Interactions between Ifenprodil and the NR2B Subunit of the N-Methyl-D-aspartate Receptor

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Ifenprodil is a atypical noncompetitive modulator of the N-methyl-D-aspartate (NMDA) receptor (NR) which demonstrates a 140-fold preference for NR2B over NR2A subunits, although the molecular basis for this subunit specificity is unknown. We have made chimeric receptors by fusing the murine forms of NR2A (ε1) and NR2B (ε2) to localize the high affinity determinants of ifenprodil inhibition on the 2B subunit. Binding experiments with [125I]-MK-801 implicated the region between prodil inhibition on the 2B subunit. Binding experiments with [125I]-MK-801 implicated the region between amino acids 198 and 356 of NR2B for high affinity ifenprodil interaction. Site-directed mutants at Arg-337 showed that this residue is absolutely required for high affinity ifenprodil inhibition. Polyamines also modulate the NMDA receptor with a preference for NR2B subunits, and the pharmacology of these agents overlaps with ifenprodil. Although the determinants of the polyamine enhancement of iodo-MK-801 binding also localize to the NH2 terminus of NR2B, the point mutants at Arg-337 form receptors that are polyamine-stimulated at wild type levels. In addition, polyamine stimulation depends on the expression of NR1 splice variants, whereas high affinity ifenprodil inhibition is independent of NR1 isoform expression. These studies provide evidence that ifenprodil and polyamines interact at discrete sites on the NR2B subunit.

The N-methyl-D-aspartate (NMDA)1 subtype of glutamate receptor is a ligand-gated ion channel that mediates the entry of Ca2+ into neurons of the central nervous system and has been linked to neurologic disorders, synaptic plasticity, and excitotoxic cell death (1–5). In addition to the natural agonist (glutamate) and coagonist (glycine), many other compounds affect NMDA receptor function, including the channel blocking agents phenocyclidine (PCP) (6), N-1-thiethylcyclohexyl)lipieridine (TCP) (7, 8), and dizocilpine (MK-801) (6, 9) and the competitive antagonists d-3-(2-carboxy-piperazine-4-yl)-propyl-1-phosphonic acid (CPP) (10) and cis(-)-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755) (11). Modulatory sites, distinct from both the glutamate and glycine sites, for polyamines (12) (such as spermidine) and for the atypical, noncompetitive antagonist ifenprodil (13) have also been characterized. Novel therapeutic agents that interact favorably at noncompetitive sites could control the excitotoxic Ca2+ influx mediated by NMDA receptor overstimulation without the psychotomimetic side effects exhibited by channel-blocking agents (5).

The cloning of several subunits of the NMDA receptor has allowed investigation of drug interactions at the molecular level. Rat NMDAR1 (NR1), NMDAR2A (NR2A), NMDAR2B (NR2B), NMDAR2C (NR2C), and NMDAR2D (NR2D) have been discovered (11, 14, 15). In addition, eight isoforms of the NR1 subunit can be generated by alternative RNA splicing, (NR1A–H) (16, 17). The murine forms of NR1 (ζ) and NR2 (ε2,ε3,ε4) have more than 99% amino acid homology with rat NR2 subunits (18–20), allowing functional coexpression of mouse and rat subunits (21). Receptors formed by coexpression of different heteromeric combinations of NR1 and NR2 subunits exhibit distinct pharmacologies and can mimic regional and developmental expression in vivo (14, 15, 18, 19, 22–25). Since recombinant receptors can be selectively mutated, they provide excellent tools for molecular mapping of drug binding sites.

I fenprodil is a structurally unique modulator of the NMDA receptor which exhibits subunit-specific affinity for NMDA receptors (24, 26). This phenylethanolamine derivative noncompetitively antagonizes the NMDA receptor either by stabilizing the closed-channel confirmation of the ion channel or by causing a modal shift in the gating of the ion pore (27, 28). By a different mechanism, ifenprodil can block NMDA receptors in a voltage-independent manner (24). Ligand binding experiments have also suggested two sites for ifenprodil interaction. Inhibition of both [125I]-MK-801 and [125I]-MK-801 and [125I]-TCP binding to rat brain by ifenprodil is biphasic (24, 27, 29), suggesting the presence of both high and low affinity ifenprodil sites. The high affinity site for ifenprodil, measured electrophysiologically, has a K, in the submicromolar range (0.2–1 μM), whereas the K, for the low affinity site is 140–300-fold higher (60–100 μM) (24). Binding and electrophysiology studies show that ifenprodil exhibits a 140-fold preference for NR1A/NR2B (ε2) receptors over NR1A/NR2A (ε1) combinations (24). Therefore, the expression of NR2 subunits may underlie the biphasic nature of ifenprodil interactions with NMDA receptors.

There is much controversy over whether ifenprodil and polyamines interact at the same site on the NMDA receptor (13, 27, 30–32). Polyamines such as spermidine or spermine interact with NMDA receptors by at least three mechanisms. Polyamines stimulate NMDA receptors by enhancing the receptor affinity for glycine (glycine-dependent stimulation) and, in saturating glycine, increase the probability of channel opening (glycine-independent stimulation) (25). At higher concentrations, polyamines can also block NMDA receptors at the channel pore in a voltage-dependent manner (25). Like ifenprodil, glycine-independent stimulation by polyamines is dependent
on NR2 subunit expression. Electrophysiologic and binding paradigms both demonstrate that in saturating concentrations of glycine, polyamines stimulate NR1A/NR2B receptors whereas NR1A/NR2A combinations are polyamine-insensitive (25, 33–36). Radioligand binding studies suggested additional linkage between ifenprodil and polyamine sites. Spermidine displaces both 125I- and [3H]ifenprodil from rat brains, although direct competition is unclear (37–40). The polyamine stimulation of 125I-MK-801 and [3H]CPP binding can also be reduced by increasing ifenprodil concentrations (13, 39), further suggesting overlapping sites. Conversely, electrophysiologic studies of ifenprodil and polyamine effects on NMDA receptors expressed in Xenopus oocytes have ruled out simple competitive interactions of ifenprodil and polyamines (24, 26–28). The molecular determination of the ifenprodil and polyamine sites on the NMDA receptor could aid in understanding the interactions between these modulators.

We have investigated the molecular interactions of ifenprodil at NMDA receptors to define the determinants of NR2B which confer subunit-specific modulation, in an attempt to provide biochemical evidence that ifenprodil and polyamines bind to discrete sites on the NMDA receptor. We designed chimeric ε1 (NR2A)/ε2 (NR2B) subunits, coexpressed them with NR1 subunits, and measured the dose-dependent inhibition of 125I-MK-801 binding by ifenprodil for these receptors in order to localize the binding determinants of ifenprodil interaction on the NR2B subunit. The NH2-terminal 464 amino acids of NR2B contained determinants for both ifenprodil and polyamine interactions, and additional chimeras permitted localization of high affinity ifenprodil inhibition to amino acids 198–356. Site-directed mutants of ε2 were then characterized to define the ifenprodil modulatory site at the molecular level. All substitutions at Arg-337 render the receptor low affinity for ifenprodil, whereas these mutant receptors maintain wild type polyamine stimulation, providing biochemical evidence for discrete modulatory sites. The distinction of polyamine and ifenprodil binding sites was corroborated by additional experiments with NR1 splice variants. Unlike polyamines (25, 36), ifenprodil inhibition was shown to be unaffected by NR1 isoform expression, further suggesting distinct binding mechanisms. The molecular characterization of modulatory sites on the NMDA receptor could provide vital information for the design of novel therapeutic agents for the prevention of the neurodegeneration following cerebral ischemia.

**EXPERIMENTAL PROCEDURES**

**Materials**

(+)-3-[125]MK-801 was purchased from DuPont NEN. Spermidine, (±)-ketamine, β-mercaptoethanol, and polyethyleneimine were obtained from Sigma. (+)-MK-801 (hydrogen maleate) and ifenprodil (tartrate) were from Research Biochemicals International. Restriction enzymes and Taq DNA polymerase were purchased from either Life Technologies, Inc. or New England Biolabs. Sequencing was performed using the Sequenase II kit from U.S. Biochemicals. Monoclonal antibodies recognizing the NH2 terminus of NR2A were purchased from Pharmingen (San Diego). Goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were purchased from Boehringer Mannheim. ECL chemiluminescence reagents were obtained from Amersham Corp. HEK293 cells were purchased from American Type Culture Collection (ATCC). All other reagents used were of the highest purity available and were obtained from standard commercial sources.

**Chimeric NMDAR2 Subunit Construction**

DNA encoding ε1 and ε2 (the murine forms of NMDAR2A and NMDAR2B, respectively) were subcloned into the Sall/EcoRI sites of the vector pRK7 downstream of the cytomegalovirus promoter, which yields high level expression of each of these subunits in mammalian cell culture systems (21, 33, 34, 41). The first chimera (CH0) (see Fig. 1) was constructed by replacing the first 1393 base pairs of ε1 with the corresponding region of ε2 by digesting ε1 with Sall and Aff1I and ligating the 1.6-kilobase fragment into the same sites of ε2. The complementary chimer (CH25) was constructed by ligating the Sall/Aff1I fragment of ε2 into ε1. A chimera (CH5) containing the first 198 amino acids of ε1 and the rest being ε2 was constructed using PCR. The two primers, SP6 (5′ primer) and REMXHO13, which introduces an Xho site into ε2 (5′-GTACCAGGTTTCCGAATTCGCC-3′), were used to amplify (95°C, 1 min; 55°C, 1 min; 72°C, 2 min; 30 cycles) the 5′ region of ε1. PCR reactions were performed with primer pair SP6 (5′) and the overlapping primer OAPAL3E1 (5′-GCAAGTACCA AACAAGCTTGGGTGACACCTTGAGCCATCTTTCAGAAAG-3′), with ε2 as the template. The second PCR reaction (PCR2) used the overlapping primer OAPALS5E2 (5′-CTTCAGTGAGGCTCGCAGGATTACCACCACCGTGGGC-3′) and a 3′ primer downstream of the Aff1I site, E1AFLI13 (5′-GTTCTGGACGAGTGTCCTTAAAGGATGTGC-3′), with ε1 as the template. The products of these PCR reactions anneal across a 44 base pair region containing the chimeric fusion point at the center. 1 μL of each of the PCR reactions (PCR1 and PCR2) are diluted 1/10 in PCR reaction buffer and are combined, allowing homologous regions to anneal. After 4 cycles of PCR, a double stranded template is formed from the annealed products. The outside primers SP6 and E1AFLI13 are then added, and a 1.7-kilobase fragment is amplified. The final fragment is ligated into the Sall/Aff1I sites of ε1. A chimera that substitutes the amino acid region 198–356 of ε1 into ε2 (CH58) was derived from CH48 by replacing the Sall/Xho fragment of CH48 with the Sall/Xho fragment of CH5. A chimera encoding ε2 only at amino acids 356–464 was created using the same overlapping primer method with the following primers and templates:

**PCR1**—The primers were as follows: 5′-E1XHOI53 (5′-GTGGGCTCAGATCGATATGCAGGATC-3′); 3′-OAPALS3E2 (5′-GAAGAGTATTCCACCGCTCCGGCCGACGCTGATAGCCTTCCTCAGTGAAG-3′). The template was ε1.

**PCR2**—The primers were as follows: 5′-OAPALS1E1 (5′-CAGTCCTGGAAAGATCTTATCAGTGCACCAGAAGCTGCATCTCTTCTTTCAGGAAAG-3′); 3′-AFLIIE23 (5′-CTTGAAGATTATTTTATTAAAGATATCAATAC-3′). The template was ε2.

**PCR3**—The primers were as follows: 5′-E1XHOL53; 3′-E1AFLI13. The templates were products of PCR1+PCR2 elongated by four cycles of PCR.

**Site-directed Mutagenesis**

The point mutations at Arg-337 were constructed using a similar three-step PCR technique. Mutagenic primers were constructed with a degeneracy at the codon for Arg-337 to allow simultaneous generation of all mutations.

**PCR 1**—The primers were as follows: 5′-SP6; 3′-E2ARXG35 (5′-GTGCAGTTGATCAATAG(TGGTATCTTACGATGAGAAGC)-3′). The template was ε2 in pRK7.

**PCR2**—The primers were as follows: 5′-E2ARXG53, 5′-CAGTCCTACAGTCTGATGACACATCTGTCACTCAAGCTGAC-3′; 3′-AFF1IE23 (see above). The template was ε2 in pRK7.

**PCR3**—The products from PCR1 and PCR2 were then digested with BclI and ligated together. A third PCR product was generated using the product of the ligation as the template and the primers: 5′-SP6; 3′-AF-LILEI23. This product was digested with Sall and Aff1I and cloned back into ε2. Sequencing the final product confirmed the mutants R337A, R337P, R337K. The mutant R337Q was made using the same mutistep PCR technique with the more restrictive primer, E2AR337EQ (5′-GTCAGTTGACAGATAGGTCACTTACGAGTTGATGACACAT-3′), substituted for the primer E2ARXG35 above.

**Cell Culture and Transfection of HEK 293 Cells**

Human embryonic kidney cells (HEK293) were propagated in minimal Eagle’s medium containing 5% horse serum and 5% fetal bovine serum, 2 mm glutamine, and 1% penicillin/streptomycin in a humid 95% air, 5% CO2 atmosphere. Cells were transfected by the calcium phosphate precipitation method of Chen and Okayama with the modification that 10 μM MK-801 is present during transfection to protect cells from NMDA-induced excitotoxicity (41, 42).
Polyclonal Antibody Production

Polyclonal antibodies were raised against the COOH terminus of the rat NMDAR2A protein. The sequence-encoding amino acids 1115–1465 was subcloned into the pRSET vector (Invitrogen) at the unique BglII site. The expression construct was transformed into the Escherichia coli strain BL21 (DE3), and high levels of fusion protein were induced with isopropyl-1-β-D-thiogalactopyranoside (Boehringer Mannheim). Fusion proteins were purified by nickel affinity chromatography, with elution in 6 M guanidine. Following further purification by SDS-polyacrylamide gel electrophoresis, isolated bands were crushed and eluted in 100 mM NaCl, 50 mM Tris buffer, pH 7.5, and subsequently sent to Rockland Inc. for polyclonal antibody production. Initial boosts of between 250 and 500 µg of fusion protein in complete Freund’s adjuvant were followed at 2-week intervals by 200-µg injections in incomplete Freund’s adjuvant. Antibodies in the sera were capable of detecting 20–50 ng of fusion protein by enzyme-linked immunosorbent assay screening. Further purification of the polyclonal antibodies was accomplished by affinity chromatography. Affinity columns were made by coupling 200 µg of fusion protein to 6 × Reacti-Gel (Pierce) in 50 mM sodium borate buffer, pH 8.5. Antiserum was applied to 3-mL columns in 20 mM Hepes, pH 7.5, and the purified antibodies were eluted with 6 M guanidine, pH 3.0, directly into 1 mL Tris to elevate the pH rapidly. The yield of purified antibody from 100 mL of antiserum was approximately 100 µg.

Western Blotting

Membranes from 293 cells transfected with combinations of NR1 and NR2 subunits were prepared by 2–3-s sonication in isosmotic medium (0.32 M sucrose, 4 mM Hepes, pH 7.5, 1 µM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 1 µM leupeptin), followed by centrifugation at 14,000 rpm for 20 min to remove the nuclear fraction. Samples were boiled in SDS sample buffer containing β-mercaptoethanol and loaded on 6% polyacrylamide gels. Protein was transferred to nitrocellulose using a submarine transfer unit (Hoeffer Scientific), and blots were incubated in either polyvalent NMDAR2A or monoclonal NMDAR1 antibodies (3 µg/mL in TBS, 2.5% dry milk) for 4–6 h with constant agitation. After excess primary antibody was removed (once, 5 min, 0.01% Triton X-100/TBS; twice, 5 min, TBS), peroxidase-conjugated goat anti-rabbit (or mouse) secondary antibody was added to a 1/30,000 dilution in 1% dry milk, 1 × TBS. Multiple washes in 0.01% Triton, 0.1% Tween 20, and TBS were followed by detection with the ECL chemiluminescence detection system.

Radioligand Binding

Cells were prepared, as described previously (33, 34), for 125I-MK-801 binding by washing in 100 mM glycine, 100 mM glutamate, 300 µM Mg2+, 20 mM Hepes, pH 7.5 (three times, 30 min, 32°C) to remove the unlabelled MK-801 used to reduce cell death during transfection. Binding assays were performed in the presence of 125I-MK-801 (150 pM), glycine (100 µM), Mg2+ (300 µM), glutamate (100 µM), and spermidine (100 µM) to facilitate channel opening and ligand binding. Increasing concentrations of either ifenprodil or spermidine were added as appropriate. Incubations were for 3 h at 37°C. Each concentration was measured in duplicate with a corresponding blank containing 10 µM unlabeled MK-801. Specific binding was measured on a Beckman gamma counter (model 5500B) following rapid filtration (Brandel).

RESULTS

Since the NMDA receptor subunits are large polypeptides (about 1,500 amino acids) the characterization of the molecular determinants of ifenprodil binding to NR2B was best accomplished using chimeric NR2A/NR2B subunits. Because the protein sequences of NR2A and NR2B are 50% identical (18–20), their sequences can be effectively fused and could be expected to retain the functional properties of wild type receptors.

The murine NR2 subunits retain the properties of their rat homologs, this validates the strategy of designing chimeric ε1/ε2 subunits to map the subunit-specific determinants of ifenprodil and polyamine interaction. In the present study we have designed seven chimeric ε1/ε2 receptors (Fig. 1), which were used to map the determinants of ifenprodil inhibition on the NR2B subunit.

Expression of NMDA Receptor Subunits by Western Blot—Western blot analysis demonstrated that NR1, NR2, ε1, and ε2 chimeras all exhibit high levels of expression in our transfection system (Fig. 2, A–C). The splice variants of NR1A-G are all recognized by the monoclonal antibody against NR1A, and membranes analyzed by Western blot demonstrate comparable levels of expression of all forms (Fig. 2A). The monoclonal against NR1 also detects the fainter breakdown products of the NMDAR1 protein, exhibiting different patterns depending on the splice variant transfectected. A polyclonal antibody raised against the COOH terminus of the NR2A was used to verify NR2B subunit expression. This antibody recognizes NR2B but not NR2B or NR2C or any of the splice variants of NR1 (Fig. 2B). Fig. 2B shows a Western blot of two of the chimeras: CH8, which contains the COOH terminus of ε1, and CH25, which contains the COOH terminus of ε2. Bands running at the predicted molecular mass (165 kDa) demonstrated high level expression of both chimeras. All of the other five chimeric NR2 subunits showed similar levels of expression by Western blot (data not shown). The total 125I-MK-801 binding for all chimeras, which ranged from 32 to 55 fmol/mg protein (with 150 pM 125I-MK-801), was comparable to wild type receptors (1A/ε1, 58 fmol/mg protein; 1A/ε2, 87 fmol/mg protein), demonstrating high level expression for chimeric NR2 subunits (Fig. 2C). Based on total radioligand binding, all eight splice variants have expression levels comparable to combinations containing NR1A (Table I).

The NH2 Terminus of ε2 Is the Site of High Affinity Ifenprodil Interaction—The region of NR2B which most likely mediates SR141716A binding in 100 mM glycine, 100 mM glutamate, 300 µM Mg2+, 20 mM Hepes, pH 7.5 (three times, 30 min, 32°C) to remove the unlabeled MK-801 used to reduce cell death during transfection. Binding assays were performed in the presence of 125I-MK-801 (150 pM), glycine (100 µM), Mg2+ (300 µM), glutamate (100 µM), and spermidine (100 µM) to facilitate channel opening and ligand binding. Increasing concentrations of either ifenprodil or spermidine were added as appropriate. Incubations were for 3 h at 37°C. Each concentration was measured in duplicate with a corresponding blank containing 10 µM unlabeled MK-801. Specific binding was measured on a Beckman gamma counter (model 5500B) following rapid filtration (Brandel).

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NR1A/CH5 receptors had IC$_{50}$ values for ifenprodil of 2.6 µM; NR1A/CH6 had an IC$_{50}$ of 3.3 µM. The retention of high affinity inhibition by CH6 localized the e$_2$-specific determinants of ifenprodil inhibition to amino acids 198–464. Three additional chimeras, CH48, CH58, and CH84, further defined the region of NR2B mediating high affinity ifenprodil inhibition. The ifenprodil inhibition curves for these chimeras are shown in Fig. 4. The IC$_{50}$ values for CH48, CH58, and CH84 were 4.2, 18, and 72 µM, respectively (Figs. 1 and 4). Only a 5-fold loss in affinity of NR1A/CH48 over NR1A/e$_2$ was observed in inhibition assays, whereas NR1A/CH58 had a 5-fold greater apparent affinity for ifenprodil than 1A/e$_2$, implicating amino acids 198–356 as an important region for high affinity ifenprodil inhibition. NR1A/CH84 receptors have an even lower IC$_{50}$ for ifenprodil (72 µM), than 1A/e$_2$ receptors (59 µM), further implicating amino acids 198–356 for high affinity ifenprodil modulation.

High Affinity Ifenprodil Inhibition Is Mediated by Arg-337—An important determinant for high affinity ifenprodil inhibition was discovered by site directed mutagenesis of e$_2$. Ifenprodil may act as an acid by losing a phenolic hydrogen in physiologic conditions; thus, efficacious ifenprodil binding could be mediated by a positively charged residue on the NR2B subunit such as lysine or arginine. The region of e$_2$ between amino acids 198 and 357 contains a single positively charged amino acid that was conserved in both e$_2$ and 2B, but not in e$_1$ or 2A, namely Arg-337. Site-directed mutants were then created at Arg-337 by the use of PCR mutagenesis, and all of these mutants showed expression levels comparable to wild type receptors (Table II). Mutation of Arg-337 to lysine (NR1A/R337K) exhibited an IC$_{50}$ for inhibition of 125I-MK-801 binding by ifenprodil of 120 µM, even lower affinity than e$_1$ (Table I and Fig. 5). Arg-337 was also changed to alanine (R337A), proline (R337P), and glutamine (R337Q), which is the conserved amino acid in e$_1$ and NR2A. The IC$_{50}$ values for R337A, R337P, and R337Q were 83, 100, 65 µM, respectively. As any residue other than arginine at position 337 attenuates high affinity ifenprodil inhibition, Arg-337 is a functional determinant of high affinity ifenprodil binding. Although the differences in IC$_{50}$ between e$_1$ and both e$_2$ and the Arg-337 mutants for ifenprodil are too great to be explained only by differences in their affinities for 125I-MK-801, saturation analysis of 1A/e$_1$, 1A/e$_2$, and R337Q was performed. The K$_d$ values for 125I-MK-801 of 1A/e$_1$, 1A/e$_2$, and R337Q were 124, 133, and 145 pM, respectively, demonstrating that the observed changes in ifenprodil potency do not result from changes in the affinity for 125I-MK-801 and that receptor integrity is preserved in these mutants.

Splice Variants of NMDAR1 Distinguish between Ifenprodil and Polyamines—Although the e$_2$-specific properties of polyamine stimulation and high affinity ifenprodil interaction map to similar regions, experiments with the splice variants of NR1 suggest that these agents bind to distinct sites. Since NR1 subunits containing the NH$_2$-terminal insert, such as NR1B, are polyamine-insensitive (25), we studied the effects of NR1 isoform expression on ifenprodil inhibition to determine whether both ifenprodil and polyamines share splice variant-specific properties. We found that NR1B/e$_1$ and NR1B/e$_2$ receptors share the same subunit-specific effects as NR1A combinations, with IC$_{50}$ values for ifenprodil inhibition of 37 and 0.52 µM, respectively (Table I and Fig. 6). Experiments with NR1C and NR1G yielded similar results (Table I), suggesting that ifenprodil interactions are not dependent on NR1 splice variant expression.

Further evidence for ifenprodil insensitivity for the splice variants of NR1 was shown by cotransfection of all eight splice variants with either CH8 or CH25. In all cases, the splice variants that were transfected with CH8 had IC$_{50}$ values close to 1 µM, whereas all splice variant combinations with CH25 demonstrated half-maximal inhibition between 46 and 89 µM (Table I), providing evidence that the high affinity ifenprodil site is not affected by the expression of any NR1 splice variant.

| Ifenprodil IC$_{50}$ (µM) | Ifenprodil IC$_{50}$ (µM) | Polyamine Stimulation |
|--------------------------|--------------------------|----------------------|
| NR1A/CH1                 | 59 ± 10                  | – – –                |
| NR1A/CH2                 | 1.0 ± 0.05               | + + +                |
| NR1A/CH3                 | 1.6 ± 0.4                | + + +                |
| NR1A/CH4                 | 65 ± 8                   | – – –                |
| NR1A/CH5                 | 2.6 ± 0.8                | + + +                |
| NR1A/CH6                 | 3.3 ± 0.8                | +                   |
| NR1A/CH7                 | 4.2 ± 0.9                | + + +                |
| NR1A/CH8                 | 18 ± 5                   | +                   |
| NR1A/CH9                 | 72 ± 12                  | –                   |

Fig. 1. Design of the e$_1$/e$_2$ chimeras. The schematic primary sequences depict how portions of e$_1$ (white regions) and e$_2$ (black regions) are fused to form chimeric NR2 subunits. The IC$_{50}$ values for ifenprodil inhibition were calculated using the best of either one- or two-site fits using PROPHET. In all cases of two-site fits the percent high affinity represented 65% of the total inhibition; low affinity was 35%. Data are representative of 4–14 experiments. Polyamine stimulation is shown by either + + + (150–170% stimulation over baseline), + (50% stimulation), – (<10% spermidine enhancement), or – – – (0% polyamine stimulation).
and 1A/CH25. Amounts loaded were: 1A/CH8 (lane 6), and 1A/CH25. The Western blot using the NR2A polyclonal shows cell expression. NR1 splice variant subunits, which vary in size based on isoform expression. The NR1 monoclonal also detected fainter breakdown products of the mass markers are in kDa. All lanes show the expected 118-kDa protein. The NR1 monoclonal also detected fainter breakdown products of the lanes with NR2A, 1A/CH8, and 1A/CH25, demonstrating that our polyclonal is specific for the rat form of NR2A but recognizes both murine forms of NR2A and NR2B. Panel C, expression levels of the NR2 chimeras. The mean total binding of $^{125}$I-MK-801, expressed as fmol/mg of protein ($n = 4-8$), is shown. All seven chimeric subunits, when coexpressed with NR1A, show comparable levels of expression with wild type receptors. Error bars are shown.

even with chimeric receptors. Unlike polyamines, the inhibition of MK-801 binding by ifenprodil is not affected by NR1 isoform expression.

NR2 Chimeras and R337 Mutants Demonstrate Discrete Modulatory Sites—The chimeras CH5, CH6, CH48, CH58, and CH84 were used to determine if polyamine stimulation and ifenprodil inhibition localize to the same region of NR2B, although the results were not as straightforward as for ifenprodil inhibition. $^{125}$I-MK-801 binding to 1A/CH5 and 1A/CH48 receptors is stimulated by spermidine to the same extent as 1A/CH8, whereas 1A/CH6 and 1A/CH58 are stimulated only 50% as much as wild type receptors (Fig. 4). This suggests that the determinants of polyamine stimulation, as for ifenprodil, are found in the region between amino acids 198 and 356, although a significant loss in polyamine stimulation occurs when the chimeric fusion point is at amino acid 198. Surprisingly, there was a slight spermidine stimulation of 1A/CH48 receptors, which suggests that the binding determinants of polyamine stimulation may involve more regions of NR2B than for ifenprodil inhibition.

Although the determinants for glycine-independent polyamine stimulation map to the same general region of NR2B, results with the Arg-337 mutants gave the most conclusive evidence that ifenprodil and polyamine sites were indeed distinct. All four mutants at Arg-337 were found to be stimulated to the same extent as $e_1$ (Fig. 5 and Table II), thus Arg-337 is not a determinant of glycine-independent stimulation by polyamines.

### DISCUSSION

Chimeric $e_1/e_2$ subunits, coupled with site-specific mutagenesis, permitted the localization of high affinity inhibition to the NH$_2$ terminus of NR2B and distinguished it from glycine-independent polyamine stimulation. To utilize the murine forms of NR2 subunits, we had to demonstrate that they exhibit the same pharmacology as their rat homologs. The murine forms show the same magnitude and subunit specificity for the ifenprodil inhibition and polyamine stimulation of $^{125}$I-MK-801 binding as pure rat receptors (24, 25, 35). The $e_1$ and $e_2$ forms of NR2 subunits thus proved to be the ideal tool for chimeras.
construction and characterization of the effects of ifenprodil and polyamines.

The expression of the murine forms of NR2 was comparable to that of NR2A and NR2B. Western blot analysis using a polyclonal antibody against NR2A demonstrated a high level of protein expression of both native e1 and e2 receptors and of all the chimeric NR2 subunits characterized. Our NR2A polyclonal was unable to discriminate between e1 and e2, which may be due to 33% homology between e2 and NR2A in the polyclonal recognition region (18–20). The peptide sequences of NR2A and NR2B in this region are less than 20% similar and share no homology to NR2C or D, possibly explaining antibody specificity for the former form of NR2A. By preabsorbing the antibodies in our polyclonal mixture on an e1 affinity column it may be possible to isolate a population that demonstrates total specificity for NR2A and e1, obtaining a valuable tool for the biochemical characterization of NMDA receptors. Definitive evidence for high level expression was confirmed by the level of MK-801 binding, which was between 30 and 110 fmol/mg of protein for all forms of murine receptors when coexpressed with rat NR1A. Thus, expression of all of the chimeric subunits and the e1 sites-directed mutants was comparable to wild type receptors. Since we have shown previously that NR1A homomeric receptors bind insignificant levels of 125I-MK-801 (34), which is generally consistent with [3H]MK-801 binding experiments (43–45), our wild type binding levels confirm that our chimeric and mutant NR2 subunits efficiently coassemble to create intact MK-801 binding sites.

The divergent effects of the splice variants of NR1 on both ifenprodil inhibition and polyamine stimulation confirmed the distinct structural determinants between these modulators. Polyamine stimulation in electrophysiologic experiments depends on which splice variant of NR1 is expressed (16, 17, 24, 46, 47). Neither NR1B homomeric receptors nor NR1-NR2 combinations with the NH2-terminal insert (such as NR1B) display glycine-independent stimulation by polyamines. The effects of ifenprodil on receptors expressed with different splice variants had not been well characterized. We have shown that the high affinity inhibition by ifenprodil is not dependent on the NR1 subunit but is regulated by the NR2 subunit. All of the NR1 splice variants (A–H), when coexpressed with NR2 subunits, formed receptors whose modulation was governed only by NR2.

### Table II

| Arg-337 mutant | Ifenprodil IC50 (μM) | Polyamine stimulation | Total 125I-MK-801 bound (fmol/mg protein) |
|----------------|----------------------|----------------------|------------------------------------------|
| R337A          | 83 ± 13              | ++                   | 45 ± 9                                   |
| R337K          | 120 ± 24             | ++                   | 38 ± 3                                   |
| R337P          | 100 ± 20             | ++                   | 38 ± 10                                  |
| R337Q          | 65 ± 11              | ++                   | 37 ± 6                                   |
expression. Conversely, polyamine stimulation was not observed for either NR1B/e1 or NR1B/e2, whereas the NR1B/e2 receptor exhibited the same 140-fold greater affinity for ifenprodil seen for combinations with NR1A. Clearly, the mechanisms by which ifenprodil and polyamines interact at discrete sites on the NR2B subunit differ.

The use of chimeric receptors facilitated the discrete mapping of the site for high affinity ifenprodil inhibition. A major determinant of high affinity ifenprodil inhibition localizes to Arg-337 on the e2 subunit. There are at least three possibilities for the mechanism of action for Arg-337. First, ifenprodil may directly bind to Arg-337 of the e2 subunit. The localization of high affinity determinants to NR2B is consistent with the dramatic differences in affinity between 1A/e2 and 1A/e3 receptors and by the lack of NR1 splice variant-specific modulation. The fact that both in situ hybridization studies of NR2B mRNA and radiolabeled ifenprodil experiments show a strong correlation between high affinity ifenprodil binding and the developmental and regional profiles of NR2B expression also strengthens the argument that residues of NR2B interact directly with ifenprodil (26, 39, 48). Electrophysiologic evidence using outside-out patches has demonstrated that the high affinity ifenprodil site is located on the extracellular portion of the NMDA receptor (28).

Theoretical models of the transmembrane architecture of the e2 subunit are consistent with Arg-337 being present on this extracellular surface (36).

FIG. 5. Arg-337 of NR2B mediates high affinity ifenprodil inhibition. All of the site-directed mutants of Arg-337 cause at least a 300-fold shift in the IC50 of ifenprodil inhibition from wild type receptors. The ifenprodil inhibition curves for 1A/e1 (○), 1A/e2 (●), 1A/R337A (▼), 1A/R337K (▲), and 1A/R337Q (▲) are shown. The lower panel shows that the mutants 1A/R337A and R337K are both polyamine-stimulated to the same extent as 1A/e2, providing direct biochemical evidence that ifenprodil and polyamines interact at discrete sites on the NR2B subunit.

FIG. 6. High affinity ifenprodil inhibition is not dependent on NR1 splice variant expression. The inhibition of 125I-MK-801 binding by ifenprodil for 1B/e1 and 1B/e2 receptors shows the same 100-fold difference in apparent ifenprodil affinity as 1A/e1 and 1A/e2. Binding data and theoretical curves for 1A/e1 (○) and 1B/e1 (▲) both demonstrated low affinity ifenprodil inhibition, whereas 1A/e2 (●) and 1B/e2 (▲) were high affinity. Conversely, neither of the 1B-containing receptors showed polyamine enhancement of iodo-MK-801 binding (lower panel), demonstrating that polyamine stimulation is dependent on NR1 isoform expression, whereas high affinity ifenprodil inhibition is not.

1A/e2 for either the agonist (glutamate) or coagonist (glycine), affecting the association of MK-801 to open channels. This is ruled out by the fact that our assay system utilizes high excess concentrations of both glutamate and glycine and that the Kd for 125I-MK-801 of all our chimeras and point mutants were identical. The binding of MK-801 acts as a good internal control for receptor integrity. Functional high affinity MK-801 binding requires the presence of both functional glycine and glutamate sites and a structurally intact channel pore (33, 34); thus the changes in ifenprodil inhibition mediated by Arg-337 must be distinct from effects on either agonist or coagonist sites. Significant reduction of either glycine or glutamate affinity in mutant receptors results in receptors that do not bind 125I-MK-801 (34, 35, 49). The glycine coagonist site has recently been localized to the aromatic residues 390, 392, 466 and the charged residues 481 and 483 of the NR1 subunit. These residues are not only distal to Arg-337, but are also present exclusively on NR1 (50, 51).

Finally, Arg-337 may interact directly with the NR1 subunit where the true binding site for ifenprodil resides. NR1A mRNA injected into Xenopus oocytes yields functional homomeric channels with a high affinity for ifenprodil (0.28 μM); thus the high affinity binding site for ifenprodil was thought to reside on the NR1 subunit or the association of multiple NR1A subunits (26). Homomeric channels, although functional, lack many of
the characteristics of native NMDA receptors and have not been conclusively shown to exist in vivo (21, 26, 33, 34). Multiple ifenprodil binding sites, present on both NR1 and NR2 subunits, could also exist. There is a considerable sequence homology between NR1 and ε2 (NR2B) near Arg-337 (11, 14, 18–20). An arginine residue exists in NR1 (Arg-344) at the comparable position of Arg-337 in ε2, and may be the site of high affinity ifenprodil inhibition found in homomeric receptors. New chimeras and site-directed mutants of both NR1 and NR2 subunits will help gain future insight into the mechanism by which high affinity ifenprodil binding occurs.

The 300-fold difference in ifenprodil affinity between NR2A- and NR2B-containing receptors can best be explained by an electrostatic interaction occurring at the high affinity ifenprodil binding site. The chemical structure of ifenprodil contains no obvious ionizable groups such as amines but does possess a phenyl ring with a hydroxyl group attached. Tyrosyl-like groups may become phenolate ions following the loss of a proton from the phenyl hydroxyl group (52–56). The O− is stabilized through conjugation with the double-bond structure of the phenyl ring. An electrostatic interaction between ionized ifenprodil and one or more basic amino acid residues of the NMDA receptor could be proposed. The energy loss from the disruption of an electrostatic interaction is believed to be approximately 3–5 kcal (57). This change in apparent binding energy would account for a change in Kd of approximately 150–4,000-fold. The 300-fold difference in IC50 is consistent therefore with the loss of a weak electrostatic interaction in ε2-bearing receptors. Arg-337 is the only basic amino acid residue that is conserved in both ε2 and 2B between amino acids 198 and 356, whereas glutamine is found at this position in ε2 and 2A. Surprisingly, even substitution of the basic residue lysine at position 337 renders the receptor low affinity, suggesting that not only is a positively charged residue necessary at residue 337, but specifically arginine. Since the orientations of the positively charged moieties of lysyl and arginyl side chains differ, it is likely that the precise positive charge alignment of Arg-337 is required for efficacious high affinity ifenprodil binding. Glutamine substitution at this position exhibits the least detrimental effect on the IC50 of ifenprodil inhibition, presumably because the glutamine side chain has a surface volume most similar to that of arginine. Additional point mutations will be necessary to define further the involvement of Arg-337 in NMDA receptor modulation.

Although some components of the NR2B-specific effects of ifenprodil and polyamines overlap, results of the NR1 splice variant experiments and the Arg-337 mutation experiments provide biochemical evidence for distinct polyamine and ifenprodil binding sites. The dissimilar structures of spermine and ifenprodil make competitive binding arguments unlikely. Even though the long aliphatic chain and amine group of spermine differ from the phenylethanolamine structure of ifenprodil, some of the pharmacologic properties of polyamines and ifenprodil overlap. Ifenprodil blocks the stimulatory effects of polyamines on both TCP and MK-801 binding and inhibits the increase in [3H]CPP binding facilitated by spermine (39). Polyamines antagonize the partial displacement of [3H]glycine by ifenprodil (32). These overlapping effects may be explained by the determinants of polyamine stimulation on the ε2 subunit being between amino acids 198 and 293, which is potentially close to the site of high affinity ifenprodil binding. Curiously, the homologous region of NR1 (amino acids 190–211) is the location for the 63-residue insertion that renders splice variants such as NR1B polyamine-insensitive (16, 17, 25). Site-directed mutants of this region of NR1A eliminate polyamine stimulation (46). Although the determinants of polyamine stimulation on ε2 have not yet been characterized at the amino acid level, it seems likely that the region from amino acids 198 to 464 will include some component of the glycine-independent polyamine stimulation region of the ε2 subunit. The binding sites for ifenprodil and polyamines are biochemically distinct, although their determinants on the NR2B subunit are at least allosterically linked if not overlapping. Since the binding of polyamines and possibly ifenprodil involves both the NR1 and NR2 subunits, biochemical information about NR1-NR2 interactions could be studied by closer examination of the allosteric linkage between ifenprodil inhibition and polyamine stimulation.

There is much current interest in the ability of ifenprodil to act as a neuroprotective agent during focal cerebral ischemia and as an anticonvulsant agent (13, 25, 58). The interaction between ifenprodil and the NMDA receptor may underlie this neuroprotective ability. Unlike many other neuroprotective agents, ifenprodil and the derivative SL 82.0715, which has a better oral bioavailability, do not cause any behavioral effects and have already been used clinically for the treatment of hypertension and cerebral ischemia (13, 58). The location and mechanism of ifenprodil action on the NMDA receptor are still not completely understood. By identifying more residues like Arg-337, which directly participate in modulating the function of NMDA receptors, and by characterizing these putative modulatory sites at the molecular level, it will be possible to design additional novel therapeutic agents to combat the neurodegeneration that follows events such as stroke. Acknowledgments—We give special thanks to Dr. Michael Robinson, Dr. Brian Bascaki, and Elfrida Grant for helpful comments on this manuscript.

REFERENCES

1. Collingridge, G. L., and Singer, W. (1990) Trends Pharmacol. Sci. 11, 290–297
2. Nakamichi, S. (1992) Science 258, 597–603
3. Monaghan, T. D., Bridges, R. J., and Cotman, C. W. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 363–402
4. Watkins, J. C., Krooggaard-Larsen, P., and Honore, T. (1990) Trends Pharma col. Sci. 11, 25–33
5. Lipton, S. (1993) Trends Neurosci. 16, 527–532
6. Ferrer-Montiel, A. V., Sun, W., and Montal, M. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 8021–8026
7. Barbour, D. W., and McNamara, J. O. (1988) Mol. Pharmacol. 34, 250–255
8. Kloo, Y., Nadler, V., and Sokolovsky, M. (1988) FEBS Lett. 230, 167–170
9. Kloo, Y., Haring, R., and Sokolovsky, M. (1988) Biochemistry 27, 843–848
10. Danyus, W., and Fadda, E. (1989) Mol. Pharmacol. 36, 912–916
11. Moriyoshi, K., Masu, M., Takahiro, I., Shigemoto, R., Mizuno, N., and Nakamichi, S. (1991) Nature 354, 31–37
12. Ransom, R. W., and Ster, L. C. (1988) J. Neurochem. 51, 830–836
13. Cartaud, C., Benavides, J., Legende, P., Vincent, J. D., Noel, F., Thuret, F., Lloyd, K. G., Arbilla, S., Zivkovic, B., Mackenzie, E. T., Scatton, B., and Langer, S. Z. (1988) J. Pharmacol. Exp. Ther. 247, 1222–1232
14. Monyer, H., Sprengel, R., Schopper, R., Herb, A., Higuchi, M., Loreli, H., Burnashev, N., Sackmann, B., and Seeburg, P. H. (1992) Science 256, 1217–1221
15. Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., and Nakamichi, S. (1993) J. Biol. Chem. 268, 2836–2843
16. Nakamichi, N., Akel, R., and Schneider, N. A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8522–8526
17. Durand, G. M., Gregor, P., Zheng, M., Bennet, M. V., Uhl, G. R., and Zukin, R. S. (1992)Proc. Natl. Acad. Sci. U.S.A. 89, 9359–9363
18. Moriyoshi, K., Mori, H., Araki, K., Kushida, E., Kutsuwada, M., Yamanouchi, K., Arakawa, M., Sakurada, K., and Mishina, M. (1992) Nature 357, 70–71
19. Kutsuwada, M., Ishii, T., Kashiwabuchi, N., Mori, H., Sakurada, K., Kushida, E., Araki, K., Moriyoshi, K., Masaki, H., Kuntzmann, H., Arakawa, M., and Mishina, M. (1992) Nature 358, 36–41
20. Ikeeda, K., Nagasawa, M., Mori, H., Araki, K., Sakurada, K., Watanabe, M., Nagasawa, Y., Uehara, S., and Mishina, M. (1992) FEBS Lett. 313, 36–38
21. Chazot, P. L., Coleman, S. K., Cik, M., and Stephenson, F. A. (1994) J. Biol. Chem. 269, 24043–24049
22. Biscoe, P. G., Gasic, G. P., Vetter, D. E., Sullivan, J. M., and Heinemann, S. F. (1993) J. Biol. Chem. 268, 22663–22671
23. Sakurai, S., Y., Penney, J. B., and Young, A. B. (1993) J. Neurochem. 60, 1344–1353
24. Williams, K. (1993) Mol. Pharmacol. 44, 851–859
25. Williams, K. (1994) Mol. Pharmacol. 45, 803–809
26. Williams, K., Russell, S. L., Shen, Y. M., and Mollinoff, P. B. (1993) Neuro 10, 267–278
27. Reynolds, I., and Miller, R. J. (1989) Mol. Pharmacol. 36, 758–765
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28. Legendre, P., and Westbrook, G. L. (1991) Mol. Pharmacol. 40, 289–298
29. Carter, C. J., Benavides, J., Dana, C., Schoemaker, H., Perrault, G., Sanger, D., and Scatton, B. (1991) in Excitatory Amino Acid Antagonists (Meldrum, B., ed) pp. 130–163, Blackwell Scientific Publications, Oxford
30. Carter, C. J., Lloyd, K. G., Zikoic, B., and Scatton, B. (1990) J. Pharmacol. Exp. Ther. 255, 475–482
31. Tamura, Y., Sato, Y., Yokota, T., Akaike, A., Sasa, M., and Takaori, S. (1993) J. Pharmacol. Exp. Ther. 265, 1017–1025
32. Ransom, R. W. (1991) Eur. J. Pharmacol. Mol. Pharmacol. 208, 67–71
33. Lynch, D. R., Anegawa, N. J., Verdoorn, T., and Pritchett, D. B. (1994) Mol. Pharmacol. 45, 540–545
34. Lynch, D. R., Lawrence, J. J., Lenz, S., Anegawa, N. J., Dichter, M., and Pritchett, D. B. (1995) J. Neurochem. 64, 1462–1468
35. Durand, G. M., Bennett, M. J., and Zukin, R. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6731–6735
36. Traynelis, S. F., Hartley, M., and Heinemann, S. F. (1995) Science 268, 873–876
37. Schoemaker, H., Allen, J., and Langer, S. Z. (1990) Eur. J. Pharmacol. 176, 249–250
38. Schoemaker, H., Allen, J., and Langer, S. Z. (1990) Br. J. Pharmacol. 100, 316P
39. Beart, P. M., Mercer, L. D., and Jarrott, B. (1991) Neurosci. Lett. 124, 187–189
40. Mercer, L. D., Jarrott, B., and Beart, P. M. (1993) J. Neurochem. 61, 120–126
41. Anegawa, N. J., Lynch, D. R., Verdoorn, T. A., and Pritchett, D. B. (1995) J. Neurochem. 64, 2004–2012
42. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
43. Grimwood, S., Le Bourdelles, B., and Whiting, P. J. (1995) J. Neurochem. 64, 525–530
44. Laurie, D. J., and Seeburg, P. H. (1994) Eur. J. Pharmacol. 268, 335–345
45. Chazot, P. L., Cik, M., and Stephenson, F. A. (1992) J. Neurochem. 59, 1176–1178
46. Zheng, X., Zhang, L., Durand, G. M., Bennet, M. V., and Zukin, R. S. (1994) Neuron 12, 811–818
47. Nicolas, C., and Carter, C. (1994) J. Neurochem. 63, 2248–2258
48. Petralia, R. S., Wang, Y. X., and Wenthold, R. J. (1994) J. Neurosci. 14, 6102–6120
49. Williams, K., Dichter, M. A., and Molinoff, P. B. (1993) Mol. Pharmacol. 42, 147–151
50. Kuryatov, A., Laube, B., Betz, H., and Kuhse, J. (1994) Neuron 12, 1291–1300
51. Wafford, K. A., Katohria, M., Bain, C. J., Marshall, G., Le Bourdelles, B., Kemp, J. A., and Whiting, P. J. (1995) Mol. Pharmacol. 47, 374–380
52. Goldberg, J. M., Zheng, J., Ding, H., Chen, Y. Q., Calender, R., and Kirsch, J. F. (1993) Biochemistry 32, 8092–8097
53. Zhao, Q., Li, Y. K., Mildvan, A. S., and Talalay, P. (1995) Biochemistry 34, 6562–6572
54. Carper, D. A., Hohman, T. C., and Old, S. E. (1995) Biochim. Biophys. Acta 1246, 67–73
55. Derst, C., Wehner, A., Specht, V., and Rohn, K. H. (1994) Eur. J. Biochem. 224, 533–540
56. Bohren, K. M., Grimshaw, C. E., Lai, C. J., Harrison, D. H., Ringe, D., Petsko, G. A., and Gabbay, K. H. (1994) Biochemistry 33, 2021–2032
57. Fersht, A. R., Leatherbarrow, R. J., and Wells, T. N. (1986) Trends Biol. Sci. 11, 321–325
58. Otomo, E., Atarashi, J., Araki, G., Ito, E., Omae, T., Kuzuya, F., Nukada, T., and Edi, O. (1985) Curr. Ther. Res. 37, 811–821
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