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Ifenprodil Discriminates Subtypes of the *N*-Methyl-D-aspartate Receptor: Selectivity and Mechanisms at Recombinant Heteromeric Receptors

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SUMMARY

The effects of the atypical *N*-methyl-D-aspartate (NMDA) receptor antagonist ifenprodil were investigated by voltage-clamp recording of *Xenopus* oocytes expressing heteromeric NMDA receptors from cloned NR1 and NR2 subunit RNAs. In oocytes voltage-clamped at −70 mV, ifenprodil inhibited NMDA-induced currents at NR1A/NR2B receptors with high affinity (IC_{50} = 0.34 μM). The affinity of NR1A/NR2A receptors for ifenprodil (IC_{50} = 146 μM) was 400-fold lower than that of NR1A/NR2B receptors. The rate of onset of inhibition by low concentrations of ifenprodil acting at NR1A/NR2B receptors was considerably slower than the onset of inhibition seen with high concentrations of ifenprodil acting at NR1A/NR2A receptors. The onset and recovery of blockade by ifenprodil at NR1A/NR2B receptors were not activity dependent. The inhibitory effects of low concentrations of ifenprodil at NR1A/NR2B receptors were not voltage dependent. In contrast, the inhibitory effects of high concentrations of ifenprodil at NR1A/NR2A receptors were partially voltage dependent, and a greater inhibition of NMDA-induced currents was seen at hyperpolarized membrane potentials than at depolarized membrane potentials. The reversal potential of NMDA currents was not altered in the presence of ifenprodil. Ifenprodil may act as a weak open-channel blocker of NR1A/NR2A receptors. The degree of inhibition seen with 100 μM ifenprodil at NR1A/NR2A receptors was not altered by changes in the concentration of extracellular glycine. However, the inhibitory effect of 1 μM ifenprodil at NR1A/NR2B receptors was reduced by increasing the concentration of glycine. Thus, part of the mechanism of action of ifenprodil at NR1A/NR2B receptors may involve noncompetitive antagonism of the effects of glycine. These results indicate that the mechanism of action of ifenprodil, as well as the potency of this antagonist, is different at NR1A/NR2B and NR1A/NR2A receptors expressed in *Xenopus* oocytes.

The NMDA subtype of glutamate receptor is involved in the generation of various forms of synaptic plasticity in the vertebrate central nervous system and in neuropathologies including ischemic neuronal cell death, epilepsy, and neurodegenerative diseases (1-5). NMDA receptors present a potential target for therapeutic intervention in these pathologies. However, because of the widespread distribution of NMDA receptors in the central nervous system, antagonists or modulators acting at these receptors are likely to have a variety of effects on nervous system functioning. Targeting particular subtypes or subpopulations of NMDA receptors may overcome this limitation. Heterogeneity in the pharmacological properties of NMDA receptors has been proposed based on regional differences in the affinity of NMDA receptors for glycine, for antagonists acting at the glutamate recognition site, and for the open-channel blocker MK-801 (6, 7). However, regional differences in the affinities of NMDA receptors for MK-801 and glutamate site antagonists are relatively modest (2-5-fold).

Ifenprodil is an atypical noncompetitive antagonist at the NMDA receptor (8, 9). The mechanism of action of ifenprodil has not been clearly defined, but it may act to promote a modal shift in channel gating properties and to decrease the affinity of the NMDA receptor for the coagonist glycine (9). We have recently shown that ifenprodil can discriminate two populations of NMDA receptors in rat brain with >100-fold selectivity (10). Ifenprodil interacts with high affinity (IC_{50} = 0.3 μM) at a homogeneous population of NMDA receptors in neonatal rat forebrain. During postnatal development, a second population of receptors having a 100-300-fold lower affinity for ifenprodil is coexpressed and represents 50% of the NMDA receptors in adult rat forebrain (10). NMDA receptors expressed in *Xenopus* oocytes injected with RNA prepared from neonatal rat brain have a high affinity for ifenprodil. Injection of oocytes with RNA prepared from adult rat brain leads to the expression of receptors having high and low affinities for ifenprodil (10). This

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 5′ UTR, 5′ untranslated region.

suggests that the delayed developmental appearance of NMDA receptors having a low affinity for ifenprodil is due to changes in the expression of mRNAs coding for subunits of the NMDA receptor during development (10).

Over the last 2 years, cDNAs coding for a number of subunits of the NMDA receptor have been cloned. These include the NMDAR1 (NR1), NR2A, NR2B, NR2C, and NR2D genes isolated from rat brain (11–13). Equivalent genes, termed ζ1, ε1, ε2, ε3, and ε4, respectively, have been cloned from mouse brain (14–16). The NR1 (ζ1) subunit can form functional homomeric NMDA receptor/channels when expressed in *Xenopus* oocytes, but whole-cell currents activated by these receptors are very small (11). Much larger NMDA-evoked currents are seen after coexpression of NR1 and NR2 subunits, suggesting that native NMDA receptors are composed of combinations of NR1 and NR2 subunits (12–15). The subunit composition of brain NMDA receptors has not yet been defined but, by analogy with other ligand-gated ion channels, the receptors are likely to be hetero-oligomeric complexes composed of four or five interacting subunits (17). Regional differences in the expression of mRNAs coding for NR2 subunits have been reported (12, 18). Furthermore, heteromeric receptors containing different NR2 subunits have differing affinities for glutamate, glycine, and open-channel blockers (13, 15). Thus, differences in NR2 subunit composition may underlie differences in the affinity of native NMDA receptors for glycine, glutamate site antagonists, and MK-801. The existence of eight isoforms of NR1, termed NR1A to NR1H, which arise through alternative splicing of RNA, has been reported (19, 20). Thus, inclusion of different NR1 splice variants in native NMDA receptors may alter their functional and pharmacological properties.

Homomeric NR1A receptors expressed in *Xenopus* oocytes have a high affinity for ifenprodil (10). In preliminary studies, it was found that heteromeric NR1A/NR2B receptors were potently inhibited by ifenprodil. However, ifenprodil was only a very weak antagonist at heteromeric NR1A/NR2A receptors, suggesting that NR1A/NR2A receptors may have a low affinity binding site for ifenprodil (10). It was proposed that delayed developmental expression of the NR2A subunit in rat brain is responsible for the late developmental appearance of NMDA receptors having a low affinity for ifenprodil (10). This proposal is supported by the observation that mRNA encoding the ε2 (NR2B) subunit is expressed in mouse forebrain at high levels throughout postnatal development, whereas mRNA encoding the ε1 (NR2A) subunit is present only at very low levels in neonatal mouse brain and expression of ε1 mRNA increases during postnatal days 7–21 (21). In the work reported in this paper, the effects of ifenprodil on recombinant heteromeric NMDA receptors expressed in *Xenopus* oocytes have been investigated. The affinities of heteromeric receptors for ifenprodil and the mechanism of action of this antagonist were studied.

Materials and Methods

NMDA receptor subunit clones. The rat brain NR1A clone (11) was a gift from Dr. S. Nakanishi (Kyoto University, Kyoto, Japan). The NR2A and NR2B clones (12) were a gift from Dr. P. H. Seeburg (University of Heidelberg, Heidelberg, Germany). Most of the 5′ UTR of the NR2 clones was removed before synthesis of cRNAs. This resulted in better expression of heteromeric channels, as assessed by the size of macroscopic NMDA-induced currents (see Results). Capped cRNAs were synthesized *in vitro* from each of the cDNA templates, as described previously (10). RNA was stored in aliquots at −80°.

***Xenopus* oocyte injection and recording.** Defolliculated stage V-VI oocytes were prepared from adult *Xenopus laevis* (Nasco, Fort Atkinson, WI) as described previously (10). Oocytes were injected in the vegetal pole with cRNAs (4 ng of NR1 plus 20 ng of NR2A or NR2B; 50-nl total volume). NR1 and NR2 RNAs were injected in a ratio of 1:5 to minimize the formation of homomeric NR1 receptors. Oocytes were maintained in a saline solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Na-HEPES, 2.5 mM sodium pyruvate, 50 μg/ml gentamycin, pH 7.6) at 18° for 3–7 days before recordings were made. The saline solution was replaced daily.

For recording, oocytes were positioned in a small Perspex chamber and continuously superfused (5–10 ml/min) with a Mg^{2+}-free saline solution (96 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, 10 mM Na-HEPES, pH 7.6). Unless otherwise indicated, the recording solution contained $BaCl_2$ rather than $CaCl_2$ to minimize a Ca^{2+}-activated Cl^- current (22). Ba^{2+} has a slightly higher permeability than Ca^{2+} through the ion channel of NMDA receptors but does not activate an endogenous Ca^{2+}-sensitive Cl^- current in *Xenopus* oocytes (22). In most experiments oocytes were injected with BAPTA on the day of recording, to eliminate a slowly activating inward current (see Results). Oocytes were injected with 50–100 nl of BAPTA (40 or 100 mM), 10–300 min before recording. Unless otherwise indicated, NMDA and ifenprodil were applied in solutions containing 10 μM glycine. Currents were recorded from oocytes using a two-electrode voltage-clamp with an OC-725 Ooclamp amplifier (Warner Instruments, Hamden, CT). Electrodes, pulled from borosilicate glass and filled with 3 M KCl, had resistances of 0.5–4 MΩ. Oocytes were voltage-clamped at a holding potential of −70 mV except in studies where the voltage dependence of NMDA responses was examined. Current and membrane potential signals were digitized using a 16-bit MacPaq-100 interface with AcqKnowledge software (Biopac Systems, Goletta, CA; World Precision Instruments, Sarasota, FL) and were stored on the hard disk of a Macintosh computer. The current signal was low-pass filtered (eight-pole Bessel) at a corner frequency less than or equal to one third of the sampling frequency, and signals were digitized at 20–100 Hz. In some experiments, data were digitally refiltered (low-pass Bessel, 2.5–5 Hz) for analysis and presentation. Current-voltage (I-V) curves were measured by using voltage ramps during steady state responses induced by NMDA or NMDA plus ifenprodil. The command potential was ramped from −100 mV to +40 mV over a period of 2.5 sec, and the membrane potential and holding current were recorded during the ramp. Leak currents, measured in control ramps applied immediately before test ramps, were digitally subtracted from currents measured in the presence of NMDA or NMDA plus ifenprodil.

Materials. Ifenprodil was a gift from Synthélabo Recherche (Bagneux, France). NMDA was purchased from Research Biochemicals Inc. (Natick, MA). Glycine was purchased from Sigma Chemical Co. (St. Louis, MO). Molecular biology reagents and restriction enzymes were purchased from Pharmacia (Piscataway, NJ), Boehringer Mannheim (Indianapolis, IN), and Promega (Madison, WI).

Results

Expression of heteromeric NMDA receptors and recording conditions. In initial studies of heteromeric NR1/NR2 receptors expressed in *Xenopus* oocytes, macroscopic currents activated by NR1A/NR2A or NR1A/NR2B receptors were 5–15-fold larger than those seen with homomeric NR1A receptors in the same batch of oocytes (10). However, macroscopic currents activated by heteromeric NR1/NR2 receptors were 5–20-fold smaller than those seen with NMDA receptors expressed from rat brain RNA.[1] It was reasoned that the level

[1] K. Williams, unpublished observations.

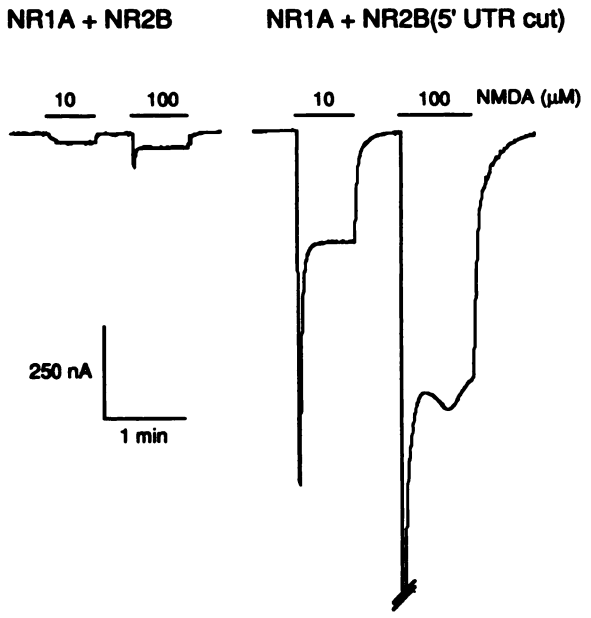

Fig. 1. Truncation of the 5′ UTR of NR2 subunits improves the expression of heteromeric NMDA receptors. Oocytes were injected with cRNAs coding for NR1A and NR2B subunits. NR2B RNA was prepared from a clone having a 5′ UTR of 1400 nucleotides (*left*) or a 5′ UTR of 33 nucleotides (*5′ UTR cut*) (*right*). Responses to NMDA (10 and 100 μM), in the presence of 10 μM glycine, were measured 3 days after injection. Inward currents are *downward*. Oocytes were voltage-clamped at −70 mV, and the extracellular solution contained 1.8 mM $CaCl_2$. The peak response to 100 μM NMDA in the NR1A/NR2B(5′ UTR cut) cell, which has been excluded for presentation, was −2.98 μA.

of expression of cloned NR2 subunits in oocytes may be low, leading to inefficient expression of subunits and assembly of heteromeric channels. The entire 5′ UTR of each of the NR2 clones was sequenced. The 5′ UTR of NR2A contains 342 nucleotides and that of NR2B contains 1400 nucleotides. In both sequences there exist multiple ATG (translation initiation) codons before the ATG that initiates the open reading frame for the NR2 protein. It is possible that the presence of these codons, or other features in the 5′ UTR of these genes, reduces the expression of full length functional NR2 proteins in oocytes. Most of the 5′ UTR was removed from the NR2A and NR2B clones, leaving a 5′ UTR of 80 nucleotides in NR2A and 33 nucleotides in NR2B. Coexpression of NR1A with NR2 subunits having a truncated 5′ UTR generated NMDA receptors that gate macroscopic currents 10–500-fold larger than those seen with the original NR2 clones (Fig. 1). After truncation of the 5′ UTR in NR2 clones, oocytes injected with cRNA from the NR2A or NR2B clones alone (without NR1) did not show any response to NMDA (30 μM; with 10 μM glycine), indicating that NR2 subunits with a truncated 5′ UTR do not form functional homomeric channels (data not shown). The NR2 clones having a truncated 5′ UTR were used in subsequent experiments for studies of heteromeric NR1/NR2 receptors.

When responses were measured in an extracellular solution containing 1.8 mM $CaCl_2$, NMDA produced inward currents consisting of a transient peak followed by a "plateau" phase (Fig. 1). In some cells the plateau had a slowly rising phase or slowly rising and falling phases. Both the quickly decaying peak and the slow waves in the plateau phase have been observed in many studies of NMDA receptors expressed from brain RNA and from cloned subunits (e.g., Refs. 10, 11, 13, and 22). Both phenomena appear to be due to activation of Ca^{2+}-dependent Cl^- currents (22). To facilitate qualitative and quantitative studies of the pharmacological properties of heteromeric NMDA receptors, conditions under which the Ca^{2+}-activated Cl^- currents were absent or negligible were sought. Replacement of extracellular $CaCl_2$ with $BaCl_2$ abolished the quickly inactivating peak in most cells. However, even in the presence of Ba^{2+}, a slowly rising phase of the inward current was seen in many cells when currents were >200 nA. Under these conditions, a steady state current was not observed even after several minutes of application of NMDA (Fig. 2, *left*). The reversal potential of these currents was −18 to −25 mV, close to the Cl^- equilibrium potential (22, 23), suggesting that a secondary Cl^- current contributes to the rising phase (data not shown). When recordings were carried out in Ba^{2+}-saline using oocytes injected with the Ca^{2+}-chelating agent BAPTA, the slowly rising phase of the inward current was not seen and steady state currents were obtained (Fig. 2, *right*). Thus, for analyses of the effects of ifenprodil, oocytes were injected with BAPTA and recordings were carried out using Ba^{2+}-saline unless otherwise indicated.

Effects of ifenprodil on heteromeric NMDA receptors. The effects of ifenprodil on heteromeric NR1A/NR2B and NR1A/NR2A receptors were investigated. Ifenprodil by itself did not induce currents or alter membrane resistance (data not shown). Coapplication of ifenprodil (0.01–300 μM) inhibited NMDA responses at NR1A/NR2B and NR1A/NR2A receptors (Fig. 3A). At NR1A/NR2B receptors, the rate of block by ifenprodil was concentration dependent, being faster with higher concentrations of ifenprodil (Fig. 3A). Ifenprodil inhibited NMDA currents at NR1A/NR2B receptors with high affinity (IC_{50} = 0.34 μM) (Fig. 3B). However, the inhibition of NR1A/NR2B receptors was incomplete, and NMDA responses were observed even in the presence of 100–300 μM ifenprodil (Fig. 3B). In contrast to NR1A/NR2B receptors, the onset of blockade by ifenprodil at NR1A/NR2A receptors was rapid and occurred within the time of the solution exchange (Fig. 3A). The affinity of NR1A/NR2A receptors for ifenprodil (IC_{50} = 146 μM) was 400-fold lower than that of NR1A/NR2B receptors

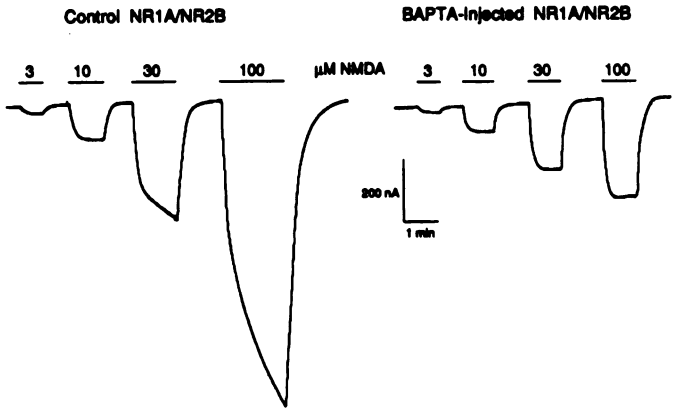

Fig. 2. BAPTA injection eliminates a slowly rising inward current. Responses to NMDA (3–100 μM), in the presence of 10 μM glycine, were measured in an extracellular solution containing 1.8 mM $BaCl_2$, 3 days after injection of oocytes with NR1A plus NR2B subunit RNAs. *Right*, currents recorded from an oocyte injected with BAPTA 60 min before recording was started; *left*, currents from a cell, from the same batch of RNA-injected oocytes, that was not injected with BAPTA (control). Inward currents are *downward*. Oocytes were voltage-clamped at −70 mV.

(Fig. 3B). The effects of ifenprodil at concentrations above 300 μM were not examined.

Experiments were carried out to determine whether the onset of blockade by ifenprodil at NR1A/NR2B receptors was activity dependent. Fig. 4 shows results from an experiment in which the inhibitory effect of 0.3 μM ifenprodil was measured during coapplication with NMDA (Fig. 4A, *left*) and during coapplication after a 3-min pre-exposure of the oocyte to ifenprodil (Fig. 4A, *right*). A steady state block of the NMDA-induced current was achieved much more quickly during coapplication of NMDA and ifenprodil when the cell had been pre-exposed to ifenprodil than without pre-exposure (Fig. 4B). Thus, the slow onset of blockade by ifenprodil at NR1A/NR2B receptors is time dependent rather than activity dependent.

The recovery from inhibition by ifenprodil at NR1A/NR2B receptors was slow, requiring 6–8 min (Fig. 5A). Experiments were carried out to determine whether the slow rate of recovery from ifenprodil inhibition at NR1A/NR2B receptors was activity dependent. Fig. 5A shows results from an experiment in which the recovery of the NMDA response from inhibition by ifenprodil was monitored for 7 min in the continued presence of NMDA (Fig. 5A, *left*) and after a 7-min wash-out in the absence of NMDA (Fig. 5A, *right*). The NMDA response recovered completely after wash-out in the absence of NMDA. Thus, the recovery from ifenprodil inhibition at NR1A/NR2B receptors is not activity dependent. Similar experiments were carried out to investigate the recovery from ifenprodil inhibition at NR1A/NR2A receptors (Fig. 5B). When inward currents were measured in the continued presence of NMDA, recovery at NR1A/NR2A receptors was biphasic, with a fast component that accounted for 60–80% of recovery and a slow component

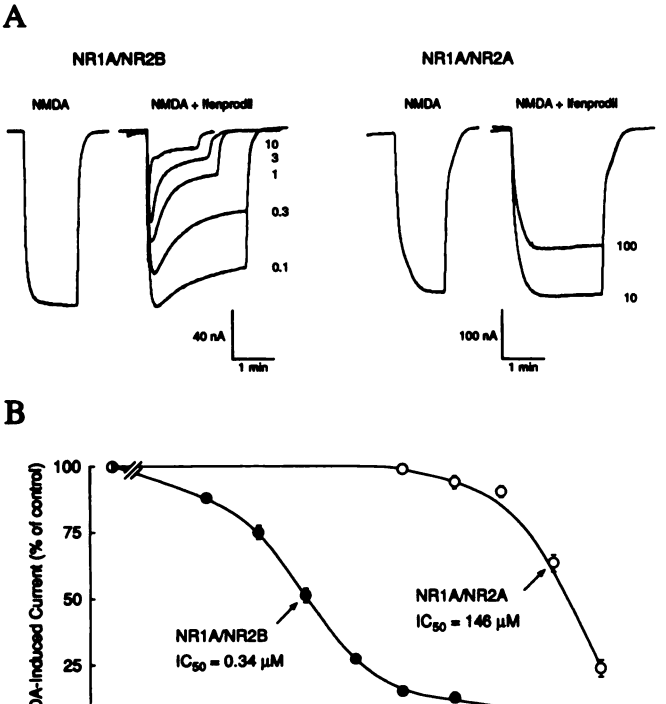

Fig. 3. Selectivity of ifenprodil at heteromeric NMDA receptors. A, Inward currents induced by NMDA (30 μM; with 10 μM glycine) in the absence or presence of 0.1–10 μM ifenprodil (*left*) or 10 and 100 μM ifenprodil (*right*). Oocytes were injected with NR1A plus NR2B (*left*) or NR1A plus NR2A (*right*) subunit RNAs. Oocytes were voltage-clamped at −70 mV, and the extracellular solution contained 1.8 mM BaCl2. The oocyte on the *left* was from the same donor frog as that on the *right*. B, Concentration-inhibition curves for ifenprodil in oocytes expressing heteromeric NR1A/NR2B (●) or NR1A/NR2A (○) receptors. Steady state currents induced by NMDA (30 μM; with 10 μM glycine) were measured in the presence of various concentrations of ifenprodil, and data are expressed as a percentage of control NMDA currents. Values are mean ± standard error from four to six cells at each concentration. Where error bars are not shown they are within the size of the symbol.

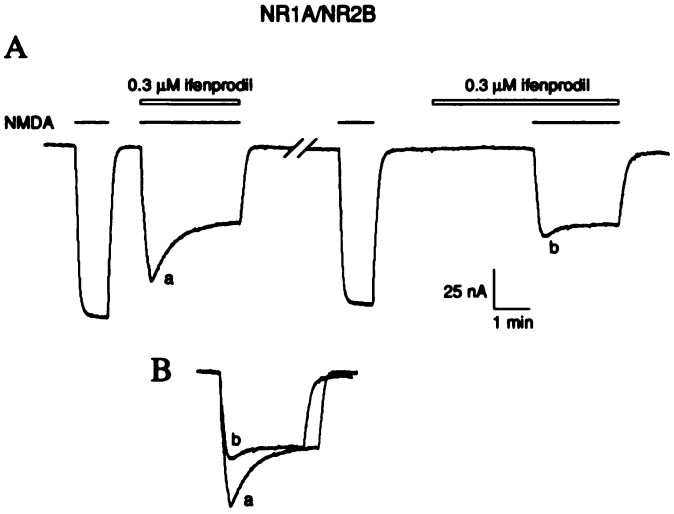

Fig. 4. The onset of blockade by ifenprodil at NR1A/NR2B receptors is not activity dependent. A, The effects of 0.3 μM ifenprodil on responses to NMDA (30 μM; with 10 μM glycine) were determined without (*trace a*) and after (*trace b*) a 3-min pre-exposure of the oocyte to ifenprodil. The break in the recording trace corresponds to a period of 5 min. The oocyte was voltage-clamped at −70 mV and the extracellular solution contained 1.8 mM $BaCl_2$. B, Traces from A have been superimposed. Note that a steady state block is achieved much more quickly after pre-equilibration with ifenprodil (*trace b*).

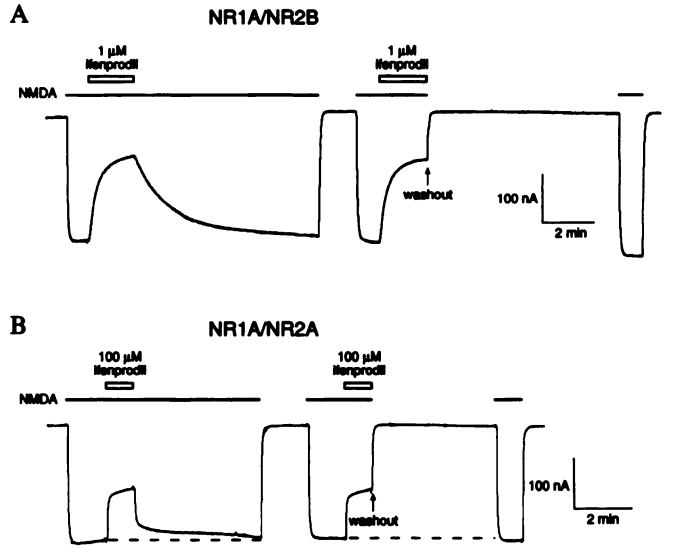

Fig. 5. Onset and recovery of inhibition by ifenprodil. A, The effects of 1 μM ifenprodil were determined in an oocyte expressing NR1A/NR2B receptors. B, The effects of 100 μM ifenprodil were determined in an oocyte expressing NR1A/NR2A receptors. NMDA (30 μM; with 10 μM glycine) and ifenprodil were applied during the times shown by the *horizontal bars*. The extracellular solution contained 1.8 mM $BaCl_2$. Inward currents are *downward*. Similar results were obtained in three or four oocytes for each combination of subunits.

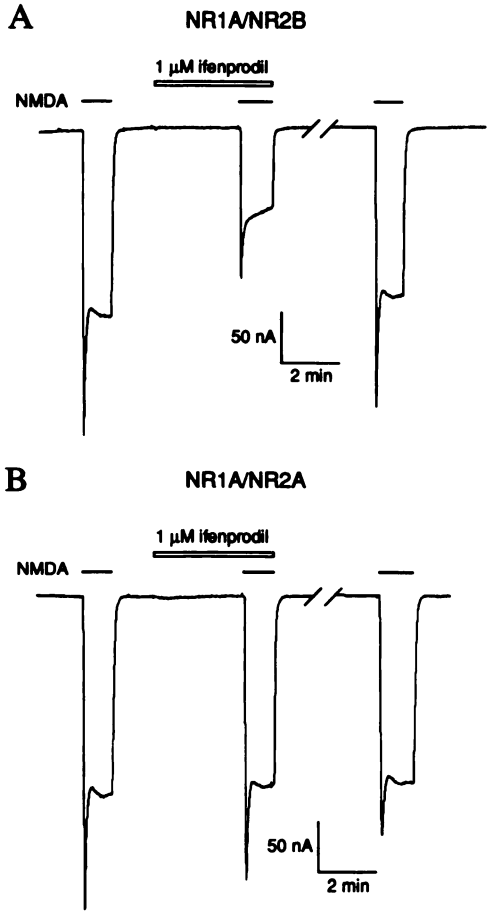

Fig. 6. Effects of ifenprodil measured in the presence of extracellular Ca^{2+}. Responses to NMDA (30 μM; with 10 μM glycine) were determined before, during, and after application of 1 μM ifenprodil in an oocyte expressing NR1A/NR2B receptors (A) and in an oocyte expressing NR1A/NR2A receptors (B). Oocytes were voltage-clamped at −70 mV and the extracellular solution contained 1.8 mM $CaCl_2$. Breaks in the recording traces correspond to a period of 5 min during wash-out of ifenprodil. Similar results were obtained with three oocytes for each combination of subunits.

requiring 4–6 min for complete recovery (Fig. 5B, *left*). Complete recovery from ifenprodil inhibition at NR1A/NR2A receptors was seen after wash-out in the absence of NMDA (Fig. 5B, *right*), indicating that the slow phase of recovery is not activity dependent.

The selectivity of ifenprodil for NR1A/NR2B versus NR1A/NR2A receptors described above was measured in the presence of extracellular Ba^{2+} (substituted for Ca^{2+}) in oocytes injected with BAPTA. The inhibitory effects of ifenprodil on NMDA receptors expressed on cultured neurons were reported to be nonadditive with Ca^{2+}-dependent inactivation (9). To determine whether ifenprodil also shows selectivity for recombinant heteromeric NMDA receptors under more "physiological" conditions, the effects of 1 μM ifenprodil on NR1A/NR2B and NR1A/NR2A receptors were examined in an extracellular solution containing 1.8 mM $CaCl_2$, using oocytes that had not been injected with BAPTA (Fig. 6). For these experiments, we selected oocytes expressing NR1A/NR2B or NR1A/NR2A receptors that produced macroscopic NMDA currents of similar magnitudes and had similar peak and plateau phases when studied in the presence of $CaCl_2$. Ifenprodil (1 μM) markedly inhibited responses of NR1A/NR2B receptors (Fig. 6A) but had no effect on responses of NR1A/NR2A receptors (Fig. 6B). This is similar to effects seen in the presence of $BaCl_2$ using cells injected with BAPTA (Fig. 3). Thus, the selectivity of ifenprodil for NR1A/NR2B receptors over NR1A/NR2A receptors is retained in the presence of physiological concentrations of extracellular Ca^{2+} and when intracellular levels of Ca^{2+} are not buffered by BAPTA.

Voltage dependence of ifenprodil inhibition. The voltage dependence of the inhibitory effects of ifenprodil on NR1A/NR2B and NR1A/NR2A receptors was investigated. Two approaches were used. In one series of experiments, the effects of ifenprodil on steady state NMDA currents were measured in oocytes voltage-clamped at different holding potentials (Figs. 7, A and B, and 8, A and B). In this paradigm, the degree of block seen with 0.5 μM ifenprodil at NR1A/NR2B receptors was the same in cells voltage-clamped at −25, −70, or −100 mV (Fig. 7, A and B). Using a similar paradigm, the degree of inhibition seen with 100 μM ifenprodil at NR1A/NR2A receptors was found to be voltage dependent (Fig. 8, A and B). A

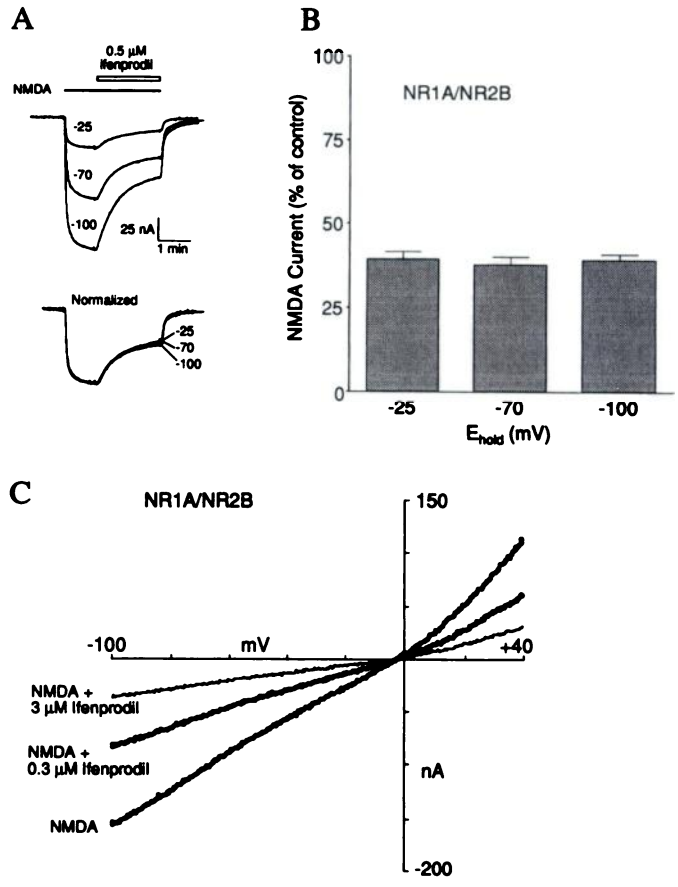

Fig. 7. The inhibitory effects of ifenprodil at NR1A/NR2B receptors are not voltage dependent. A, *Upper traces*, responses to NMDA (30 μM; with 10 μM glycine) were measured in the absence or presence of 0.5 μM ifenprodil in an oocyte voltage-clamped at −25, −70, and −100 mV. *Lower traces*, superimposed responses measured at −25, −70, and −100 mV have been normalized to the peak control response seen at each holding potential. B, Steady state NMDA currents measured in the presence of 0.5 μM ifenprodil are expressed as a percentage of control NMDA responses. Values are mean ± standard error from five oocytes. C, I-V curves were constructed by voltage ramps applied during steady state responses induced by NMDA (30 μM; with 10 μM glycine) or NMDA plus ifenprodil. Leak currents have been subtracted. The extracellular solution contained 1.8 mM $BaCl_2$.

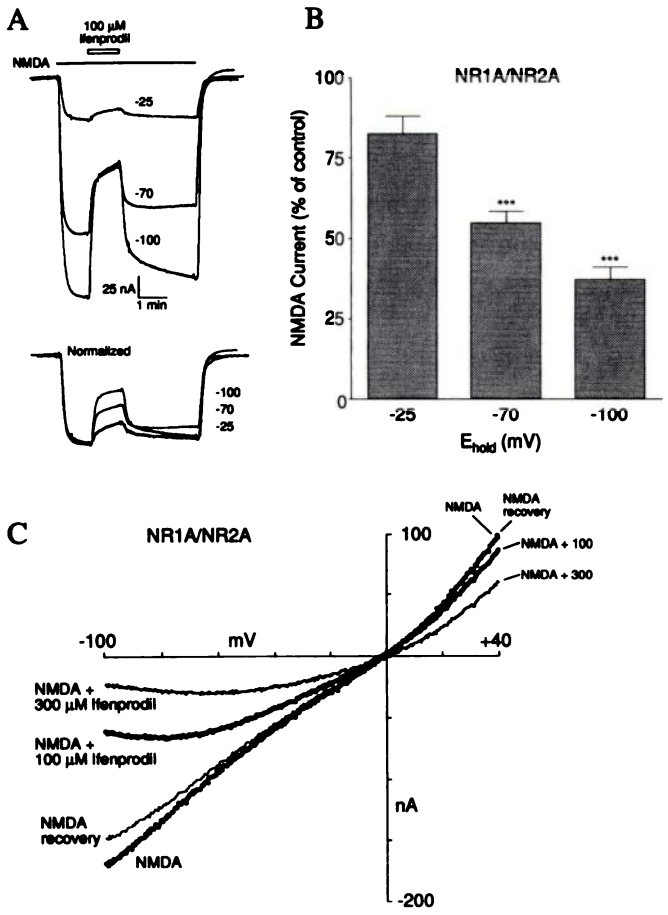

Fig. 8. The inhibitory effects of ifenprodil at NR1A/NR2A receptors are voltage dependent. A, *Upper traces*, responses to NMDA (30 μM; with 10 μM glycine) were measured in the absence or presence of 100 μM ifenprodil in an oocyte voltage-clamped at −25, −70, and −100 mV. *Lower traces*, superimposed responses measured at −25, −70, and −100 mV have been normalized to the peak control response seen at each holding potential. B, Steady state NMDA currents measured in the presence of 100 μM ifenprodil are expressed as a percentage of control NMDA responses measured at each holding potential. Values are mean ± standard error from five oocytes; ***, $p < 0.001$, compared with −25 mV (repeated-measures analysis of variance with *post hoc t* test and Bonferroni correction). C, I-V curves were constructed by voltage ramps applied during steady state responses induced by NMDA (30 μM; with 10 μM glycine) or NMDA plus ifenprodil. The NMDA recovery curve was measured 6 min after wash-out of NMDA plus 300 μM ifenprodil. Leak currents have been subtracted. The extracellular solution contained 1.8 mM $BaCl_2$.

larger inhibition of NMDA currents was seen at more hyperpolarized membrane potentials (−70 and −100 mV) than at −25 mV (Fig. 8, A and B). The rates of onset and recovery of ifenprodil inhibition at NR1A/NR2A receptors were similar at different holding potentials (Fig. 8A).

The second approach used to investigate the effects of membrane potential on the inhibition by ifenprodil was to construct I-V curves by ramping the command potential from −100 to +40 mV during steady state responses (Figs. 7C and 8C). In the absence of ifenprodil, the I-V relationship for NMDA currents at NR1A/NR2B and NR1A/NR2A receptors was nearly linear, with a reversal potential of 0 mV to −8 mV (Figs. 7C and 8C). The inhibition by ifenprodil (0.3–3 μM) of NMDA currents through NR1A/NR2B receptors was not voltage dependent (Fig. 7C). In contrast, the inhibitory effects of 100–300 μM ifenprodil at NR1A/NR2A receptors were voltage dependent. A greater inhibition of NMDA currents was seen at hyperpolarized membrane potentials than at depolarized membrane potentials (Fig. 8C). However, inhibitory effects of ifenprodil were apparent even at +40 mV, suggesting a weak voltage dependence or an additional voltage-independent action of ifenprodil at NR1A/NR2A receptors (Fig. 8C). The reversal potential of NMDA-induced currents at NR1A/NR2B and NR1A/NR2A receptors was not altered in the presence of ifenprodil (Figs. 7C and 8C). The results obtained using voltage ramps are consistent with those seen in studies of steady state NMDA currents. Thus, in two experimental paradigms, the inhibitory effects of low concentrations of ifenprodil acting at NR1A/NR2B receptors were not voltage dependent, whereas those of high concentrations of ifenprodil acting at NR1A/NR2A receptors were voltage dependent.

Interaction with glycine. The inhibitory effects of high concentrations of ifenprodil on NMDA receptors expressed on cultured neurons have been proposed to be due in part to a decrease in the affinity of the NMDA receptors for glycine (9). Experiments were carried out to determine whether the effects of ifenprodil on heteromeric NR1A/NR2B and NR1A/NR2A receptors were dependent on the concentration of glycine. For NR1A/NR2B receptors, responses were measured in the presence of 0.1 μM, 0.3 μM, and 30 μM glycine (Fig. 9). Glycine at 30 μM is suprasaturating at NR1A/NR2B receptors (data not shown). The degree of inhibition by 1 μM ifenprodil was reduced with increasing concentrations of glycine (Fig. 9). Thus, part of the inhibitory effect of ifenprodil at NR1A/NR2B receptors may be due to a noncompetitive antagonism of the effects of glycine.

Higher concentrations of glycine are required to activate NR1A/NR2A receptors than to activate NR1A/NR2B receptors (13, 15). The inhibition by 100 μM ifenprodil of NMDA responses at NR1A/NR2A receptors was determined in the presence of 1 μM and 30 μM glycine (Fig. 10). Because the inhibitory effects of ifenprodil at NR1A/NR2A receptors are voltage dependent (see Fig. 8), experiments were carried out using oocytes voltage-clamped at −25 and −70 mV, to avoid possible masking of glycine-dependent effects of ifenprodil by a separate voltage-dependent inhibition. In oocytes voltage-clamped at −25 or −70 mV, the degree of inhibition by 100 μM ifenprodil was the same when NMDA currents were measured in the presence of 1 μM or 30 μM glycine (Fig. 10). This is in contrast to the glycine-dependent inhibition by ifenprodil seen at NR1A/NR2B receptors (Fig. 9).

Discussion

The effects of ifenprodil on heteromeric NMDA receptors expressed from cloned subunits have been investigated. These studies were prompted by the observation that ifenprodil can distinguish two subtypes of NMDA receptors that are differentially expressed in rat brain during development (10). Ifenprodil has also been reported to inhibit NMDA responses of cultured neurons in a biphasic manner, with high and low affinity components similar to those seen in binding assays with adult rat brain membranes (9). We have proposed that the biphasic inhibition seen with ifenprodil in radioligand binding assays and in oocytes expressing rat brain NMDA receptors (10) reflects interactions at two distinct populations

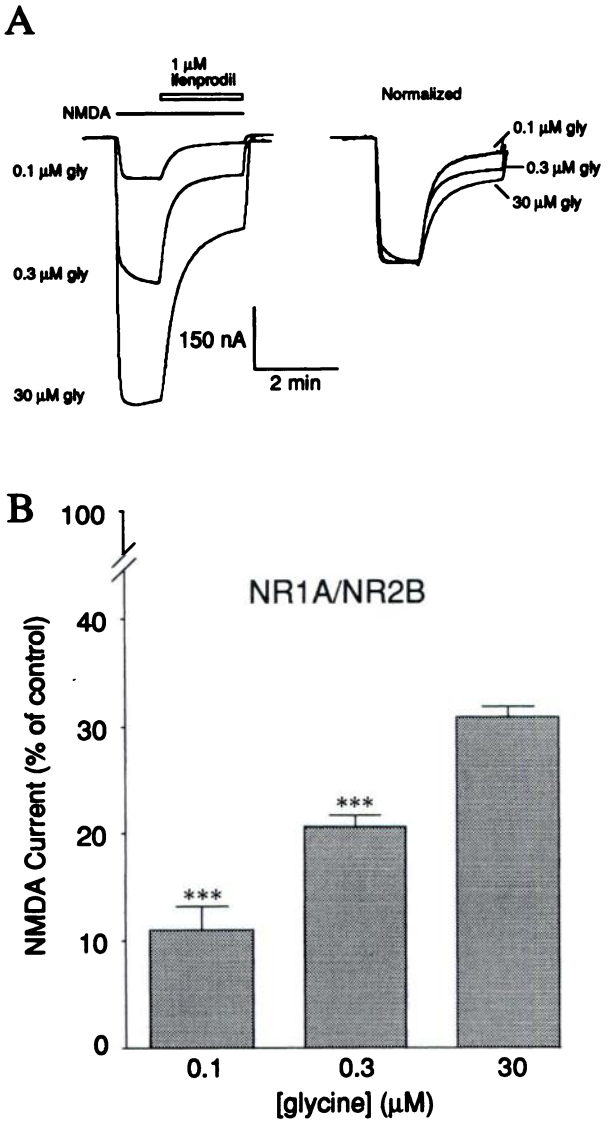

Fig. 9. The effects of ifenprodil at NR1A/NR2B receptors are dependent on the concentration of glycine (*gly*). A, *Left*, inward currents elicited by NMDA (100 μM) and NMDA plus ifenprodil in the presence of 0.1, 0.3, and 30 μM glycine in an oocyte expressing NR1A/NR2B receptors and voltage-clamped at −70 mV. *Right*, superimposed currents measured in the presence of 0.1, 0.3, and 30 μM glycine have been normalized to the peak control responses. B, Steady state NMDA currents measured in the presence of 1 μM ifenprodil are expressed as a percentage of control responses measured in the presence of 0.1, 0.3, and 30 μM glycine. Values are mean ± standard error of seven cells. ***, $p < 0.001$, compared with 30 μM glycine (repeated-measures analysis of variance with *post hoc t* test and Bonferroni correction).

of NMDA receptors, one having a high affinity binding site and the other a low affinity binding site for ifenprodil. Recombinant NR1A/NR2B receptors have a high affinity for ifenprodil, whereas NR1A/NR2A receptors have a low affinity for this antagonist. The affinities measured at recombinant NR1/NR2 receptor subtypes are very similar to those seen in studies of native NMDA receptors (9, 10). Thus, NR1/NR2B and NR1/NR2A receptors may correspond to the two major subtypes of NMDA receptor expressed in adult rat forebrain (10).

One of the objectives of the present work was to compare the mechanisms of action of ifenprodil that have been reported in studies of cultured neurons (9), which express mixed populations of NMDA receptors (9, 10), with the action of ifenprodil at cloned heteromeric NMDA receptors having high or low affinities for this antagonist. Some of the characteristics of the effects of ifenprodil on cloned NMDA receptors were similar to those seen with native NMDA receptors. However, some of the effects of ifenprodil on cloned NMDA receptors were unexpected and differed from those predicted on the basis of studies of native NMDA receptors.

At NR1A/NR2B channels, which have a high affinity for ifenprodil, the apparent rate of block by ifenprodil was concentration dependent. The onset of block was slow in the presence of low concentrations of ifenprodil, requiring several minutes to reach a steady state inhibition. These effects are similar to those of low concentrations (0.1–10 μM) of ifenprodil seen in whole-cell patch-clamp recordings of cultured neurons (9). The effects of ifenprodil at NR1A/NR2B receptors do not appear to be activity dependent. This observation is consistent with results obtained in electrophysiological and biochemical studies of native NMDA receptors, where the effects of ifenprodil were mechanistically different from those of activity-dependent

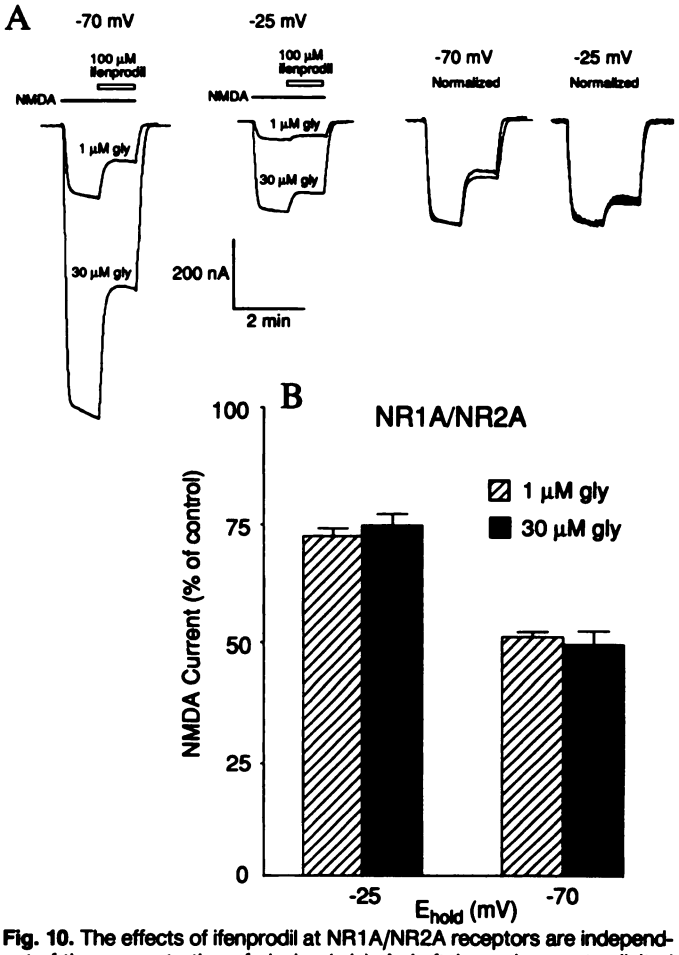

Fig. 10. The effects of ifenprodil at NR1A/NR2A receptors are independent of the concentration of glycine (*gly*). A, *Left*, inward currents elicited by NMDA (100 μM) and NMDA plus ifenprodil, in the presence of 1 and 30 μM glycine, in an oocyte expressing NR1A/NR2A receptors and voltage-clamped at −70 and −25 mV. *Right*, superimposed currents measured in the presence of 1 and 30 μM glycine have been normalized to the peak control response seen at each holding potential. B, Steady state NMDA currents measured in the presence of 100 μM ifenprodil are expressed as a percentage of control responses measured in the presence of 1 and 30 μM glycine at each holding potential. Values are mean ± standard error of four cells.

channel blockers such as MK-801 and ketamine (9, 10, 24–26). Although NR1A/NR2B channels have a high affinity for ifenprodil, the inhibition was incomplete even at a concentration of ifenprodil 1000 times higher than that required to cause 50% inhibition of macroscopic currents. It is possible that injection of oocytes with NR1A plus NR2B subunit RNAs leads to the formation of a mixed population of receptors, with a small proportion having a low affinity for ifenprodil. However, this cannot be due to the formation of homomeric NR1A receptors, which have a high affinity for ifenprodil similar to that of NR1A/NR2B receptors (10). It remains possible that mixed populations of receptors having different NR1A/NR2B stochiometries are coexpressed and have different affinities for ifenprodil. A more likely explanation for the incomplete inhibition seen at NR1A/NR2B receptors is that ifenprodil alters the gating properties of these receptors such that the channel open time or gating frequency is reduced but channel opening is not abolished by the antagonist. In this regard, low concentrations of ifenprodil, presumably acting at high affinity sites, were reported to cause a shift in channel gating properties by reducing long- but not short-duration channel openings in outside-out patches from cultured hippocampal neurons (9). Such a mechanism could account for the incomplete inhibition of macroscopic NMDA currents at NR1A/NR2B receptors seen in oocytes.

An unexpected finding from the present study was that the inhibitory effects of high concentrations of ifenprodil at NR1A/NR2A receptors were partially voltage dependent. Effects of ifenprodil on native NMDA receptors were reported to be independent of membrane potential (9). In the present work, the degree of inhibition seen with ifenprodil was greater at hyperpolarized than at depolarized membrane potentials. This may suggest that ifenprodil acts as a weak voltage-dependent open-channel blocker of NR1A/NR2A receptors and that the degree of inhibition is sensitive to the transmembrane potential. In that case, the binding site for ifenprodil may be located within or close to the membrane-spanning regions of the receptor, and the inhibitory effect of ifenprodil may be similar to that of MK-801. The recovery from block at NR1A/NR2A receptors was biphasic, having fast and slow components. The slow component was similar to the slow recovery seen at NR1A/NR2B channels and was not use dependent, suggesting that it is unrelated to a voltage-dependent open-channel block. The fast phase of recovery at NR1A/NR2A receptors may thus correspond to recovery from a weak open-channel blocking effect of ifenprodil. Because the reversal potential of NMDA currents through NR1A/NR2A receptors was not altered in the presence of ifenprodil, the voltage dependence of inhibition is unlikely to be due to changes in the ionic selectivity of the channel. In contrast to NR1A/NR2A receptors, inhibition of NR1A/NR2B receptors by ifenprodil was not voltage dependent. This suggests that not only do NR1A/NR2B receptors have a higher affinity for ifenprodil than do NR1A/NR2A receptors, but that the mechanism and possibly the site of action of ifenprodil are different for the two receptor types. It remains possible that the voltage-dependent inhibition seen at NR1A/NR2A receptors is a relatively nonspecific effect and that high concentrations of ifenprodil can also block NR1A/NR2B channels in a low affinity, voltage-dependent manner. However, such effects occurring at NR1A/NR2B receptors would be masked by the high affinity inhibitory effect of ifenprodil, which is not voltage dependent.

Results of studies of the effects of glycine also suggest that there are differences in the mechanism and site of action of ifenprodil at NR1A/NR2B and NR1A/NR2A receptors. The degree of inhibition by ifenprodil at NR1A/NR2B receptors but not at NR1A/NR2A receptors was sensitive to changes in the concentration of glycine. Increasing the extracellular concentration of glycine reduced the degree of inhibition by ifenprodil at NR1A/NR2B receptors. Thus, part of the mechanism of action of ifenprodil may be to reduce the affinity of these receptors for glycine. An increase in the concentration of glycine would therefore overcome part of the inhibition caused by ifenprodil. These results are consistent with the reported effects of ifenprodil on the binding of [^{3}H]glycine to NMDA receptors on rat brain membranes (27). Ifenprodil was found to partially inhibit the binding of [^{3}H]glycine to NMDA receptors, with an apparent affinity for ifenprodil of <1 μM (27).

In patch-clamp studies of cultured hippocampal neurons, high concentrations of ifenprodil were reported to reduce the affinity of NMDA receptors for glycine (9). This may suggest an action of ifenprodil on glycine sensitivity at receptors having a low affinity for ifenprodil (i.e., NR1A/NR2A-like receptors). However, in the present work, inhibition by ifenprodil was glycine-sensitive at receptors having a high affinity for ifenprodil (NR1A/NR2B) but not at receptors having a low affinity for ifenprodil (NR1A/NR2A). Thus, the decrease in the affinity for glycine seen at NMDA receptors on cultured neurons may be due to effects at high affinity ifenprodil sites on native receptors containing the NR2B subunit. The subunit composition of native NMDA receptors has not yet been defined. It is possible that ifenprodil has different actions at some native NMDA receptors, compared with NR1A/NR2A or NR1A/NR2B receptors. Inclusion of different NR1 splice variants, of NR2C and NR2D subunits, or of other, unknown, subunits in NMDA receptors may also alter the pharmacological properties of noncompetitive antagonists such as ifenprodil. In this regard, homomeric NR1A receptors expressed in *Xenopus* oocytes were found to have a high affinity for ifenprodil, suggesting either that the high affinity ifenprodil site is localized on the NR1 subunit or that the interaction of two or more NR1 subunits is sufficient to form a high affinity site (10). Inclusion of different NR2 subunits in heteromeric complexes may therefore alter the properties of a site on the NR1 subunit, or the interaction of NR1 with NR2A and NR2B subunits may form binding sites with differing affinities for ifenprodil.

Ifenprodil is the first NMDA receptor antagonist that has been shown to discriminate subtypes of both native and recombinant NMDA receptors with >100-fold selectivity (this work and Refs. 9 and 10). Ifenprodil is thus a potentially useful tool for fingerprinting different subtypes or isoforms of the NMDA receptor in central nervous system tissues and for studies of the role of NMDA receptors in synaptic transmission and plasticity. Because of its ability to discriminate particular subtypes of the NMDA receptor, ifenprodil or related analogs may prove to be useful for targeting NMDA receptors in a therapeutic setting. However, ifenprodil also binds with high affinity to α_1-adrenergic receptors and so-called "σ sites" (28, 29). Strategies to develop analogs of ifenprodil with enhanced selectivity for NMDA receptors over non-NMDA receptor sites (29) may result in analogs with enhanced therapeutic usefulness and

with potential value as selective probes to study the structural and functional properties of subtypes of the NMDA receptor.

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