An NR2B Point Mutation Affecting Haloperidol and CP101,606 Sensitivity of Single Recombinant N-Methyl-D-Aspartate Receptors

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ABSTRACT
Haloperidol and ifenprodil are N-methyl-D-aspartate (NMDA) receptor (NR) antagonists with preference for the NR1/NR2B subunit combination. Previous investigations utilizing 125I-MK801 binding assays with recombinant receptors distinguished certain structural determinants on the NR2B subunit for these two drugs, with glutamate 201 being critical for haloperidol sensitivity and arginine 337 being important for ifenprodil block. Other studies, however, suggested that these two sites pharmacologically overlap. In an attempt to resolve these discrepancies, we have characterized the actions of haloperidol and CP101,606, an ifenprodil analog, on the single-channel properties of NR1/NR2B(E201R) receptors transiently expressed in Chinese hamster ovary cells, because receptors formed by NR1/NR2B(R337K) appear to be nonfunctional. Haloperidol (10 μM) inhibited wild-type NR1/NR2B channels by decreasing the frequency of channel opening, whereas CP101,606 (0.5 μM) antagonized NR1/NR2B channel activity by decreasing both the open dwell time and the frequency of channel opening. The inhibitory actions of both drugs were virtually absent in the mutant NR1/NR2B(E201R) receptors. These results suggest that glutamate 201 is critical for both haloperidol and CP101,606 inhibition, thus demonstrating common features in the action of these two antagonists.

The NMDA receptor is a ligand-gated ion channel involved in excitatory neurotransmission, synaptic plasticity and neuronal cell death. Although the precise subunit composition and stoichiometry of this receptor are still being debated (Béhé et al., 1995; Ferrer-Montiel and Montal, 1996; Premkumar and Auerbach, 1997), it is generally agreed that receptors in mammalian cells are formed by the co-assembly of the NR1 subunit with at least one type of NR2 subunit (Wafford et al., 1993; Boeckman and Aizenman, 1994; Chazot et al., 1994; Sheng et al., 1994; McIlhinney et al., 1996; Luo et al., 1997). The type of NR2 subunit present in a functional receptor confers unique pharmacological and biophysical properties upon NMDA receptors (Kutsuwada et al., 1992; Monyer et al., 1992; Stern et al., 1992; Williams, 1993; Williams et al., 1994; Burnashev et al., 1995; Kuner and Schoepfer, 1996; Brimecombe et al., 1997). For example, there are a number of substances that are more effective antagonists of the recombinant NR1/NR2B subunit combination when compared with NR1/NR2A receptors (Williams, 1993; Lynch et al., 1995; Lynch and Gallagher, 1996; Avenet et al., 1996). An example of such a drug is ifenprodil (fig. 1), a noncompetitive NMDA receptor antagonist (Carter et al., 1988) and neuroprotective agent (Gotti et al., 1988). This agent decreases single-channel activity and reduces channel open dwell time in a voltage-independent manner in native hippocampal NMDA receptors (Legendre and Westbrook, 1991). Studies utilizing recombinant receptors have revealed that this drug is highly selective for NR1/NR2B-containing receptors, as measured in the frog oocyte system (Williams, 1993) and in transfected HEK 293 cells (Gallagher et al., 1996). Because ifenprodil also possesses nanomolar affinity for other neurotransmitter receptors such as adrenergic and serotoninergic receptors (Chenard et al., 1991), several related analogs with increased specificity for the NMDA receptor were recently synthesized. We have previously investigated the effects of one such compound, CP101,606 (Chenard et al., 1995; fig. 1), on recombinant NMDA receptors expressed in CHO-K1 cells (Boeckman and Aizenman, 1996; Brimecombe et al., 1997). This drug protected cells expressing NR1/NR2B, but not NR1/NR2A, receptors from the cytotoxicity that ensues after functional NMDA receptor expression, in a dose-
dependent manner. Similar to the actions of ifenprodil on native receptors, CP101,606 inhibited NR1/NR2B channel activity by decreasing both the open dwell time of the channel and the frequency of channel opening. This drug did not drastically alter either NR1/NR2A or NR1/NR2C channel activity. Therefore, CP101,606 selectively inhibits NMDA receptors composed of NR1 and NR2B in a manner analogous to ifenprodil.

Haloperidol (Fig. 1), a commonly used antipsychotic agent that antagonizes dopamine D2 receptors, can also inhibit NMDA receptor activity in a subunit-selective manner, with NR2B-containing receptors displaying more sensitivity for the drug (Lynch and Gallagher, 1996; Ilyin et al., 1996; Whittemore et al., 1997). Haloperidol inhibits NMDA-induced native channel activity in rat cortical neurons by decreasing both the mean open dwell time and the frequency of channel opening in a voltage-independent manner (Ilyin et al., 1996). Haloperidol and native NMDA receptors. Recent125I-MK801 binding assays using site-specific mutants have localized the putative sites of action of these two antagonists on the NR2B subunit, with arginine 337 being important for ifenprodil sensitivity and glutamate 201 being critical for haloperidol block (Gallagher et al., 1996; Gallagher et al., 1998). Other studies, however, have demonstrated that haloperidol inhibits [3H]ifenprodil binding in adult rat brain membranes, suggesting that these two drugs may have pharmacologically overlapping binding sites (Coughenor and Cordon, 1997). In an effort to elucidate the pharmacological sites of action of these drugs, we have investigated whether mutations at arginine 337 and glutamate 201 in NR2B similarly affect the actions of haloperidol and CP101,606 at the single-channel level.

Materials and Methods

Tissue culture and transfection protocol. CHO-K1 cells (ATTC CCL61) were grown in Ham’s F-12 nutrient medium with 10% fetal bovine serum and 1 mM glutamine (CHO medium) in 50- or 200-ml flasks. Cells were passaged at a 1:10 dilution at 80% confluence, approximately every 2 days, no more than 40 times. The cDNAs for the NMDA subunits NR1 and NR2B (e2) were previously subcloned into mammalian expression vectors (Boeckman and Aizenman, 1994; Boeckman and Aizenman, 1996; Gallagher et al., 1996; Gallagher et al., 1997). The expression vector for a positive transfection marker protein, green fluorescent protein (GFP), was also generated previously (Brimecombe et al., 1997). Recombinant NMDA receptors were transiently expressed in CHO cells by using LipofectAMINE (Gibco-BRL) reagent. Cells were seeded at 3  10^5 cells per well in six-well plates ~24 hr before transfection with 1.1 μg of total DNA and 5 μl of lipofectAMINE in 1 ml of serum-free CHO medium per 35-mm dish. The ratio of marker plasmid (pCIGFP) to total DNA was 1:4.3, and the ratio of NR1 to NR2 subunits transfected was 1:3 (Cik et al., 1993). After a 4- to 5-hr incubation at 37°C with the transfection solution, cells were refed with CHO medium containing 1 mM 5,7-dichlorokynurenic acid to prevent the cell death that accompanies NMDA receptor expression (Boeckman and Aizenman, 1996). Cells were used for recording ~40 to 50 hr after the start of the transfection.

Patch-clamp recordings. Electrophysiological measurements were performed at room temperature (25°C) with the outside-out configuration of the patch-clamp technique by utilizing 10- to 15-MΩ silicon-coated electrodes. Current signals were amplified by using an Axopatch 200 patch-clamp amplifier (Axon Instruments), filtered at 2 kHz with an 80-dB/decade low-pass Bessel filter, stored with a videotape system (Neuro Data), and later replayed and digitized at 10 kHz with a computer interface system (Digidata 1200, Axon Instruments). The reference electrode was a Ag-AgCl wire connected to the extracellular solution by a 2 M KCl/1% agarose bridge. The extracellular recording solution was nominally Mg2+-free and contained (in mM): NaCl, 150; KCl, 2.8; CaCl2, 1.0; HEPES, 10 and glycine 0.01 (pH adjusted to 7.2 with 0.3 N NaOH). The intracellular pipette solution contained (in mM): CsF, 140; ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 10; CaCl2, 1.0 and N2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 10 (pH adjusted to 7.2 with CsOH). Drugs and chemical agents were used at concentrations that were within the range of those used in previous studies (Gallagher et al., 1997; Brimecombe et al., 1998). Other drugs were diluted in the patch-clamp recording solution and were added to the solution at concentrations of 10 μM or lower. The effects of haloperidol and CP101,606 on NMDA receptor current were analyzed using the ClampFit software (Axon Instruments) and were statistically evaluated using ANOVA with a Student-Newman-Keuls post hoc test.
dissolved in the extracellular solution. NMDA (10 \mu M), haloperidol (10 \mu M) and (1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol (CP101,606; 0.5 \mu M) were applied to the patch by complete bath exchange.

Data analysis. Single-channel analysis was performed by using pClamp6 software (Axon Instruments) that utilized a 50% threshold criterion. In total, data were gathered from 25 patches obtained from transfected CHO cells. Data from other patches lost before the completion of a treatment protocol were not utilized, because each patch served as its own control. Normally, 200 to 500 events (range of 30-sec to 2-min traces) were analyzed per single treatment, although many records contained a much larger number of events. Multiple openings, when present, constituted <3% of all openings for a given treatment. Amplitude histograms obtained from idealized traces were most commonly fit with a single Gaussian function. Most (72%) open dwell-time histograms were best fit with a single exponential function by using a simplex maximum-likelihood fitting routine on log-transformed binned data (six bins per decade). An F statistic obtained from a \chi^2 analysis was utilized to determine the simplest fit of the data. When an open dwell-time histogram was better fit by the sum of two exponentials (28% of all histograms), the weighted mean open time was utilized for the necessary calculations. In our previous work with recombinant NMDA receptors expressed in CHO cells, the vast majority of the open dwell-time histograms were best fit with a single exponential function (Brimecombe et al., 1997) when NMDA was used as the agonist. Previous work on recombinant receptors expressed in oocytes or HEK 293 cells (Stern et al., 1994) have reported multiple open dwell times when glutamate is used as the ligand, although the majority of events (70%) have an open dwell time that is very similar to our time constant. Events briefer than 180 \mu sec (twice the rise time of the filter) were ignored. We did not obtain recordings after removal of the antagonists used, because CP101,606 in particular is quite difficult to wash out completely. We therefore conducted a series of experiments aimed at determining the stability of our patches. Three patches excised from cells transfected with NR1/NR2B were continuously exposed to 10 \mu M NMDA alone. Recordings were obtained for 8 min (a time period actually longer than the normal experimental protocol performed in the present paper) in the continuous presence of agonist. Traces were analyzed in 1-min intervals, with the open dwell time and frequency of channel opening analyzed for each segment. As demonstrated in figure 2, both parameters were relatively stable throughout the

**Fig. 3.** Representative single-channel events elicited by NMDA in the absence or presence of haloperidol. Single-channel recordings were obtained from outside-out patches excised from cells transfected with either NR1/NR2B or NR1/NR2B(E201R). Events were elicited by 10 \mu M NMDA in the absence or presence of 10 \mu M haloperidol at -60 mV.
recording period, with no evidence of patch rundown. Results are expressed as means ± S.E.M.

Results

We sought to determine the effects of haloperidol and CP101,606 on the single-channel properties of both NR1/NR2B(R337K) and NR1/NR2B(E201R) receptors. Previous studies utilizing 125I-MK801 binding assays suggested that ifenprodil has an ~150-fold higher affinity for wild-type NR1/NR2B receptors than for the arginine 337-mutated receptors (Gallagher et al., 1996). Surprisingly though, attempted single-channel electrophysiological studies on recombinant NR1/NR2B(R337K) receptors expressed in CHO cells seemed to indicate that these mutant receptors were not functional, in spite of their having normal 125I-MK801 affinity in the binding studies. The lack of functional channels was confirmed by both whole-cell recordings and intracellular Ca++ measurements (data not shown). We therefore could only investigate the NR1/NR2B(E201R) mutant, which has a 10-fold reduced sensitivity to haloperidol when compared with wild-type receptors, as revealed by binding assays (Gallagher et al., 1998).

NMDA (10 μM)-activated channels were recorded from patches of cells transfected with either NR1/NR2B (n = 4) or NR1/NR2B(E201R) (n = 7), both in the absence or presence of 10 μM haloperidol (fig. 3). For the two receptor combinations (fig. 4), haloperidol slightly, albeit significantly, decreased the single-channel amplitudes at a holding voltage of −60 mV. Hence, NR1/NR2B channel amplitudes decreased from −3.7 ± 0.2 in NMDA to −3.4 ± 0.2 pA in NMDA and haloperidol (P < .05, paired t test), whereas NR1/NR2B(E201R) channel amplitudes similarly decreased from −3.6 ± 0.1 to −3.4 ± 0.1 pA (P < .05, paired t test). Haloperidol also produced a substantial decrease in the frequency of channel opening of wild-type NR1/NR2B channels by 77% but did not significantly decrease their open dwell time.
TABLE 1
Single-channel parameters for NR1/NR2B and NR1/NR2B(E201R) receptors in the absence and presence of the two antagonists

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<tr>
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<th>Control</th>
<th>Drug</th>
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<tr>
<td>NR1/NR2B</td>
<td></td>
<td></td>
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<tr>
<td>Amplitude (pA)</td>
<td>3.7 ± 0.2</td>
<td>3.4 ± 0.2*</td>
</tr>
<tr>
<td>Open time (msec)</td>
<td>3.0 ± 0.7</td>
<td>2.6 ± 0.5</td>
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<tr>
<td>% frequency</td>
<td>77 ± 1.5%*</td>
<td></td>
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<tr>
<td>NR1/NR2B(E201R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>-3.6 ± 0.1</td>
<td>-3.4 ± 0.1*</td>
</tr>
<tr>
<td>Open time</td>
<td>3.4 ± 0.5</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>% frequency</td>
<td>77 ± 1.5%*</td>
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<tr>
<td>NR1/NR2B</td>
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<tr>
<td>Amplitude (pA)</td>
<td>3.7 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Open time (msec)</td>
<td>3.9 ± 0.6</td>
<td>2.3 ± 0.3*</td>
</tr>
<tr>
<td>% frequency</td>
<td>67 ± 6.3%*</td>
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<tr>
<td>NR1/NR2B(E201R)</td>
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<tr>
<td>Amplitude</td>
<td>-3.9 ± 0.1</td>
<td>-3.8 ± 0.1</td>
</tr>
<tr>
<td>Open time</td>
<td>3.0 ± 0.4</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>% frequency</td>
<td>up 9 ± 48%</td>
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* Significant difference (P < .05) in parameter between control condition versus either 10 μM haloperidol or 0.5 μM CP101,606.

(3.0 ± 0.7 control versus 2.6 ± 0.5 msec drug). In contrast, NR1/NR2B(E201R) receptors were relatively insensitive to haloperidol, with this drug only slightly decreasing the frequency of channel opening (by 28%). The effects of haloperidol on channel opening frequency were significantly different between wild-type and mutant channels (P < .05, unpaired t test). Similar to the NR1/NR2B channels, the mean open dwell time of the mutant channel was not altered by haloperidol (3.4 ± 0.5 control versus 3.7 ± 0.8 msec drug). Furthermore, the mutation itself did not affect the amplitude or the open dwell time of the channel (table 1).

The actions of CP101,606 on wild-type and haloperidol-insensitive receptors were also examined at the single-channel level. Patches excised from cells transfected with either NR1/NR2B (n = 5) or NR1/NR2B(E201R) (n = 6) were exposed to 10 μM NMDA in the absence or presence of 0.5 μM CP101,606 (fig. 5). Similar to the results seen in a previous study (Brimecombe et al., 1997), this drug decreased both the open dwell time and the frequency of channel opening of NR1/NR2B channels without altering the amplitude of the currents. The open dwell time of NR1/NR2B channels decreased from 3.9 ± 0.6 msec in NMDA alone to 2.3 ± 0.3 msec in NMDA and CP101,606 (P < .005, paired t test). When the open dwell-time histograms were best fit with two exponentials, the antagonist did not seem to affect one time constant over the other. In addition, the frequency of channel opening was decreased by 67% in the presence of the drug (fig. 6). In contrast, CP101,606 had no antagonistic effects on NR1/NR2B(E201R) receptors. The open dwell time of the channel was not altered by the drug (3.0 ± 0.4 control versus 3.4 ± 0.9 msec drug). Moreover, the frequency of channel opening did not decrease at all but in fact slightly increased in the presence of CP101,606 (fig. 6), opposite to what was seen in the wild-type channels. Therefore, in addition to being less sensitive to haloperidol, NR1/NR2B(E201R) channels are not altered by CP101,606, suggesting that this glutamate residue is critical for the effects of both classes of drugs (table 1).

**Discussion**

The single-channel data described here suggest that haloperidol and the ifenprodil analog CP101,606 have pharmacologically overlapping sites of action. Haloperidol, at the concentration tested, inhibited recombinant wild-type NR1/NR2B channel activity by decreasing the frequency of channel opening, whereas CP101,606 antagonized these channels by decreasing both the open dwell time and the frequency of channel opening. Haloperidol (3 μM) decreases NMDA receptor activity in young (<10 days in vitro) rat cortical neurons by decreasing the frequency of channel opening, whereas higher concentrations (30 μM) decrease both the frequency of channel opening and the open dwell time (Ilyin et al., 1996). This is similar to the observed actions of 3 μM ifenprodil on native NMDA receptors in hippocampal neurons, because this drug inhibits channel activity by decreasing both channel parameters (Legendre and Westbrook, 1991). Here we show that a single amino acid change in the amino terminus of the NR2B subunit (E201R) substantially decreased both the haloperidol and CP101,606 sensitivity of the receptor. These results are in apparent conflict with 125I-MK801 binding results that implicated two separate amino acids as being solely responsible for the two antagonists’ actions, viz., arginine 337 for ifenprodil inhibition and glutamate 201 for haloperidol sensitivity (Gallagher et al., 1996; Gallagher et al., 1998). Our data, however, are in support of previous competition binding assays that have demonstrated that haloperidol can inhibit [3H]ifenprodil binding to rat brain membranes (Coughenour and Cordon, 1997).

There are several possible scenarios that could account for these discrepancies. 125I-MK801 binding assays are sometimes thought to assess interactions with the desensitized state of the receptor, given its high affinity for this ligand and the long time needed to approach equilibrium. A binding assay can also measure receptors that are not present at the cell surface. Consequently, the actual receptor populations studied by both methods may differ and perhaps be modulated in different manners. This could be relevant to this study because ifenprodil, and presumably CP101,606, has a slightly higher affinity with desensitized states of the receptor when compared with the open states (Kew et al., 1996). In addition, ligand-binding assays are performed with much higher concentrations of glycine and glutamate and in the presence of 100 μM spermidine. Because spermidine itself may interact with glutamate 201 (Gallagher et al., 1997), it is possible that the binding of antagonists whose effects are also mediated by this region of NR2B can be altered by the presence of spermidine. This scenario could be similar to the recently noted changes in the median inhibitory concentration (IC50) values of ifenprodil and CP101,606 produced by alterations in extracellular pH (Pahk and Williams, 1997; Zhang et al., 1997).

Another unexpected finding is the absence of electrophysiologically detectable channels in receptors formed by NR1 and the NR2B(R337K) mutant. Cells transfected with this subunit combination have been previously shown to bind 125I-MK801 with high affinity, which is stimulated by polyamines but insensitive to ifenprodil (Gallagher et al., 1996), although the total number of binding sites in these cells is apparently lower (M. J. Gallagher and D. R. Lynch, unpublished observations). Although MK801 binding is activity dependent and enhanced by the same factors that augment physiological responses, agents that block the channel do not always produce identical effects in electrophysiological mea-
measurements when compared with assays examining the inhibition of MK801 binding (Monaghan and Larsen, 1997). A viable, albeit yet untested, possibility is that the mutation at position 337 produces a permanently desensitized state of the receptor that can still bind MK801. The present results do suggest, however, that the structural requirements for MK801 binding in vitro may not translate to functional electrophysiological channels and stress the need to compare results between these techniques with caution.

Experiments demonstrating that haloperidol inhibits NR1/NR2B channel activity by decreasing the frequency of channel opening provides similar results to what was reported for native NMDA receptors in immature cortical neurons (Ilyin et al., 1996), which primarily express the NR1 and NR2B subunits (Zhong et al., 1994). Mutation of glutamate 201 in the amino terminus of the NR2B subunit decreases the ability of haloperidol to inhibit channel activity. In both the wild-type and mutant receptors, haloperidol slightly, but significantly, decreased the single-channel amplitudes. A similar decrease in the amplitude of single-channel responses was seen by Ilyin et al. (1996) on native NMDA receptors, although these authors attributed this effect to a possible measurement error. Although such an effect is suggestive of an unresolved open channel block effect, haloperidol’s effect on native NMDA receptors is not voltage dependent (Ilyin et al., 1996). However, the effect on channel amplitude is very small in comparison to the effects of the drug on open dwell time and frequency of channel opening and thus likely accounts for a very minor component of the total block. Furthermore, this slight decrease in amplitude was also present in the mutated NR1/NR2B(E201R) channels, consistent with an action at an additional site, such as the ion channel.

As a final note, glutamate 201 on the NR2B subunit has recently been shown to be important for glycine-independent polyamine stimulation and proton sensitivity of recombinant NR1/NR2B receptors (Gallagher et al., 1997). Mutants with the positively charged arginine in place of the glutamate rendered the receptors insensitive to glycine-independent spermidine stimulation and to proton inhibition. Interestingly, very recent evidence has revealed that CP101,606 may antagonize NR1/NR2B receptors by enhancing proton inhibition, i.e., by shifting the IC50 of hydrogen ions from pH 7.4
to 9.3 (Zhang et al., 1997). Other investigators have demonstrated that mutation of aspartate 669 in the NR1 subunit, located in the extracellular M3 to M4 loop, abolishes glycine-independent polyamine stimulation, reduces proton inhibition and decreases ifenprodil block in NR1/NR2B receptors (Kashiwagi et al., 1996). Clearly then, many residues within the NMDA receptor protein are likely participating in the formation of the binding sites for all of these different modulators, including both ifenprodil and haloperidol.

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