Subtype-Selective Antagonism of N-Methyl-D-Aspartate Receptors by Felbamate: Insights into the Mechanism of Action

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ABSTRACT

Felbamate is an anticonvulsant used in the treatment of seizures associated with Lennox-Gastaut syndrome and complex partial seizures that are refractory to other medications. Its unique clinical profile is thought to be due to an interaction with N-methyl-D-aspartate (NMDA) receptors, resulting in decreased excitatory amino acid neurotransmission. To further characterize the interaction between felbamate and NMDA receptors, recombinant receptors expressed in Xenopus oocytes were used to investigate the subtype specificity and mechanism of action. Felbamate reduced NMDA- and glycine-induced currents most effectively at NMDA receptors composed of NR1 and NR2B subunits (IC$_{50}$ = 0.93 mM), followed by NR1-2C (2.02 mM) and NR1-2A (8.56 mM) receptors. The NR1-2B-selective interaction was noncompetitive with respect to the coagonists NMDA and glycine and was not dependent on voltage. Felbamate enhanced the affinity of the NR1-2B receptor for the agonist NMDA by 3.5-fold, suggesting a similarity in mechanism to other noncompetitive antagonists such as ifenprodil. However, a point mutation at position 201 (E201R) of the ε2 (mouse NR2B) subunit that affects receptor sensitivity to ifenprodil, haloperidol, and protons reduced the affinity of NR1-ε2 receptors for felbamate by only 2-fold. Furthermore, pH had no effect on the affinity of NR1-2B receptors for felbamate. We suggest that felbamate interacts with a unique site on the NR2B subunit (or one formed by NR1 plus NR2B) that interacts allosterically with the NMDA/glutamate binding site. These results suggest that the unique clinical profile of felbamate is due in part to an interaction with the NR1-2B subtype of NMDA receptor.

Felbamate is an anticonvulsant drug used in the treatment of seizures associated with Lennox-Gastaut syndrome in children and complex partial seizures in adults (reviewed in Pellock and Brodie, 1997). It has been found to interact with several sites within the brain, which may be responsible for its efficacy against a broad spectrum of seizure disorders in animals (Rogawski and Porter, 1990). Like some of the older antiepileptic drugs, felbamate inhibits voltage-sensitive sodium channels, probably by prolonging inactivation (Srinivasan et al., 1996; Taglialatela et al., 1996), and decreases the firing rate of neurons (White et al., 1992). Voltage-sensitive calcium channels are also blocked by felbamate (Stefani et al., 1996). These in vitro effects occur at concentrations of felbamate similar to those measured in the plasma and brain tissue of rats and humans after the administration of anticonvulsant doses of the drug (McCabe et al., 1993; Adusumalli et al., 1994; Troupin et al., 1997). Felbamate also potentiates γ-aminobutyric acid-mediated chloride currents (Rho et al., 1994), which might enhance inhibition of neurons, although these actions require high concentrations of drug and are of uncertain clinical significance.

Felbamate also has a novel site of action compared with other antiepileptic drugs, interacting with NMDA receptors that are known to be involved in animal models of epilepsy (reviewed in McNamara, 1994). However, controversy has surrounded determination of the site of action of felbamate on NMDA receptors. Early reports suggested that felbamate competitively inhibited the binding of the glycine antagonist $[^{3}$H]$5,7$-dichlorokynurenic acid to brain sections (McCabe et al., 1993; Wamsley et al., 1994). Glycine was also shown to reverse the anticonvulsant effects of felbamate in the electroshock and NMDA-induced seizure models (Coffin et al., 1994), the inhibition of Ca$^{2+}$ influx by felbamate after NMDA/glycine exposure in cultured cells (White et al., 1995), and the neuroprotective effects of felbamate after hypoxia in

**ABBREVIATIONS:** NMDA, N-methyl-D-aspartate; MBS, modified Barth’s solution; TCP, N-[1-(2-thienyl)cyclohexyl]piperidine.

886
hippocampal slices (Wallis and Panizzon, 1993). Although these studies suggest that felbamate competes with glycine, subsequent studies have not supported this. Subramanian et al. (1995) showed that felbamate competitively inhibited [3H]MK-801 binding but did not inhibit 5,7-dichlorokynurenic acid binding. In addition, the excitoprotective effects of felbamate on cultured cortical neurons exposed to NMDA or glutamate could not be overcome by glycine (Kanthasamy et al., 1995). Furthermore, no competitive interactions between felbamate and glycine have been observed in studies of NMDA/glycine-mediated channel currents (Rho et al., 1994). Instead, a channel-blocking mechanism was suggested due to the appearance of felbamate-induced flickering of single NMDA channel currents (Rho et al., 1994; Subramanian et al., 1995). It has been suggested that these channel-blocking effects are only observed at high felbamate concentrations not normally observed in patients undergoing felbamate therapy (Coffin et al., 1994).

Some of the controversy surrounding the mechanism of action of felbamate at NMDA receptors may be due to differences in the models systems used. Native NMDA receptors are thought to exist in a variety of subtypes whose compositions are not completely understood. It is likely that at least four subunits must assemble around a central pore (Laube, 1998) and that these subunits are recruited from two classes, NMDA-R1 (NR1) and NMDA-R2 (NR2). Each of these classes has multiple members, with NR1 subunits existing in several splice variants, and NR2 subunits coded for by at least four separate genes (reviewed by McBain and Mayer, 1994). The NR2 subunit or subunits present are believed to confer most of the differential pharmacological properties of native receptors (Kutsuwada et al., 1992; Monyer et al., 1992; Buller et al., 1994; Lynch et al., 1995). Several noncompetitive antagonists, such as ifenprodil, have been found to interact selectively with receptors containing NR2B subunits (Williams, 1993; Lynch et al., 1995). Ifenprodil was also shown to inhibit different subtypes of NMDA receptor by distinct mechanisms; glycine-dependent inhibition by ifenprodil was observed for receptors containing NR1 and NR2B subunits, whereas inhibition of NR1-NR2A receptors exhibited voltage dependence (Williams, 1993). By analogy, different mechanisms of felbamate action might be expected for different NMDA receptor subtypes.

This report describes the subtype selectivity of felbamate interactions with recombinant NMDA receptors expressed in Xenopus laevis oocytes and offers insights into its mechanism of action. Similar to ifenprodil and other noncompetitive NMDA receptor antagonists, we found felbamate to be moderately selective for the NR1-NR2B subtype of NMDA receptor. We also found felbamate to be noncompetitive with respect to NMDA and glycine at all NMDA receptor subtypes tested and to have a voltage-independent mechanism of action at a unique site on NR1-NR2B receptors.

**Experimental Procedures**

**RNA Transcription.** Plasmids containing the cDNA coding sequences for the NR1a, NR1α(N616R), NR2A, NR2B, NR2C, e2, and e2E201R) subunits were linearized via restriction digestion. Transcription of RNA from the linearized templates was performed using the Stratagene RNA Transcription kit (La Jolla, CA) with additions of RNasin RNase inhibitor (Promega, Madison WI), the m7G(ppp)G capping analog, and rCT2-p (Amersham-Pharma, Piscataway, NJ). Transcripts were stored at concentrations of 20 to 200 ng/μl in diethylpyrocarbonate-treated water.

**Preparation and Injection of Oocytes.** X. laevis females (Nasco, Fort Atkinson, WI, or Xenopus 1, Ann Arbor, MI), 3 to 5 inches in length, were anesthetized with 0.12 to 0.16% tricaine (3-aminobenzoic acid ethyl ester; Sigma Chemical Co., St. Louis, MO) in artificial spring water (17 mM NaCl, 0.054 mM KCl, and 0.041 mM CaCl2 in deionized water). Oocytes were removed surgically from a small incision in the abdomen, and placed in modified Barth’s solution (MBS) culture media [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 10 mM HEPES, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.91 mM CaCl2, and 0.01 mg/ml penicillin and streptomycin or 0.05 mg/ml gentamicin, pH 7.4]. Follicle cells were removed by incubation of the oocytes for 2 h in 2 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN) in Ca2+-free OR-2 media (82 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM MgCl2, 0.1 mM EDTA, and 0.77 mM NaH2PO4, pH 7.5). Defolliculated oocytes were rinsed in Ca2+-free OR-2 media.

On the day of the surgery, stage V or VI oocytes were injected with approximately 50 nl of RNA. The amount of RNA varied depending on the subunit combination. Typically, 1 to 2 ng of a 1:1 ratio of NR1α and NR2A was injected. For all other subunit combinations, approximately 10 ng of RNA was injected into each oocyte with a ratio of 1:3 to 1:5 of NR1α and either an NR2 or e2 subunit RNA. Control oocytes were injected with diethylpyrocarbonate-treated water or were not injected. Injected oocytes were incubated at 19°C in MBS culture media supplemented with 100 μM d-2-amino-5-phosphonovaleric acid for at least 40 h before use to allow for expression of receptors. In some cases, oocytes were injected with 50 nl of 25 mM 1,2-bis(2-amino-phenoxy)ethane-N,N,N’,N’-tetraacetic acid 15 min to 1 h before recording to minimize run-up of NMDA/glycine-mediated currents.

**Electrophysiology.** Recording electrodes were pulled from EN-1 glass (Garner Glass, Claremont, CA) on a vertical two-stage puller (Narashigi PP-83, Tokyo, Japan) or in three stages on a horizontal puller (PMP-100D; MicroData Instruments, Woodhaven, NY) to resistances of 0.5 to 1.2 MΩ for current passing electrodes and 0.5 to 2.5 MΩ for voltage-recording electrodes. Oocytes were perfused continuously at a rate of 3.4 ml/min with recording MBS (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 10 mM HEPES, and 2 mM BaCl2, pH 7.4) and voltage-clamped (OC-725B; Warner, Hamden, CT) to ~60 mV unless otherwise indicated. Currents were filtered at 1 kHz before recording on a chart recorder (Dash 4; Astromed, Warwick, RI) and digitizing for analysis with DataPacIII software (Laguna Hills, CA).

Drug solutions were diluted into recording MBS from frozen stocks (NMDA, glycine) or dissolved from powder directly into MBS (felbamate, haloperidol) on the day of the experiment. Felbamate was dissolved in MBS to the highest concentration used (up to 3 mM) by heating to 50°C followed by vigorous stirring. The process was repeated once or twice, as necessary, until the felbamate dissolved. Serial dilutions to lower concentrations were then made in MBS. Haloperidol was dissolved in dimethyl sulfoxide and diluted 1000- to 100,000-fold in MBS. Drug solutions were bath applied by switching from MBS alone to MBS plus drug solutions, and the current change monitored until a plateau was reached, usually in 40 to 60 s. From 4 to 5 min were allowed for recovery between drug perfusions.

**Statistical Analysis.** Agonist and antagonist concentration-response curves were analyzed with Axum (MathSoft, Cambridge, MA). Currents generated by the application of increasing concentrations of agonist were fitted with a nonlinear least-squares method to the logistic equation:

$$ I = I_{\text{max}}/[1 + (EC_{50}/[A])^n] $$

where I is the current at agonist concentration [A], $I_{\text{max}}$ is the maximum current, $EC_{50}$ is the concentration of agonist that pro...
duces 50% of the maximum current, and \( n \) is the Hill coefficient. Estimates of \( I_{\text{max}} \), \( EC_{50} \), and \( n \) were generated during the fit. The computer generated estimate of \( I_{\text{max}} \) was then used to normalize the currents. Final curves were generated by fitting all of the normalized data points to the above equation. Fitted curves were superimposed on the mean ± S.E.M. normalized current for each concentration. For agonist concentration-response curves generated in the presence of felbamate, the same process was used except that the \( I_{\text{max}} \) from the concentration-response curve without felbamate present was used to normalize the data (except where otherwise indicated). Geometric means for agonist \( EC_{50} \) measurements were compared in the presence and absence of felbamate with paired t tests. Arithmetic mean values are reported for clarity.

Antagonist inhibition curves were generated by fitting currents generated by fixed concentrations of agonists in the presence of antagonist at increasing concentration with another form of the logistic equation:

\[
I = I_{\text{max}}/(1 + ([B]/IC_{50})^n)
\]

where \( I \) is the agonist-induced current in the presence of antagonist at concentration \([B]\), and the \( IC_{50} \) is the concentration of antagonist that reduces the agonist-generated current (\( I_{\text{max}} \)) by 50%. Computer-generated estimates of \( I_{\text{max}} \) were used to normalize the currents, and final fits were made with all of the normalized data points. Geometric mean values of antagonist \( IC_{50} \) measurements were compared for different solution pH with a one-way ANOVA. Arithmetic mean values are reported for clarity.

**Materials.** Bluescript plasmids containing the cDNA coding sequences for NR1a, NR2A, and NR2C were obtained from S. Nakamichi (Kyoto University, Kyoto, Japan), for NR2B from Jane Sullivan (Salk Institute, La Jolla, CA), and NR1a(N616R) and NR1a(2E201R) mutants, including the e2 coding sequences in pRK7, from Ray Dingledine (Emory University, Atlanta, GA). e2 coding sequences in prk7, including the e2(E201R) mutant, were obtained from David Lynch (University of Pennsylvania, Philadelphia, PA). Felbamate was obtained from Dr. D. Sophia and Dr. H. Mortgo (Wallace Laboratories, Cranbury, NJ). NMDA and haloperidol were obtained from Research Biochemicals, Inc. (Natick, MA), and all other chemicals were from Sigma Chemical Co.

**Results**

The application of 100 \( \mu \)M NMDA and either 1 or 10 \( \mu \)M glycine induced inward currents in oocytes expressing the NR1 subunit plus one of the NR2 or e2 subunits. The amplitude of the currents depended on subunit combinations (NR1-NR2A currents tended to be the largest), batch of oocytes, and RNA preparations used. Despite variability in current amplitude between batches of oocytes for a given receptor subtype, no major differences in agonist and antagonist interactions with receptors were apparent. Some variability in the potency of felbamate across experiments was observed and is attributable to the relative insolubility of the drug in aqueous solution. Unless otherwise indicated, all experiments include data from two or three separate batches of oocytes and several batches of felbamate solution, which was made daily from powder.

**Subtype Selectivity of Felbamate.** To investigate the subtype selectivity of felbamate inhibition of NMDA receptors, felbamate concentration-response curves were constructed for oocytes injected with RNA encoding NR1a and NR2A subunits (NR1-2A receptors), NR1a and NR2B subunits (NR1-2B receptors), or NR1a and NR2C subunits (NR1-2C receptors). Concentrations of felbamate ranging from 0.1 to 3 mM in MBS were superfused onto oocytes before the application of 100 \( \mu \)M NMDA and 1 \( \mu \)M glycine in the presence of felbamate. Preperfusion of felbamate was used for these experiments because no inhibition was observed on NR1-2A receptors in preliminary experiments with acute exposure to felbamate at the same time as NMDA/glycine application (data not shown). Subsequent experiments showed that preperfusion increased felbamate (3 mM) block of NR1-2A receptors by 18% (\( n = 6; P < .003 \)) and increased felbamate (0.9 mM) block of NR1-2B receptors by 11.5% (\( n = 13; P < .001 \)).

Felbamate showed the greatest inhibition of currents induced by 100 \( \mu \)M NMDA and 1 \( \mu \)M glycine in oocytes expressing the NR1-2B receptors (Fig. 1). The lowest concentration with detectable inhibition of NR1-2B receptors by felbamate was 100 \( \mu \)M, and the \( IC_{50} \) was 0.93 ± 0.14 mM (\( n = 7 \)). NR1-2A and NR1-2C receptors were inhibited by felbamate with \( IC_{50} \) measurements of 8.56 ± 2.1 mM (\( n = 8 \)) and 2.02 ± 0.30 mM (\( n = 6 \)), respectively. These results indicate that the selectivity ratios of NR1-2B/NR1-2A and NR1-2B/NR1-2C were 9.2 and 2.2, respectively. The \( IC_{50} \) value reported for NR1-2A receptors is only a rough estimate because the highest concentration of felbamate possible, 3 mM, inhibited the receptor by only about 35%. In addition, precipitation of felbamate was sometimes observed at this concentration.

**Competition with Agonists.** Several studies have suggested that the therapeutic effects of felbamate are mediated through an interaction with the strychnine-insensitive glycine site on NMDA receptors (McCabe et al., 1993; Coffin et al., 1994). To investigate whether felbamate blocks one or more of the receptor subtypes by competing with either agonist, agonist concentration-response curves were constructed by applying increasing concentrations of either glycine or NMDA in the absence and presence of a fixed concentration (1 mM) of felbamate (Fig. 2A). If felbamate...
were competitive with either agonist, a shift to the right of that agonist concentration-response curve would be expected, with a corresponding increase in the agonist EC$_{50}$.

Felbamate (1 mM) decreased the maximum response achieved by 100 mM NMDA and increasing concentrations of glycine without significantly altering the glycine EC$_{50}$ value for all three receptor subtypes tested (Fig. 2, B–D). As expected, felbamate inhibited NR1-2B receptors the most, reducing the maximum response by 70% (Fig. 2B). EC$_{50}$ measurements for glycine of 0.43 ± 0.14 and 0.26 ± 0.06 mM in the absence and presence of felbamate were not significantly different (n = 5; P = .26). The maximum response for NR1-2A receptors was reduced by only about 30% (Fig. 2C). Glycine EC$_{50}$ measurements of 3.91 ± 0.49 and 4.25 ± 0.31 mM were not significantly different from each other (n = 5; P = .57). The mean EC$_{50}$ measurements for NR1-2C receptors were 0.38 ± 0.04 and 0.34 ± 0.04 mM (n = 6; P = .19), and the maximum response was reduced by 41% (Fig. 2D).

The concentrations of felbamate typically observed in brain tissue are lower than the 1 mM concentration we tested in these studies; therefore, we repeated the glycine concentration-response curve in 0.5 mM felbamate, a concentration where block of the NR1-2B receptor showed no voltage dependence (Fig. 2B; voltage dependence of a J-shaped current-voltage relation in the presence of 1 mM felbamate (Fig. 4A)). No significant effect of voltage was found with a corresponding increase in the agonist EC$_{50}$ without an increase in the NMDA EC$_{50}$ measurements for all three subtypes (Fig. 3, A, C, and D). Interestingly, for NR1-2B receptors, felbamate produce a significant 3.5-fold shift to the left in the NMDA concentration-response curve (EC$_{50}$ = 18.8 ± 1.5 mM; EC$_{50}$ + felbamate = 5.3 ± 1.4 mM; n = 6, P < .002), indicating an enhancement of receptor affinity for NMDA (Fig. 3B). No changes in the NMDA EC$_{50}$ measurements were observed for NR1-2A (Fig. 3C) or NR1-2C (Fig. 3D) receptors with exposure to felbamate (NR1-2A: EC$_{50}$ = 23.7 ± 2.63 mM, EC$_{50}$ + felbamate = 20.4 ± 2.39 mM, n = 6, P = .48; NR1-2C: EC$_{50}$ = 25.4 ± 4.74 mM, EC$_{50}$ + felbamate = 19.7 ± 4.5 mM, n = 3, P = .61).

**Voltage Dependence.** The voltage dependence of felbamate inhibition was investigated in different subtypes of NMDA receptor. Previous evidence suggests strong voltage dependence of block by many antagonists that presumably bind to sites within the channel. Ifenprodil has been shown to block the NR1-2A receptor in a voltage-dependent manner, whereas block of the NR1-2B receptor showed no voltage dependence (Williams, 1993). Current-voltage curves for NR1-2B receptors were generated by stepping the voltage from −100 to +10 mV in increments of 10 mV. No voltage sensitivity of block was apparent, as determined by the lack of a J-shaped current-voltage relation in the presence of felbamate (Fig. 4A). No significant effect of voltage was found on the fractional block induced by 0.3 mM (F = 0.92, P = .47) or 1 mM felbamate (F = 0.16, P = .92) at four selected voltages (Fig. 4B; n = 3). In separate experiments with NR1-2B receptors in which percent block was examined at three voltages (−100, −60, and −30 mV), no effect of voltage was observed on block by 0.9 mM felbamate (n = 7, data not shown). Oocytes expressing the NR1-2A and NR1-2C receptor subtypes were tested at three or four voltages for the fractional block of NMDA/glycine-induced currents produced without significantly altering the glycine EC$_{50}$ measurements for glycine (see text).
Felbamate inhibition of mutant receptors. Felbamate is similar to the NMDA receptor antagonists ifenprodil and haloperidol in that it interacts selectively with the NR1-2B receptor compared with the other subtypes (Williams, 1993; Ilyin et al., 1996; Lynch and Gallagher, 1996). It is possible, therefore, that felbamate interacts with NR1-2B receptors at a site similar to the ifenprodil- and/or haloperidol-binding sites. To investigate this possibility, a mutant of the e2 subunit [e2(E201R)] that is less sensitive to inhibition by haloperidol (Gallagher et al., 1998) and the ifenprodil analog CP101,606 (Brimecombe et al., 1998) was expressed with rat NR1a in oocytes. The sensitivity of this receptor to felbamate was compared with NR1-2B receptors and receptors composed of NR1 and wild-type e2. Additionally, because Rho et al. (1994) and Subramanian et al. (1995) suggested that felbamate acts within the ion channel, the NR1a (N616R) mutant that is less sensitive to blockade by Mg2+ and N-[1-(2-thienyl)cyclohexyl]piperidine (Kawajiri and Dingledine, 1993) was expressed along with the NR2B subunit [NR1(N616R)-2B receptors].

In preliminary experiments, the extent of block produced by 1 mM felbamate was reduced by 58% in NR1-e2(E201R) receptors compared with NR1-2B. The extent of felbamate block of NR1(N616R)-2B receptors, on the other hand, was reduced by only 30% compared with NR1-2B receptors. Therefore, NR1-e2(E201R) receptors were further investigated for sensitivity to felbamate and were compared with wild-type NR1-e2 receptors.

The currents induced by 100 μM NMDA and 10 μM glycine in oocytes expressing the NR1-e2 and mutant receptors were quite large. To minimize run-up of currents common to responses this large, 50 nl of 25 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid was injected into each cell before recording (Williams, 1993), and the felbamate concentration-response curves were carried out differently from previous experiments. NMDA and glycine were applied, and the currents were allowed to plateau before switching to solutions containing NMDA, glycine, and felbamate (Fig. 5, A and B). The currents in the presence of felbamate were expressed as a fraction of the NMDA/glycine current just before felbamate application.

Felbamate decreased NMDA/glycine-induced current in NR1-e2 receptors in a concentration-dependent manner with an IC50 value of 0.78 ± 0.07 mM, similar to that shown for NR1-2B. Felbamate was slightly less effective on NR1-2B receptors. The concentration-response curve was shifted to the right, with a 2-fold increase in the IC50 to 1.56 ± 0.16 mM (Fig. 5C; n = 10, P < .001). The rightward shift produced by the mutation indicates a significant disruption of the ability of felbamate to inhibit these receptors.
Interestingly, haloperidol inhibition of NMDA/glycine-mediated currents was only slightly and nonsignificantly affected by the mutation. The IC50 value for inhibition of NR1-e2 receptors was 13.2 ± 2.2 μM, whereas that for the mutant was 17.3 ± 1.9 μM (n = 4, P = .16; data not shown).

**Effect of pH on Felbamate Inhibition of NR1-2B Receptors.** Another characteristic of ifenprodil inhibition of NR1-2B receptors is dependence on protons. Protons have been shown to enhance the ifenprodil IC50 value by 30-fold over a range of pH from 8.5 to 7.0 (Pahk and Williams, 1997). We tested the effect of pH on felbamate inhibition of NR1-e2 receptors by constructing felbamate concentration-response curves in extracellular solutions of differing pH (6.8, 7.4, and 8.3). Unlike their effects on ifenprodil inhibition, protons had no effect on the IC50 value for felbamate inhibition of currents through NR1-e2 receptors (Fig. 5D; F = 1.54, P = .25). IC50 values were 0.72 ± 0.13, 0.8 ± 0.16, and 0.98 ± 0.06 mM for pH 6.8, 7.4, and 8.3, respectively.

**Discussion**

**Subtype Selectivity and Clinical Relevance.** A major finding of this study is that felbamate interacts with moderate selectivity with the NR1-2B subtype of NMDA receptor at concentrations that are clinically relevant. The reported IC50 measurements from this study (0.93 mM for NR1-2B and 0.78 mM for NR1-e2) are only slightly higher than reported brain concentrations of felbamate in animals (McCabe et al., 1993) and humans (Adusumalli et al., 1994; Troupin et al., 1997) after the administration of doses that show anticonvulsant effects. However, our measurements probably overestimate the actual IC50 values (underestimate affinity) because of the poor solubility of the drug. Although all felbamate solutions were heated and stirred until dissolved, by the end of some experiments, solutions containing the highest concentration of felbamate used, 3 mM, had formed precipitates. At least in some cases, therefore, the concentration of felbamate applied to cells was lower than expected. Dimethyl sulfoxide was not used in these experiments because it did not consistently aid in dissolving felbamate in our hands. With this in mind, our reported IC50 values for NR1-2B receptors are consistent with measurements from binding studies (0.3–0.46 mM; McCabe et al., 1993, 1998; Wamsley et al., 1994) and with inhibitory concentrations (0.1–1.3 mM) used in physiological experiments of NMDA receptors from cortical neurons (Kanthasamy et al., 1995), hippocampal neurons (Taylor et al., 1995), and hippocampal slices (Wallis and Panizzon, 1993; Pugliese et al., 1996).

The interaction of felbamate with NR1-2B receptors at...
clinically relevant concentrations may reflect its therapeutic actions. Felbamate is used predominantly for seizures associated with the Lennox-Gastaut syndrome and in cases of medically refractory complex partial seizures (reviewed in Pellock and Brodie, 1997). The NR2B subunit of NMDA receptors has been reported to be present at high levels in developing rat brain (Monyer et al., 1994). Furthermore, the presence of NR2B during early development (and the relative absence of NR2A) correlates with high glycine affinity and increased sensitivity to the NR2B-selective antagonist ifenprodil (Kew et al., 1998). The usefulness of felbamate in Lennox-Gastaut syndrome, a form of epilepsy occurring primarily in children, might therefore be a consequence of the predominance of the NR2B subunit at this developmental stage. However, caution is warranted in this interpretation because the developmental distribution of human NR2B subunits is unknown. In adult rats, NR2B receptors are distributed, albeit at lower levels, in the hippocampus and cortex of adults (Monyer et al., 1994; Kew et al., 1998). The hippocampus is implicated in medically refractory complex partial seizures in adults (Engel and Pedley, 1998).

NR2B-selective interactions have been observed for several other modulators of NMDA receptor function, including ifenprodil, polyamines, protons, and haloperidol. For example, glycine-independent stimulation of NMDA/glycine-induced currents by spermidine is limited to recombinant NMDA receptors containing the NR2B subunit (Gallagher et al., 1997). In addition, ifenprodil has been found to be 140- to 400-fold selective for receptors composed of NR1 and NR2B subunits compared with those containing the NR2A subunit (Williams, 1993; Gallagher et al., 1996). Similarly, haloperidol has been shown to have 100-fold selectivity for inhibition of currents through NR1-2B receptors expressed in X. laevis oocytes (Ilyin et al., 1996) and 8- to 10-fold selectivity for inhibition of [3H]MK-801 binding to transfected human embryonic kidney 293 cells (Lynch and Gallagher, 1996). The 9.2-fold selectivity of felbamate for NR1-2B versus NR1-2A receptors places it into the category of NR2B-selective drugs and suggests a possible similarity in the mechanisms of action of these drugs.

Mechanism of Action of Felbamate on NMDA Receptors. The data presented here suggest that felbamate is a noncompetitive inhibitor of NMDA receptors with respect to the coagonists NMDA and glycine. Felbamate reduced the maximum currents generated by NMDA and glycine without causing the increase in the agonist EC50 measurements expected of a competitive antagonist. Previous reports indicating a lack of the ability of glycine to overcome inhibition of Ca2+ influx by felbamate (Kanthasamy et al., 1995; Taylor et al., 1995) or to shift the glycine EC50 for NMDA receptors (Rho et al., 1994) also support a noncompetitive mechanism. Although it is possible that the inability of glycine to overcome felbamate inhibition is attributable to the high concentrations used in some studies (1–3 mM), we found that inhibition of NR1-2B receptors by 0.5 mM felbamate, a concentration within the therapeutic window (McCabe et al., 1993), could not be overcome by glycine. Furthermore, a recent study showed that felbamate did not inhibit, but
rather enhanced, binding of [3H]glycine to rat brain membranes (McCabe et al., 1998). Therefore, we conclude that felbamate does not compete with glycine for the strychnine-insensitive glycine site on recombinant NMDA receptors.

A channel site of action for felbamate has been suggested based on single-channel evidence showing a flickering block induced by 3 mM felbamate in NMDA receptors from rat hippocampal neurons (Rho et al., 1994; Subramanian et al., 1995). Felbamate (1 mM) has also been shown to inhibit binding of [3H]MK-801 to rat forebrain membranes (Subramanian et al., 1995, but see White et al., 1992). Our results, which indicate no voltage dependence of felbamate inhibition at any receptor subtype tested, do not necessarily contradict this evidence for a channel site. Although many channel blockers do show voltage dependence of inhibition [Mg2+], MK-801, N-[1-(2-thienyl)cyclohexyl]piperidine, tricyclic antidepressants], these compounds are charged at physiological pH. Felbamate, a dicarbamate, has no ionizable groups and is therefore not charged at physiological pH (H. J. Mortgo, personal communication). In our hands, felbamate did not exhibit activity or use dependence (data not shown), which, if present, would also suggest a channel site. However, Fig. 5 shows that felbamate dissociates from NR1-e2 receptors more rapidly than NMDA or glycine (indicated by the transient off responses in Fig. 5, A and B), so we would be unlikely to observe use dependence in this system.

The lack of voltage and glycine dependence of felbamate block at NR1-2B receptors suggests a similarity in the mechanisms of action of felbamate and other noncompetitive, NR2B-selective antagonists, such as haloperidol and ifenprodil. A similar mechanism is also suggested by the observed shift in the NMDA affinity by felbamate, which also has been shown for ifenprodil (Kew et al., 1996). To further assess the mechanism, we tested an NR2B mutant [e2 (E201R)] that has been reported to have a reduced sensitivity to haloperidol, the ifenprodil analog CP-101,606, polyamine stimulation, and protons (Gallagher et al., 1997; Brimicombe et al., 1998; Gallagher et al., 1998). Receptors composed of NR1 and e2(E201R) subunits showed a 2-fold reduction in sensitivity to felbamate compared with NR1-e2 receptors, suggesting that the glutamate at amino acid 201 (E201) influences felbamate binding, albeit only to a small degree. The small magnitude of effect suggests that E201 is not likely to be an integral part of the felbamate-binding site.

One mechanism by which noncompetitive, NR2B-selective antagonists have been suggested to act is by increasing proton inhibition (Zhang et al., 1997). Protons have been shown to reciprocally enhance ifenprodil sensitivity as well (Pahk et al., 1999). Therefore, we suggest that felbamate might interact with a unique portion of a complex of overlapping sites to which other modulators also bind (Gallagher et al., 1998). The felbamate site or sites also affect channel properties (Rho et al., 1994; Subramanian et al., 1995), suggesting some form of association with the channel pore as well.

In conclusion, the selectivity of felbamate for NMDA receptors containing the developmentally regulated NR2B subunit indicates that the unique anticonvulsant profile of this drug may be due to its interaction with these receptors. Furthermore, this evidence strengthens the need for continued targeted drug design of compounds that interact with specific subtypes of NMDA receptors.

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