 | 14        |
| RI/F554A/2A      | 1012 ± 244                | 8         |
| RI/F558A/2A      | 159 ± 34                  | 11        |
| RI/W563A/2A      | 181 ± 30                  | 9         |
| RI/W631A/2A      | 552 ± 143                 | 6         |
| RI/W636A/2A      | =                        | —         |
| RI/F639A/2A      | 1778 ± 280                | 23        |
| RI/F639W/2A      | 279 ± 54                  | 9         |
| RI/M641A/2A      | 211 ± 36                  | 20        |
| RI/F817A/2A      | =                        | —         |
| RI/L819A/2A      | =                        | —         |
| RI2A(F637A)      | =                        | —         |
| RI(F639A/2A/F637A) | =                     | —         |

* Receptor did reliably produce currents >50 nA.

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Ethanol sensitivity of NMDA receptors carrying mutations in the pre-TM1 domain of the NR1 subunit. Oocytes expressing the NR2A subunit and either wild-type or mutant NR1 subunits were stimulated with 100 μM NMDA and 10 μM glycine in the absence or presence of ethanol. Data represent the mean ± S. E. percent inhibition by ethanol and are from 4–14 (mutants) or 28–30 (wild-type) oocytes for each ethanol concentration.

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Ethanol sensitivity of wild-type NR1/2A, TM1 mutant NR1(W563A)/2A (A), and TM2 mutant NR1(W611A)/2A (B) expressed in oocytes. Oocytes were stimulated with 100 μM NMDA and 10 μM glycine in the absence or presence of ethanol. Data represent the mean ± S. E. percent ethanol inhibition and are from 5–16 oocytes for each ethanol concentration.
In contrast, expression of the TM3 mutant NR1(F639A) with the NR2A subunit yielded receptors that were significantly less sensitive to ethanol than wild type (Fig. 4A). This effect was manifested as a rightward and downward shift in the ethanol dose-response curve and persisted over ethanol concentrations from 10 to 200 mM (Fig. 4B). Concentrations of ethanol >200 mM produced unstable responses in voltage-clamped oocytes and were not tested. Mutation of the nearby methionine (Met641) to alanine did not alter the inhibitory effect of ethanol compared with wild-type receptors (Fig. 4C). In addition, mutation of NR1(F639A) to the larger tryptophan residue (F639W) produced receptors that were slightly more sensitive to ethanol than wild-type receptors (Fig. 4D). To determine whether the effects of the F639A mutation on ethanol sensitivity were NR2 subunit-dependent, NR1(F639A) was co-expressed with either NR2B or NR2C subunits. The ethanol sensitivity of both NR1(F639A)/2B and NR1(F639A)/2C receptors was also significantly less than that determined for their respective wild-type counterparts (Fig. 5A and B). Expression of NR1(F639A) with NR2A, NR2B, or NR2C subunits in HEK293 cells also significantly reduced the inhibitory effects of 100 mM ethanol compared with wild-type receptors (Fig. 5C).

Current-voltage experiments revealed no differences in the reversal potential or slope conductance between NR1(F639A)/2A receptors and wild-type receptors (data not shown). In addition, expression of NR1(F639A) with the NR2A subunit did not significantly alter the ability of the physiological agonist glutamate to activate the receptor (Fig. 6A). However, the F639A substitution in the NR1 subunit shifted the concentration response for glycine to the left of that of the wild-type receptor (Fig. 6B). Calculated EC50 values for the wild-type (NR1/2A) and mutant (NR1(F639A)/2A) receptors were 0.94 μM (Hill slope, 1.58) and 0.38 μM (Hill slope, 1.40), respectively. This effect of the F639A mutation on glycine potency was even more pronounced in receptors co-expressing NR2B subunits (Fig. 6C). The glycine EC50 value for wild-type NR1/2B receptors was 0.18 μM (Hill slope, 2.00). When NR1(F639A) was co-expressed with the NR2B subunit, significant receptor activation was observed even in the absence of added glycine. This effect prevented an accurate calculation of the EC50 value for this subunit combination. The activation of NR1(F639A)/2B receptors in solutions lacking added glycine was blocked by the glycine site antagonist 5,7-dichlorokynurenic acid (5,7-DCK; data not shown). The glycine sensitivity of receptors expressing NR1(F639A) and NR2C subunits (EC50, 0.18 μM; Hill slope, 1.44) was not significantly different from wild-type NR1/2C receptors (EC50, 0.31 μM; Hill slope, 1.96; Fig. 6D).

To investigate possible mechanisms underlying the shift in apparent glycine sensitivity with the mutant receptor, the sensitivity of wild-type and mutant receptors to a competitive...
glycine antagonist and a glycine partial agonist were determined. At a fixed glycine concentration of 10 μM, the competitive antagonist, 5,7-DCK dose-dependently inhibited NMDA-stimulated currents from oocytes expressing wild-type NR1/2A receptors with an IC50 value of 0.68 μM (Fig. 7A). Expression of NR1(F639A)/2A receptors shifted the concentration-response curve for 5,7-DCK to the right and increased the IC50 value to 2.37 μM. The effect of the F639A mutant on glycine efficacy was examined by using (+)-HA-966, a high-affinity, low-efficacy agonist at the glycine site. Oocytes expressing either wild-type NR1/2A or NR1(F639A)/2A receptors were stimulated with NMDA (100 μM) and increasing concentrations of (+)-HA-966 in the absence of any added glycine. The currents obtained in the presence of each concentration of (+)-HA-966 were normalized to the current produced in each oocyte by a maximum concentration of NMDA and glycine. In the absence of any added glycine, NMDA application resulted in currents from both wild-type and NR1(F639A)/2A receptors that were 5–10% of the response obtained in the presence of saturating concentrations of NMDA and glycine (Fig. 7B). Addition of (+)-HA-966 up to 300 μM to NMDA-containing solutions lacking added glycine did not significantly increase the amplitude of currents from wild-type receptors. In contrast, (+)-HA-966 dose-dependently increased the amplitude of NMDA-stimulated currents in oocytes expressing NR1(F639A)/2A subunits, reaching a maximum of ~30% at 300 μM.

**DISCUSSION**

The major goal of this study was to test the hypothesis that the inhibition of NMDA receptor currents by ethanol is mediated via an interaction with one or more amino acids contained within transmembrane domains of the receptor. Because ethanol inhibition of NMDA receptor currents does not resemble that of channel-blocking drugs such as MK801 or ketamine, we initially selected amino acids that were not thought to be pore-facing (29). Subsequent cysteine scanning studies revealed that some of the pre-TM1 residues tested (Phe554 and Phe558) were accessible to sulfhydryl-modifying agents, suggesting that the pre-TM1 domain contains amino acids that contribute to the outer vestibule of the channel (30). The pre-TM1 domain of NR2 subunits also contains amino acids involved in regulating part of the glycine-independent desensitization of the NR2A- and NR2B-containing receptors (31). In the present study, none of the NR1 subunit pre-TM1 mutants tested modified the NMDA receptor sensitivity to ethanol inhibition. Although it is possible that changes in ethanol sensitivity may have been seen by modifying NR2 residues specifically involved in regulating glycine-independent desensitization, nondesensitizing receptor subtypes (e.g. NR1/2C) are still significantly inhibited by ethanol, suggesting that these amino acids are not likely sites of ethanol interaction.

Mutation of Phe639 to alanine in the TM3 domain of the NR1 subunit significantly decreased the ethanol inhibition of NMDA receptors expressed in either oocytes or HEK293 cells. Substitution of the slightly larger tryptophan residue at Phe639 resulted in receptors that were slightly more sensitive to ethanol inhibition than wild-type receptors, suggesting that some physical or chemical property of the amino acid substitution at this position may be an important determinant of ethanol sensitivity.

This has been more carefully studied in mutagenesis experiments on GABA_A and glycine receptors. In those receptors, residues in TM2 and TM3 have been shown to influence the degree of potentiation of receptor function by ethanol and vol-

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**Fig. 6.** Agonist sensitivity of NR1 TM3 mutant NR1(F639A)/2A receptors expressed in oocytes. Shown are dose-response curves for glutamate (A) and glycine (B–D) in oocytes expressing either wild-type NR1/2A or NR1(F639A) plus an NR2 subunit. For the glutamate dose response, the glycine concentration was held at 10 μM, whereas glycine dose responses were obtained using 100 μM NMDA. Data represent the mean ± S.E. from 5–10 oocytes for each condition and are expressed as percent maximal current obtained in each oocyte at a saturating concentration of agonist.
TM3 Phenylalanine and Ethanol

The leftward shift in the concentration-response curve for glycine, which resulted in receptors that were either potentiated (small amino acids), inhibited (large amino acids), or insensitive to these compounds (25, 32). Whether these amino acids are involved in defining a receptor agonist and are expressed as percent maximum current obtained in the absence of any added glycine. Data obtained from 6–11 oocytes at each concentration and are expressed as percent control current obtained in the absence of antagonist for each oocyte. A, dose-response relationship for inhibition of wild-type and NR1(F639A)/2A receptors by the competitive glycine site antagonist 5,7-DCK. Currents were activated by 100 μM NMDA and 10 μM glycine in the presence of increasing concentrations of 5,7-DCK. Data represent the mean ± S. E. obtained from 6–11 oocytes at each concentration and are expressed as percent control current obtained in the absence of antagonist for each oocyte. B, dose-response relationship for the glycine site competitive antagonist (+)-HA-966 in oocytes expressing wild-type NR1/2A or NR1(F639A)/2A receptors. Currents were activated by 100 μM NMDA and increasing concentrations of (+)-HA-966 in the absence of any added glycine. Data represent the mean ± S. E. obtained from 8–12 oocytes at each concentration and are expressed as percent maximum current obtained in each oocyte with 100 μM NMDA and 10 μM glycine.

Fig. 7. Modulation of wild-type NR1/2A and NR1 TM3 mutant NR1(F639A)/2A receptors by glycine site compounds. A, dose-response relationship for inhibition of wild-type and NR1(F639A)/2A receptors by the competitive glycine site antagonist 5,7-DCK. Currents were activated by 100 μM NMDA and 10 μM glycine in the presence of increasing concentrations of 5,7-DCK. Data represent the mean ± S. E. obtained from 6–11 oocytes at each concentration and are expressed as percent control current obtained in the absence of antagonist for each oocyte. B, dose-response relationship for the glycine site competitive antagonist (+)-HA-966 in oocytes expressing wild-type NR1/2A or NR1(F639A)/2A receptors. Currents were activated by 100 μM NMDA and increasing concentrations of (+)-HA-966 in the absence of any added glycine. Data represent the mean ± S. E. obtained from 8–12 oocytes at each concentration and are expressed as percent maximum current obtained in each oocyte with 100 μM NMDA and 10 μM glycine.

The Phe639 residue in the TM3 domain of the NR1 subunit is one of several that were found to be insensitive to cysteine-modifying agents, suggesting that it does not face the pore of the ion channel (30). In contrast, a long stretch of TM3 residues at the C-terminal end of TM3 was accessible to these modifying agents, and this domain (SYTANLAAAF) is the most highly conserved among all ionotropic glutamate receptors. This has led to the suggestion that the TM3 domain may be located near the central axis of the NMDA receptor ion channel and that amino acids in the outer portion of this domain interact with the aqueous lumen of the channel, whereas residues deeper within the membrane face amino acids located on other transmembrane domains. A mutation in the N-terminal end of TM3 of the glutamate 82 subunit gives rise to the Lurcher mouse phenotype that is characterized by constitutive channel activation (34). Mutation of homologous sites in various ionotropic glutamate receptors, including the NMDA receptor, alters channel properties and kinetics, suggesting that the TM3 domain may be an important regulator of receptor gating (35).

Finally, although the F639A mutation reduced the ethanol sensitivity of all heteromeric NMDA receptors tested in this study, it did not fully eliminate ethanol inhibition. However, because NMDA receptors probably contain at least two copies of an NR1 and NR2 subunit, mutation of both NR1 and NR2 subunits at the Phe639 site may be required to create an ethanol-insensitive NMDA receptor. Interestingly, all NMDA and non-NMDA subunits have a phenylalanine at the site homologous to Phe639 of the NR1 subunit, suggesting a key role for this residue. In the present study, expression of wild-type NR1 or NR1(N639A) subunits with NR2A subunits carrying the phenylalanine to alanine substitution (F637A) did not yield functional receptors. Further analysis of NMDA receptors carrying other amino acid substitutions at this site may reveal additional determinants of ethanol sensitivity.

Acknowledgment—We thank T. Blevins for assistance with the mutation experiments.

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J. Biol. Chem. 2001, 276:44729-44735.
doi: 10.1074/jbc.M102800200 originally published online September 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102800200

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