Memantine Blocks α7* Nicotinic Acetylcholine Receptors More Potently Than N-Methyl-d-aspartate Receptors in Rat Hippocampal Neurons

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ABSTRACT

The N-methyl-d-aspartate (NMDA) receptor antagonist memantine is an approved drug for treatment of Alzheimer’s disease (AD). Other such treatments are cholinesterase inhibitors and nicotinic acetylcholine receptor (nAChR)-sensitizing agents such as galantamine. The present study was designed to test whether memantine exerts any effect on the cholinergic system, in particular the Ca\(^{2+}\)-conducting α7* nAChR, in cultured hippocampal neurons. Memantine caused a concentration-dependent reduction of the amplitudes of whole-cell currents evoked by the α7* nAChR-selective agonist choline (10 mM) or by N-methyl-d-aspartate (NMDA) (50 μM) plus glycine (10 μM). It also inhibited tonically activated NMDA receptors. Memantine was more potent in inhibiting α7* nAChRs than NMDA receptors; at −60 mV, the IC\(_{50}\) values for memantine were 0.34 and 5.1 μM, respectively. Consistent with an open-channel blocking mechanism, memantine-induced NMDA receptor inhibition was voltage and use-dependent; the Hill coefficient (n\(_H\)) was ~1. Memantine-induced α7* nAChR inhibition had an n\(_H\) < 1 and showed a variable voltage dependence; the effect was voltage-independent at 0.1 μM, becoming voltage-dependent at ≥1 μM. Thus, memantine interacts with more than one class of sites on the α7* nAChRs. One is voltage-sensitive and therefore likely to be within the receptor channel. The other is voltage-insensitive and therefore likely to be in the extracellular domain of the receptor. It is suggested that blockade of α7* nAChRs by memantine could decrease its effectiveness for treatment of AD, particularly at early stages when the degrees of nAChR dysfunction and of cognitive decline correlate well.

Alzheimer’s disease (AD) is a neurodegenerative disorder that is characterized by progressive decline of intellectual abilities, is fatal, and afflicts millions worldwide. It is estimated that by the year 2050, approximately 14 million Americans will have AD (Hebert et al., 2003). Although the cause of AD remains undetermined, the magnitude of cognitive impairment observed as the disease progresses from mild-to-moderate stages correlates well with the degree of loss of 1) nAChRs, particularly those composed of α4, β2, and α7 subunits, in various brain areas (Francis et al., 1985; Perry et al., 2000; Nordberg, 2001); and 2) basal forebrain cholinergic neurons (Geula, 1998). At more advanced stages of the disease, this correlation does not seem to exist (Sabbagh et al., 2001). To date, one of the most successful therapeutic approaches to delay the advancement of AD consists of increasing brain nicotinic cholinergic functions with the anticholinesterase and nicotinic allosteric-potentiating ligand galantamine, or the anticholinesterases donepezil and rivastigmine (Maelicke and Albuquerque, 1996; Pereira et al., 2002; Doody, 2003).

There have been reports that increased tonic (Jiménez-Jiménez et al., 1998) and decreased synaptic (Lin et al., 2003) NMDA receptor activities also contribute to the insufficiency of the AD brain. Excessive tonic and decreased synaptic NMDA receptor activities are known to cause impairment of one of the proposed cellular mechanisms for memory storage, long-term potentiation (Frankiewicz and Parsons, 1999). In addition, excitotoxicity resulting from excessive tonic NMDA receptor activation (Hardingham and Bading, 2002) can be one of the processes underlying neuronal loss in AD. Thus, drugs that selectively reduce tonic without altering synaptic NMDA receptor activity in the brain emerged as a promising therapeutic approach for AD. One such drug is the low-affinity, noncompetitive NMDA receptor antagonist memantine (Frankiewicz and Parsons, 1999; Shimono et al., 2002).

ABBREVIATIONS: AD, Alzheimer’s disease; NMDA, N-methyl-d-aspartate; nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine.
A recent placebo-controlled clinical study reported that memantine improves global clinical impression of change, daily performance, and cognition of patients with moderate-to-severe AD (Reisberg et al., 2003). Improvements of global clinical impression and functional performance had also been reported in an earlier placebo-controlled study of memantine in patients with moderately severe-to-severe AD or vascular dementia (Winblad and Porits, 1999). In contrast, in placebo-controlled studies of patients with mild-to-moderate vascular dementia, a complex disorder in which the cholinergic system is also compromised (Gratham and Geerts, 2002), memantine caused a small, albeit significant, cognitive improvement that was not clinically perceived, given that it was not accompanied by amelioration of the global clinical impression of change (Wilcock, 2003). Furthermore, there is evidence that, resembling nicotinic antagonists (Levin, 2002), memantine impairs cognition in laboratory animals (Willmore et al., 2001) and healthy human subjects (Schugens et al., 1997).

In particular, a single dose of memantine (30 mg) administered orally to young, healthy humans impairs the eyeblink classical conditioning (Schugens et al., 1997), a form of associative learning that is modulated by the nicotinic cholinergic system and differentiates cognitive deficits in normal aging and probable AD (Woodruff-Pak, 2001). Therefore, the question is raised as to whether memantine has actions in the brain nicotinic cholinergic system that would preclude its use when reduction of nicotinic cholinergic activity is accompanied by an increase in the degree of cognitive decline.

In the cerebrospinal fluid of patients receiving the recommended maintenance dose of 20 mg/day, the concentrations of memantine range from 0.2 to 0.3 μM (Kornhuber and Quack, 1995). However, concentrations as high as 1 μM may be reached in the extracellular brain compartment (Danyasz and Parsons, 2003). At this concentration, in addition to blocking NMDA receptors, memantine blocks human α7 nAChRs and human α4β2 nAChRs ectopically expressed in Xenopus oocytes (Buisson and Bertrand, 1998; Maskell et al., 2003). At ~100 to ~60 mV, the IC50 values for memantine to inhibit native NMDA receptors (Chen and Lipton, 1997), recombinant α4β2 nAChRs (Buisson and Bertrand, 1998), and recombinant α7 nAChRs (Maskell et al., 2003) range from 1 to 7 μM.

This study was designed to comparatively examine the effects of memantine on native α7* nAChRs (asterisk next to the nAChR subunit is intended to indicate that the exact subunit composition of the receptor is not known; Lukas et al., 1999) and NMDA receptors in rat hippocampal neurons. Evidence is provided that memantine blocks α7* nAChRs noncompetitively by interacting with voltage-sensitive and voltage-insensitive sites on the receptors. At all membrane potentials studied, memantine inhibited more potently α7* nAChRs than NMDA receptors. At ~60 mV, for instance, memantine blocked α7* nAChRs and NMDA receptors with IC50 values of 0.34 and 5.1 μM, respectively. Therefore, should similar memantine-induced inhibition of α7* nAChRs take place in the human brain, it would be counterproductive for the treatment of AD and other dementias at stages when there is a correlation between the magnitude of nAChR dysfunction and the degree of cognitive impairment.

Materials and Methods

Primary hippocampal cultures were prepared according to the procedure described previously (Alkondon and Albuquerque, 1993). Briefly, hippocampi of 16- to 19-day-old fetal rats (Sprague-Dawley) were dissected out and mechanically dissociated after trypsinization (0.25% trypsin; Invitrogen, Carlsbad, CA) for 30 min. The dissociated cells were plated at a density of approximately 700,000 cells/2 ml on collagen-coated, 35-mm petri dishes. During the first 24 h after plating the cells, the culture medium was composed of minimum essential medium (Invitrogen) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, 2 mM glutamine, and deoxyribonuclease II (20 μg/ml, type V; Sigma-Aldrich, St. Louis, MO). After 24 h and twice a week thereafter, the culture medium was replaced with minimal essential medium containing only 10% heat-inactivated horse serum and 2 mM glutamine. At the 8th day after plating, the antimetabolic mix of 5'-fluoro-2'-deoxyuridine (2 μg/ml) and uridine (13 μg/ml) was added to the medium for 24 h to halt glial cell proliferation. Recordings were obtained from neurons cultured for 3 to 8 weeks.

Electrophysiological recordings were obtained from cultured neurons by means of the whole-cell mode of the patch-clamp technique using an LM-EP-C7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). After being filtered at 1 kHz (8-pole Bessel filter), the signals were digitized at 200 μs and acquired on-line into a microcomputer using the pCLAMP software (Axon Instruments Inc., Union City, CA). The extracellular solution bathing the neurons consisted of 165 mM NaCl, 5 mM KCl, 2 mM CaCl2, 5 mM HEPES, and 10 mM glucose. The pH was adjusted with NaOH to 7.3, and the osmolality was ~340 mOsM. Atropine (1 μM) and tetrodotoxin (0.1–0.3 μM) were added to the external solution to block muscarinic receptors and voltage-gated Na+ channels, respectively. Cells in culture were perfused with the external solution at a rate of 2.5 to 3 ml/min. Recording pipettes were filled with internal solution that consisted of 60 mM CsCl, 60 mM CsF, 10 mM HEPES, 20 mM Tris-phosphate, and 5 mM ATP, in addition to 50 μM creatine phosphokinase (pH adjusted to 7.3 with CsOH). The ATP-regenerating compounds in the internal solution diminished the rundown of α7* nAChR-mediated whole-cell currents. When filled with the internal solution, the recording pipettes had a resistance of ~3 MΩ. No compensation for access resistance was made during the recordings. Results obtained from cells in which access resistance changed by >15% were discarded. The agonist solutions, with or without memantine, were applied to the neurons through a glass U-tube (Alkondon and Albuquerque, 1993). Whenever stated, memantine, and various concentrations, was also added to the bathing solution.

Data Analysis. The peak amplitude and decay-time constant (τd,decay) of agonist-evoked currents were analyzed using the pCLAMP6 software package (Axon Instruments Inc.). Rundown of the amplitudes of currents triggered by α7* nAChR activation had a fast and a slow component. The fast component, which was discarded, was finished after the first 3 to 4 min after the GΩ seal was achieved. To correct for the slowly developing rundown of whole-cell currents triggered by activation of α7* nAChRs, after the first 4 min following achievement of the GΩ seal, at least 10 agonist pulses were applied to the neurons before and after their exposure to memantine. The amplitudes of currents evoked by the agonist alone, before and after the first 4 min, were plotted against the log of the agonist concentration and the resulting plots were fitted by linear regression. Then, the expected amplitudes of agonist-evoked currents at any given time were estimated and used to normalize the actual amplitudes of currents recorded in the presence of memantine and during the washing phases. Full reversibility of the effects of memantine was indicated by the recovery of the peak current amplitudes to estimated control levels. Concentration-response curves were fitted by the Hill equation: $I = I_{\text{max}} \times [A]^{n_{H}}/(I_{\text{max}} + EC_{50}^{n_{H}})$, where $I_{\text{max}}$ and $I_{\text{max}}$ are, respectively, the measured and maximum current amplitude, [A] is the agonist concentration, $n_{H}$ is the Hill coefficient,
and EC_{50} is the agonist concentration producing half-maximum response.

**Drugs Used.** Memantine hydrochloride, choline chloride, acetylcholine (ACh) chloride, NMDA, glycine, atropine sulfate, and tetrodotoxin were all purchased from Sigma-Aldrich. All chemicals were dissolved in double-distilled water, and the stock solutions were kept frozen until ready to use.

**Statistical Analysis.** Data are presented as mean ± S.E.M., and differences between results obtained from a group of cells during their exposure to memantine and those obtained from the same cells before or after that treatment were statistically evaluated using the paired Student’s t test.

**Results**

**Memantine Blocks α7* nAChRs in Hippocampal Neurons.** More than 80% of the hippocampal neurons in culture responded to 1-s pulses of ACh (1 mM) or choline (10 mM) with whole-cell currents that 1) decayed to the baseline while the agonist was present (Fig. 1A), and 2) were reversibly blocked by 1 nM methyllycaconitine and irreversibly blocked by 10 nM α-bungarotoxin (data not shown). The pharmacological and kinetic profile of these currents, which are herein referred to as type IA currents (Aikondon and Albuquerque, 1993), are characteristic of responses mediated by α7* nAChRs.

When neurons that responded to choline (10 mM) or ACh (1 mM) with type IA currents were exposed to memantine (0.01–30 μM), there was a concentration-dependent reduction of the amplitude of the agonist-evoked currents (Fig. 1A and B). Fitting the concentration-response relationship for memantine-induced inhibition of α7* nAChR activation by choline (10 mM) at −60 mV yielded an IC_{50} of approximately 0.34 ± 0.22 μM and an n_{H} of 0.47 ± 0.08 (Fig. 1B). The magnitude of the inhibitory effect of memantine on type IA currents evoked by choline (10 mM) was not significantly different from that observed when ACh (1 mM) was used as the agonist to activate the α7* nAChRs (Fig. 1B).

**Memantine-Induced Blockade of α7* nAChRs Is Use-Dependent and Quickly Reversible.** To analyze the time it takes for the onset and termination of the effect of memantine on α7* nAChRs and the use dependence of the interactions between memantine and the receptors, two sets of experiments were performed.

The protocol in one set of experiments was the same as that used in the majority of the experiments performed in this study. It consisted of applying 10 consecutive pulses of choline (10 mM) via the U-tube to hippocampal neurons starting at 5 min after establishment of the whole-cell configuration. The agonist pulses lasted 1 s and the interpulse intervals were maintained constant at 30 s. Recovery of the α7* nAChRs from agonist-induced desensitization was maximal between the agonist pulses and the amplitudes of choline-evoked currents were stable during the recording time (Fig. 2A). Subsequently, at approximately 2 min after the neurons were subsequently perfused with external solution containing 1 μM memantine, pulses of choline (10 mM) plus memantine (1 μM) were applied to the neurons via the U-tube until maximal reduction of the peak current amplitude was achieved. Finally, after a 1-min washing of the neurons with memantine-free external solution, pulses of choline (10 mM) alone were delivered via the U-tube to the neurons for as long as needed to reverse the effect of memantine. Even after the neurons were equilibrated with memantine, reduction of the peak amplitude of choline-evoked currents increased with repetitive exposure to choline plus memantine, becoming maximal at the fourth pulse in neurons held at −60 mV (Fig. 2A). These results suggest that memantine-induced inhibition of α7* nAChRs is use dependent. Full reversibility of the memantine-induced inhibition of α7* nAChRs was achieved within 10 min after washing of the neurons with memantine-free external solution.

The other set of experiments used the same protocol as that described above, except that memantine was applied to the neurons exclusively in admixture with choline via the U-tube. In neurons that were not in equilibrium with memantine, reduction of the peak amplitude of choline-evoked currents by the drug increased and became maximal after the fourth pulse application of choline plus memantine to the neurons (Fig. 2B). Therefore, inhibition of α7* nAChR development quickly and did not reverse, significantly within the 30-s interpulse intervals. The time course of development and the degree of maximal α7* nAChR inhibition achieved when memantine was applied to the neurons exclusively via the U-tube were the same as those observed when neurons...
were equilibrated with the drug (Fig. 2, A and B). Finally, after maximal inhibition of the choline-evoked currents had been achieved, it took approximately 10 min for the amplitudes of currents evoked by pulses of choline alone to reach predicted control levels (Fig. 2B).

Inhibition of type IA currents by all tested concentrations of memantine was use-dependent. As the concentration of memantine increased, fewer exposures of the neurons to the drug were necessary for attaining maximal inhibition of the α7* nAChRs. For instance, at −60 mV, four consecutive pulses of choline plus 1 μM memantine were necessary to cause maximal blockade of type IA currents, whereas only two pulses of choline plus 10 μM memantine were needed to induce the maximal effect.

**Memantine-Induced Inhibition of α7* nAChRs Is Noncompetitive with Respect to the Agonist: Analysis of Voltage Dependence.** To determine whether memantine acts as a competitive or noncompetitive antagonist at α7* nAChRs, type IA currents were recorded from neurons voltage clamped at −60 mV and evoked by increasing concentrations of choline in the absence or presence of 1 μM memantine. Calibration bars: vertical, 125 (10 mM), 200 (3 mM), 140 (1 mM), and 45 pA (0.3 mM); horizontal, 50 (top currents) and 100 ms (bottom currents). Arrows indicate the beginning of the 1-s agonist pulses. B, double reciprocal plot of the concentration-response relationship for choline in the absence and presence of memantine. The rundown-corrected amplitudes of currents evoked by 10 mM choline were taken as 1 and used to normalize the amplitudes of currents evoked by choline in the absence (control) or presence of 1 μM memantine. Points and error bars represent mean and S.E.M., respectively, of results obtained from four neurons. C, effects of memantine (0.1–3 μM) on the decay of choline (1–10 mM)-activated currents. Under control conditions, the average τdecay for currents evoked by 1, 3, and 10 mM choline was 154 ± 39.3, 54.5 ± 5.8, and 17.7 ± 2.4 ms, respectively. Graph and error bars are mean and S.E.M., respectively, of results obtained from at least four neurons. According to the paired Student’s t test, results obtained in the presence of memantine are significantly different from those obtained under control conditions (*, p < 0.05; **, p < 0.01). Holding potential, −60 mV.

Inhibitory effect of memantine on α7* nAChRs could not be surmounted by increasing the agonist concentration (Fig. 3B). Memantine decreased to 43.4 ± 0.85% the efficacy of choline to activate α7* nAChRs. In contrast, the EC50 for
choline in activating α7* nAChRs was not altered significantly by memantine (Fig. 3B); in the absence and in the presence of 1 μM memantine, the EC50 values for choline were 1.04 ± 0.38 and 0.86 ± 0.043 mM, respectively. Likewise, the cooperativity between the agonist recognition sites on the α7* nAChRs was not altered by memantine; in the absence and in the presence of 1 μM memantine, the nH values for choline were 1.25 ± 0.47 and 1.47 ± 0.10, respectively. These results indicate that blockade by memantine of α7* nAChRs is noncompetitive with respect to the agonist.

In addition to decreasing the amplitude, memantine accelerated the decay phase of choline-evoked type IA currents (Fig. 3C), and the magnitude of this effect increased as the agonist concentration decreased. For instance, although memantine at 0.1 and 1 μM caused a significant reduction of the amplitude of currents triggered by 10 mM choline (Figs. 1 and 3A), only at 3 μM did it cause a significant acceleration of the decay phase of these currents (Fig. 3C). As the concentration of choline was decreased to 3 and 1 mM, acceleration of the decay phase became significant with 1 μM memantine (Fig. 3C).

To investigate the voltage dependence of memantine-induced block of α7* nAChRs, type IA currents were evoked by application of 1-s pulses of choline (10 mM) to neurons held at membrane potentials ranging from −20 to −80 mV. Subsequently, the neurons were perfused for 6 to 8 min with physiological solution containing one of various concentrations of memantine (0.1–30 μM), and 1-s pulses of choline plus that concentration of memantine were applied to the neurons until the amplitude of type IA currents decreased to a plateau (Fig. 4A). Memantine at 0.1 μM caused approximately 30% reduction of the amplitude of type IA currents recorded from neurons voltage clamped between −20 and −80 mV (Fig. 4B). In contrast, the magnitude of the effect of ≥1 μM memantine on type IA currents was voltage-dependent such that for any given concentration of the drug, reduction of the amplitude of type IA currents increased as the membrane potential was made more negative (Fig. 4B). Considering that α7* nAChR desensitization is voltage-independent (Mike et al., 2000), the voltage dependence of the inhibition of type IA currents by ≥1 μM memantine was suggestive of an open-channel blocking mechanism.

Plots of percentage of inhibition of type IA currents versus membrane potential for each concentration could be fitted by linear regression (Fig. 4B). The angular coefficients of the regressions varied with memantine concentrations; for 1, 10, and 30 μM, the angular coefficients of the linear regressions revealed approximately 4.0, 4.0, and 2.3% inhibition per 10-mV step, respectively. Thus, the voltage dependence of the inhibition decreased as the concentration of memantine increased from 1 to 30 μM. This finding could be explained by assuming that memantine interacts with distinct and independent sites on the α7* nAChRs, a voltage-sensitive and a voltage-insensitive site, and that the relative contribution of the interaction of memantine with the voltage-insensitive site to the total effect of memantine increases as its concentration is raised from 1 to 30 μM. Alternatively, it could be accounted for by the interactions of memantine with distinct classes of sites that show some degree of negative cooperativity. Either explanation is consistent with the nH < 1 estimated from the concentration-response relationship for memantine-induced inhibition of type IA currents.

**Comparative Analysis of the Apparent Potency of Memantine in Blocking NMDA Receptors and α7* nAChRs in Hippocampal Neurons.** To trace a direct comparison of the effects of memantine on NMDA receptors to those observed on α7* nAChRs, whole-cell currents were evoked by U-tube application of NMDA plus glycine to the cultured hippocampal neurons in the absence and in the presence of memantine. At −60 mV, memantine caused a concentration-dependent reduction of the amplitude of whole-cell currents evoked by the NMDA (50 μM) plus glycine (10 μM). Fitting the concentration-response relationship for memantine-induced block of NMDA receptors under this experimental condition yielded an IC50 and an nH of 5.1 ± 1.7 μM and 1.1 ± 0.30, respectively (Fig. 5). The nH close to 1 suggests that memantine interacts with a single class of sites on the NMDA receptors. In addition, a direct comparison of the memantine-induced block of NMDA receptors and α7* nAChRs in hippocampal neurons held at −60 mV revealed that the α7* nAChRs are more sensitive than the NMDA receptors to inhibition by memantine (Fig. 5).
Mechanism of Action of Memantine on NMDA Receptors. To determine the mechanism by which memantine inhibits NMDA receptors in the cultured hippocampal neurons, whole-cell currents were evoked by increasing concentrations of NMDA plus 10 μM glycine in the absence and in the presence of a fixed concentration of memantine (Fig. 6A). With 10 μM glycine, the EC$_{50}$ value for NMDA in inducing whole-cell currents at −60 mV in the absence and in the presence of 5 μM memantine was 9.5 ± 3.4 and 12.4 ± 0.51 μM, respectively (Fig. 6B). The $n_H$ value obtained from the fitting of the concentration-response relationship for NMDA in evoking whole-cell currents was also not altered by 5 μM memantine. In the absence and in the presence of the drug, the $n_H$ values were 1.5 ± 0.6 and 1.1 ± 0.33, respectively. Thus, memantine caused no significant changes in the apparent potency or cooperativity for NMDA. However, it decreased significantly the efficacy of NMDA in activating NMDA receptors. As shown in Fig. 6B, increasing the concentrations of NMDA did not surmount memantine-induced NMDA receptor inhibition. The efficacy of NMDA estimated from the fitting of the NMDA concentration-response relationship in the absence and in the presence of 5 μM memantine was 100 ± 18 and 61.5 ± 11.4%, respectively. These findings confirmed the noncompetitive mechanism of action for memantine on NMDA receptors (Rogawski and Wenk, 2003, and references therein).

To investigate the voltage dependence of the memantine-induced inhibition of NMDA receptors, whole-cell currents were activated by NMDA (50 μM) plus glycine (10 μM) at various membrane potentials using a nominally Mg$^{2+}$-free physiological solution (Fig. 7). At all negative membrane potentials tested (−100 to −30 mV), memantine (1−30 μM) caused a significant reduction of the amplitude of the NMDA-evoked currents (Fig. 7). For any given concentration of memantine, the magnitude of the effect increased as the membrane potential was made more negative (Fig. 7). At +30 mV, only at concentrations >10 μM did memantine reduce significantly the amplitude of currents evoked by NMDA plus glycine (Fig. 7).

Using the Hill equation to fit the concentration-response relationships for memantine-induced inhibition of NMDA-evoked whole-cell currents at various membrane potentials
revealed that the apparent potency for memantine decreased significantly as the neurons were depolarized (Fig. 8A). The average IC₅₀ values for memantine in blocking the NMDA receptors in hippocampal neurons held at −100, −60, −30 and +30 mV were approximately 1.9, 5.1, 8.0, and 96.3 μM, respectively (Fig. 8B). The estimated nₑ values were close to 1 at all tested membrane potentials (Fig. 8B). From the lowest to the highest test concentration of memantine, inhibition of NMDA receptors was always sensitive to the membrane potential (Fig. 8C). Plots of percentage of inhibition versus membrane potential for each memantine concentration could be fitted by linear regression (Fig. 8C). The angular coefficient of all regressions revealed approximately 5% inhibition per 10-mV step. The finding that there was parallelism between the regression lines revealed that the voltage dependence of the magnitude of inhibition of NMDA receptors was independent on the drug concentration.

**Use Dependence and Reversibility of Memantine-Induced Blockade of NMDA Receptors at Different Membrane Potentials.** Experiments similar to those described above to analyze the kinetics of the interactions between memantine and α7 nAChRs were performed to evaluate the use dependence and the reversibility of the inhibitory effect of memantine on NMDA receptors.

The protocol used in one set of experiments was the same as that used for most experiments throughout the present study. Thus, 10 consecutive pulses of NMDA (50 μM) plus glycine (10 μM) were applied via the U-tube to hippocampal neurons. With 1-s agonist pulses and 30-s interpulse intervals, recovery of the NMDA receptors from agonist-induced desensitization between the agonist pulses was maximal and the amplitudes of the currents evoked by the admixture of NMDA plus glycine were stable during the recording time (Fig. 9A). Subsequently, starting at 2 min after the neurons had been perfused with external solution containing 3 μM memantine, pulses of NMDA plus glycine plus memantine (3 μM) were applied to the neurons via the U-tube until maximal reduction of the peak current amplitude was

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**Fig. 8.** Quantitative analysis of the voltage dependence of memantine-induced blockade of NMDA receptors. A, concentration-dependent plots of memantine-induced blockade of currents evoked by 50 μM NMDA plus 10 μM glycine at various membrane potentials. The amplitudes of NMDA plus glycine-triggered currents were taken as 100% and used to normalize the amplitudes of currents evoked by NMDA plus glycine in the presence of memantine. B, plots of log of IC₅₀ and nₑ versus holding potential (millivolts). There was an e-fold change of IC₅₀ for each 75-mV step. C, plots of percentage of inhibition versus holding potential for various memantine concentrations. At each membrane potential, the amplitudes of NMDA plus glycine-evoked currents recorded in the presence of a given concentration of memantine were expressed as percentage of the control, rundown-correct amplitudes of agonist-evoked currents recorded in the absence of memantine. Percentage of inhibition was calculated as (100%) − (percentage of control). In A–C, points and error bars represent mean and S.E.M., respectively, of results obtained from at least four neurons.

**Fig. 9.** Use dependence and reversibility of memantine-induced inhibition of NMDA plus glycine-triggered currents. A, plot of amplitudes of currents triggered by 1-s pulses of 50 μM NMDA plus 10 μM glycine with or without 3 μM memantine versus recording time. Recordings were obtained from a neuron at −60 mV. Memantine was applied to the neuron via the U-tube and bath perfusion. The solid line indicates the time the neuron was perfused with memantine-containing physiological solution. B, plot of amplitudes of currents triggered by 1-s pulses of 50 μM NMDA plus 10 μM glycine with or without 10 μM memantine versus recording time. Recordings were obtained from a neuron at −100 mV. Memantine was applied to the neuron exclusively via the U-tube in admixture with the agonists. In A and B, interpulse intervals were 30 s.
achieved. Finally, after a 2-min wash of the neurons with memantine-free external solution, pulses of NMDA plus glycine were delivered via the U-tube to the neurons for as long as needed to reverse the effect of memantine. Consistent with reports that memantine-induced inhibition of NMDA receptors is use dependent was the finding that even after the neurons were equilibrated with memantine the magnitude of the inhibitory effect of the drug on NMDA receptors increased with consecutive exposure of the neurons to agonists plus memantine, becoming maximal at the fourth pulse application to neurons held at −60 mV (Fig. 9A). In addition, in neurons held at −60 mV, it took approximately 4 min to reverse the inhibition of the NMDA receptors by 3 μM memantine (Fig. 9A).

The protocol used in the other set of experiments was the same as that described above, except that memantine was applied to the neurons exclusively via the U-tube in admixture with NMDA plus glycine. Maximal inhibition of the NMDA receptors by 10 μM memantine was achieved when the fourth pulse of agonists plus memantine were applied to neurons held at −100 mV (Fig. 9B). Thus, memantine-induced inhibition of NMDA receptors developed quickly upon agonist binding to the receptors and did not reverse significantly within the 45-s interpulse intervals. The degree of maximal NMDA receptor inhibition by memantine observed in neurons that had been allowed to equilibrate with the drug were similar to those seen in neurons under nonequilibrium conditions. For example, at 10 μM, memantine reduced by approximately 80% the amplitude of whole-cell currents triggered by NMDA (50 μM) plus glycine (10 μM), regardless of whether it was delivered to the neurons exclusively via the U-tube or both via the U-tube and the bath perfusion (Figs. 5 and 9B).

The degree of reversibility of memantine-induced inhibition of NMDA receptors depended on the membrane potential at which the recordings were obtained. At −60 mV, full reversibility was achieved within 10 min after removal of memantine from the agonist-containing solution, whereas at −100 mV, only partial reversibility could be achieved after removal of memantine (Fig. 9, A and B). The lower degree of reversibility of memantine-induced inhibition of NMDA receptors observed at the more negative membrane potentials is believed to reflect trapping of the drug within the NMDA receptor channel (Chen and Lipton, 1997).

**Memantine-Induced Inhibition of NMDA Receptors Tonically Activated in Hippocampal Neurons.** The on/off kinetics and the voltage dependence of memantine’s actions on NMDA receptors favor the selective block of receptors that are tonically activated by low ambient levels of glutamate. During synaptic transmission, the instantaneous rise of the synaptic glutamate concentration and robust depolarization of the postsynaptic neuron help the removal of memantine from its site of action in the NMDA receptor channels, rendering the postsynaptically located NMDA receptors fully active (Danyasz and Parsons, 2003).

In the present study, a U-tube was used to deliver a bulky amount of agonist-containing solutions to the entire surface of voltage-clamped neurons. The agonist concentrations used in the experiments were sufficiently high to activate a large number of receptors synchronously on the neuronal surface, and, consequently, trigger whole-cell currents with well-defined peaks. Because voltage clamp of large neurons with a single electrode is not perfect, the membrane potential in areas far from the recording electrode cannot be appropriately controlled. It is, therefore, conceivable that NMDA receptor activation by high agonist concentrations in poorly clamped and/or unclamped areas was sufficient to cause a shift of the membrane potential in those areas toward more positive values, which would favor unbinding of memantine from the receptor channels. Thus, to evaluate the possibility that the apparent potency for memantine-induced block of NMDA receptors was underestimated to any significant extent, tonic NMDA receptor activity elicited by continuous perfusion of the neurons with low NMDA concentrations was analyzed in the absence and in the presence of 1, 3, and 10 μM memantine.

In the nominal absence of Mg2+ and in the presence of tetrodotoxin, the baseline current was recorded from cultured hippocampal neurons voltage clamped at −60 mV and continuously bathed with physiological solution containing 1 μM glutamate and 1 μM glycine. Subsequently, the baseline current was recorded from the same neurons exposed to 1, 3, and 10 μM memantine (Fig. 10A). At 3 and 10 μM, memantine caused a significant reduction of the S.D. of the baseline current (Fig. 10C). Similar results were obtained when 1 mM Mg2+ was added to the physiological solution (Fig. 10D). The concentration dependence of the effect of memantine on tonically activated NMDA receptors was similar to that observed on whole-cell currents evoked by bulky application of high concentrations of NMDA to the neurons. Thus, the S.D. of the baseline current recorded in the continuous presence of 1 μM glutamate and 1 μM glycine was decreased by 1, 3, and 10 μM memantine to 92.4 ± 4.85% (not significantly different from control), 64.7 ± 3.43% (p < 0.001 according to the paired Student’s t test), and 32.8 ± 3.77% (p < 0.001 according to the paired Student’s t test), respectively (data are presented as mean ± S.E.M. of results obtained from six to seven neurons).

**Discussion**

**a7* nAChRs as Targets for Memantine’s Actions in the Brain.** The comparative analyses performed on rat hippocampal neurons in the present study demonstrated that NMDA receptors are not selective targets for memantine in the brain. At clinically relevant concentrations, memantine blocked more potently a7* nAChRs than NMDA receptors; the estimated IC50 values at −60 mV were 0.34 and 5.1 μM, respectively. Memantine-induced inhibition of NMDA receptors and a7* nAChRs was noncompetitive with respect to the agonists; memantine reduced the efficacies without affecting the apparent potencies for agonists to activate the receptors. The concentration-response relationship for memantine-induced inhibition of NMDA receptors at various membrane potentials showed an nH of approximately 1, indicating that memantine interacts with a single class of sites on NMDA receptors (Chen and Lipton, 1997). Consistently with the previously described open-channel blockade of NMDA receptors by memantine (Chen and Lipton, 1997), inhibition by memantine of NMDA receptors in the hippocampal neurons was found to be use and voltage-dependent, with an e-fold change of IC50 values occurring per 75-mV step. The use
inhibition of type IA currents suggested that the drug interacts with more than one class of sites on α7* nAChRs. Memantine at 0.1 μM caused a voltage-independent inhibition of type IA currents that could be explained by the drug’s interaction with sites on the extracellular domain of the receptor. A voltage-dependent inhibition of α7* nAChRs became evident with ≥1 μM memantine and was most likely the result of the drug’s interaction with sites within the receptor channel. The finding that the voltage dependence of the α7* nAChR inhibition decreased as the membrane concentration increased from 1 to 30 μM suggested that these multiple classes of sites are distinct and independent, affording different relative contributions to the total effect of memantine within an overlapping range of concentrations, or that these sites, although distinct, show some degree of negative cooperativity.

Inhibition of α7* nAChRs by memantine at all tested concentrations was use-dependent, and the time course for development of the maximal effect was not affected by pre-equilibration of the neurons with the drug. These results indicated that the interactions of memantine with α7* nAChRs depended on agonist binding and/or channel opening. Considering that α7* nAChR desensitization is voltage-independent (Mike et al., 2000), the use dependence of the voltage-independent inhibition of type IA currents could be explained by memantine acting as an enhancer of agonist-induced receptor desensitization. In contrast, the use dependence of the voltage-dependent inhibition of type IA currents could be in part due to open-channel blockade by memantine of α7* nAChRs.

Analysis of the decay phase of type IA currents lent support to the notion that open-channel blockade contributes to the α7* nAChR inhibition by ≥1 μM memantine. The rate of α7* nAChR desensitization is the primary determinant of the decay phase of type IA currents triggered by saturating agonist concentrations, whereas the rate of α7* nAChR closure (1/channel open time) contributes more significantly to the decay phase of type IA currents elicited by nonsaturating agonist concentrations (Mike et al., 2000). Accordingly, 1 μM memantine caused no change in the decay phase of currents evoked by 10 μM choline and accelerated the decay phase of currents evoked by 1 or 3 μM choline.

Studies from different laboratories have shown that memantine 1) inhibits voltage dependently recombinant human α4β2 nAChRs and rat α9α10 nAChRs expressed in oocytes with IC50 values of approximately 7 and 1.5 μM, respectively (Buisson and Bertrand, 1998; Oliver et al., 2001); and 2) inhibits voltage-independent recombinant human homomeric α7 nAChRs expressed in oocytes and recombinant human 5HT3 receptors expressed in mammalian cell lines with IC50 values of about 5 and 2 μM, respectively (Maskell et al., 2003, and references therein). The discrepant potencies and voltage dependence of memantine-induced inhibition of native rat α7* nAChRs and recombinant human α7 nAChRs could be accounted for by the facts that folding and assembly of α7 nAChRs is host-cell specific (Cooper and Mil1ar, 1997) and that the pharmacological properties of nAChRs vary with the system in which the receptor is expressed (Lewis et al., 1997). For instance, α-conotoxin EpI selectively blocks homomeric rat α7 nAChRs expressed in oocytes, while having no effect on native rat α7* nAChRs (Nicke et al., 2003). However, one cannot rule out that the above-noted discrepancies are due to species-specific differences in the α7 nAChR pharmacology (Papke and Porter Papke, 2002).

Possible Clinical Implications of Memantine-Induced Inhibition of α7* nAChRs and NMDA Receptors. Since it has long been recognized that excessive activation of NMDA receptors in central nervous system neurons leads to an intracellular Ca2+ overload that triggers cell death, NMDA receptor antagonists have been considered for treat-
ment of neurodegenerative disorders, including AD (for review, see Rogawski and Wenk, 2003). Earlier, the serious psychotomimetic effects, cognitive deficits and potential disruption of neuritogenesis and synaptogenesis triggered by such high-affinity NMDA receptor antagonists as dizocilpine and phencyclidine hampered their clinical development (Rogawski and Wenk, 2003). Memantine revived the therapeutic approach based on the NMDA receptor role in neurodegeneration to treat AD and other types of dementia (Rogawski and Wenk, 2003, and references therein) and is currently approved for treatment of patients with moderate-to-severe AD.

There are two major forms of NMDA receptor activities that regulate neuronal survival and plasticity. One is a tonic activity that is not regulated by nAChRs (Alkondon et al., 2003) and is maintained by repetitive activation of extrasynaptic NMDA receptors by ambient extracellular levels of glutamate (Sah et al., 1989). Another is a phasic (or synaptic) activity that is tightly regulated by $\alpha 7^*$ and non-$\alpha 7^*$ nAChRs (Alkondon et al., 2003; Pereira et al., 2002, and references therein) and is maintained by glutamate released from glutamatergic neurons onto postsynaptic densities rich in NMDA receptors. Glutamate-induced excitotoxicity, which is primarily mediated by excessive tonic NMDA receptor activity, has been hypothesized as a pathomechanism of neurodegenerative disorders, including AD and vascular dementia. Thus, it has been proposed that selective inhibition of the tonic NMDA receptor activity may account for the memantine-induced memory improvement seen in patients with moderate-to-severe AD and vascular dementia (for review, see Danysz and Parsons, 2003). This effect does not seem to result from a selective interaction of memantine with the NR2B-containing NMDA receptors that prevail in extrasynaptic compartiments of central nervous system neurons (Tovar and Westbrook, 1999). Instead, the rapid blocking kinetics, in addition to the high voltage- and use-dependent NMDA receptor inhibition by memantine seem to favor the blockade of extrasynaptic NMDA receptors (Danyzs and Parsons, 2003; Rogawski and Wenk, 2003) that are likely to be repetitively activated by the elevated extracellular levels of glutamate in the brain of AD patients (Jiménez-Jiménez et al., 1998).

The magnitude of memantine-induced block of NMDA-evoked whole-cell currents in the cultured hippocampal neurons was found to be similar to that of memantine-induced inhibition of tonically activated NMDA receptors. The effect of memantine on tonically activated NMDA receptors was evidenced by the reduction of the S.D. and the enhancement of the number of events caused by memantine was likely a result of the rapid flickering of the NMDA receptor channels between open, conducting and blocked, nonconducting states. The lower degree of trapping of memantine on NMDA receptors compared with that of other NMDA receptor antagonists seems to contribute to its better therapeutic index because it decreases the probability of long-lasting NMDA receptor inhibition (Chen and Lipton, 1997; Danyzs and Parsons, 2003; Rogawski and Wenk, 2003).

It has been consistently reported that $\alpha 7$ nAChR agonists protect neurons against NMDA-induced excitotoxicity and improve cognition (Levin, 2002; Gahring et al., 2003). The neuroprotective effects of $\alpha 7^*$ nAChR activation are inhibited by $\alpha 7$ nAChR antagonists, including the amyloid $\beta$ peptide 1-42 (Li and Buccafusco, 2003), which are also known to impair cognition (Levin, 2002). Taking these facts into account, memantine-induced $\alpha 7^*$ nAChR inhibition could explain the report that in healthy, young human subjects memantine impairs eyeblink conditioning (Schugens et al., 1997), a response that is modulated by the cholinergic system and is known to discriminate between aging related and probable AD cognitive deficits (Woodruff-Pak, 2001). It could also underlie the impairment of short-term memory processing observed in rats treated with memantine (Willmore et al., 2001). Thus, $\alpha 7^*$ nAChR inhibition by memantine could be counterproductive to its effectiveness in AD, particularly at early stages of the disease, when deterioration of cognitive functions correlates well with the degree of nicotinic cholinergic dysfunction (Francis et al., 1985; Perry et al., 2000; Nordberg, 2001; Sabbagh et al., 2001); the benefit of inhibiting excessive tonic NMDA receptor activity with memantine could be counteracted and eventually outweighed by the comntaminant $\alpha 7^*$ nAChR inhibition.

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