Amantadine Inhibits Nicotinic Acetylcholine Receptor Function in Hippocampal Neurons

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Accepted for publication January 27, 1997

ABSTRACT

The effects of amantadine on nicotinic acetylcholine receptors (nAChRs) of hippocampal neurons were studied by recording three types of acetylcholine (ACH)-evoked currents, using the whole-cell patch-clamp technique. The rapidly desensitizing type IA nicotinic current, which is α-bungarotoxin-sensitive and is mediated by nAChRs bearing α7 subunits, was inhibited by application of amantadine to neurons for 10 min (IC50 = 6.5 μM), but the potency of ACh (EC50 = 0.27 mM) was not affected by the drug. Amantadine (30–50 μM) attenuated the peak current amplitude in a voltage-dependent manner, with greater effect at negative than at positive membrane potentials. In contrast, the decay phase of the currents was shortened in a voltage-independent manner. When amantadine was coapplied briefly with ACh, the drug was markedly less potent (IC50 = 130 μM). Thus, the noncompetitive effects of amantadine on the type IA nicotinic current are complex, involving actions on the closed and desensitized states of the α7 nAChR. The slowly desensitizing, α-bungarotoxin-insensitive nicotinic currents of type II, which is inhibited by dihydro-β-erythroidine and is mediated by α4β2 nAChRs, and of type III, which is inhibited by mecamylamine and is mediated by α3β4 nAChRs, were also sensitive to inhibition by amantadine. The peak amplitude of type II current was reduced only slightly by 10 μM amantadine coapplied with ACh, but the decay-time constant and amplitude of the sustained current were markedly reduced. Type III current was also inhibited when amantadine was briefly coapplied with ACh. In contrast to its effects on nicotinic currents, amantadine at 10 μM did not affect currents evoked by N-methyl-D-aspartate plus glycine, γ-aminobutyric acid, glycine or kainate. Thus, on cultured hippocampal neurons, amantadine preferentially inhibits nicotinic currents.

Amantadine hydrochloride is recommended for the treatment of influenza and Parkinson’s disease (Davies et al., 1964; Schwab et al., 1972; Standaert and Young, 1996). The effectiveness of the drug against influenza A virus appears to be via the blockade of pH-sensitive cation channels formed by the M2 integral membrane protein (Duff et al., 1994; Wang et al., 1994). In comparison, the mechanisms accounting for the mild efficacy of amantadine against symptoms of Parkinson’s disease are not clear. Amantadine enhances the side effects of anticholinergic agents used in treating parkinsonism (Franz, 1975), and a direct inhibition of neurotransmission mediated by NMDA receptors in the caudate nucleus may be beneficial (Lupp et al., 1992; Stoof et al., 1992). In contrast, the NMDA-activated currents in superior colliculus neurons and the experimental seizures in mice were antagonized only by doses of amantadine well above the usual therapeutic levels (Parsons et al., 1995). Nevertheless, evidence that amantadine may favorably affect the limbic system in some cases was shown by the response of several individuals with refractory absence epilepsy to treatment with this compound (Shahar and Brand, 1992). Although large groups of elderly patients are treated with amantadine, raising concern about potential clinical effects on cognitive processes, amantadine has been found not to impair cognition (McEvoy, 1987; Hitri et al., 1987; Van Putten et al., 1987).

The question of whether amantadine alters the function of nAChRs in the brain has not been addressed, even though amantadine has been known for many years to modulate the function of nAChRs in muscle. In nerve-muscle preparations, amantadine decreases the amplitude of the EPC. The effect on the decay-time constant of the EPC is more complex, with shortening at negative membrane potentials but lengthening at positive potentials (Albuquerque et al., 1978; Tsai et al.,

ABBREVIATIONS: ACh, acetylcholine; ATP-RS, ATP-regenerating solution; α-BGT, α-bungarotoxin; DHβE, dihydro-β-erythroidine; EPC, end-plate current; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate.

Received for publication November 18, 1996.

1 A preliminary report of this study appeared previously (Matsubayashi and Albuquerque, 1995). This study was supported by National Institute of Neurological Diseases and Stroke Grant NS22986 and grants from Financiadora de Estudos e Projetos (FINEP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Brazil).
1978). This shortening of the decay-time constant of the EPC led to the suggestion that amantadine is a noncompetitive blocker at the muscle-type nAChR. Furthermore, amantadine was found to activate nAChR single-channel currents in isolated muscle fibers (Dumbill and Albuquerque, 1987), leading to the suggestion that the compound also may activate opening of the nAChR channel by acting as a weak noncompetitive agonist, as does physostigmine (Shaw et al., 1985; Pereira et al., 1993; Schrattenholz et al., 1993; Albuquerque et al., 1997).

Several types of nAChRs present in the mammalian brain differ sufficiently in their molecular structures, from each other and from nAChRs in muscle, to yield diverse functional and pharmacological properties (Alkondon and Albuquerque, 1993; Sargent, 1993; Albuquerque et al., 1995a,b; Lindstrom, 1995). The neuronal nAChRs in rat hippocampal neurons mediate three main types of current (Alkondon and Albuquerque, 1993). A fast desensitizing nicotinic current, named type IA current, is the most frequently observed type of nicotinic current in hippocampal neurons and is mediated by a nAChR that is highly permeable to Ca⁺⁺ and seems to bear α7 subunits (Alkondon and Albuquerque, 1993; Alkondon et al., 1994; Castro and Albuquerque, 1995; Bonfante-Cabarcas et al., 1996). MLA, α-BGT or α-conotoxin-ImI potently inhibits the activation of type IA current (Alkondon et al., 1992; Pereira et al., 1996). The nicotinic currents of types II and III are observed infrequently in cultured hippocampal neurons and desensitize more slowly (Alkondon and Albuquerque, 1993). The type II current is inhibited by DHβE and seems to be carried by α4β2-bearing nAChRs. On the other hand, the type III current is inhibited by mecamylamine and seems to be mediated by α3β4-bearing nAChRs.

The possibility that amantadine has an action on neuronal nAChR ion channels is supported by homology between the critical amino acids of transmembrane fragments that are proposed to line the ion channel pore of nAChRs (Séguéla et al., 1993; Unwin, 1995) and the amantadine-sensitive channel of the influenza virus M2 protein (Duff et al., 1994; Wang et al., 1994). In each case, a critical serine or threonine residue may be present where the open channel is most constricted, and hydrophobic amino acids are likely to line the inner wall of the channel in the closed state. The present study evaluated the effects of amantadine on neuronal nAChRs and a few other ligand-gated (GABA, glycine, kainate and NMDA) ion channels. The results demonstrate that amantadine has a weak antagonist effect that may signify ion channel blockade of the open state and a potent noncompetitive action at closed and/or desensitized states of the nAChRs in the mammalian central nervous system.

**Materials and Methods**

**Neuron culture.** Hippocampal neurons in culture were prepared by a procedure similar to that described previously (Aracava et al., 1987; Alkondon and Albuquerque, 1993). Briefly, the hippocampi of 16- to 18-day-old rat fetuses (Sprague-Dawley) were dissected out, minced and incubated with 0.25% trypsin (Gibco-BRL, Grand Island, NY) for 30 min at 36°C. Using a sterile Pasteur pipette, neurons were dispersed and then plated at a density of ~700,000 cells/35-mm culture dish ( precoated with Vitrogen 100 collagen; Celsis Laboratories, Palo Alto, CA). The cells were incubated at 36°C in a water-saturated, 10% CO₂/90% air atmosphere. The medium surrounding the cells was replaced twice each week. On the seventh day after plating, uridine and 5-fluoro-2'-deoxyuridine (final concentrations, 6.7 and 13.3 μM, respectively) were added to the culture medium for 24 hr to inhibit the proliferation of non-neuronal cells. For this study, the neurons were cultured for 14 to 30 days.

**Whole-cell current recording.** Whole-cell currents were recorded from hippocampal neurons with the standard patch-clamp technique (Hamill et al., 1981), using an LM-EPAC-7 patch-clamp system (List Electronics, Darmstadt, Germany). The patch pipettes were pulled from borosilicate capillary glass, and their resistance when filled with internal solution was 2 to 5 MΩ. The series resistance of the patches was 8 to 20 MΩ and was not compensated. The signals were filtered at 3 kHz and either were recorded on tape for later analysis or were sampled directly by a microcomputer using the program pCLAMP (Axon Instruments, Foster City, CA). All experiments were performed at room temperature (20–22°C).

The external bath solution (340 mOsm) consisted of 165 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM glucose, 5 mM HEPES, 0.001 mM atropine and 0.0003 mM tetrodotoxin, and the pH of the solution was adjusted to 7.3 with NaOH. The neurons were superfused continuously with external solution flowing at 1.5 to 2.0 ml/min.

In many studies, the pipettes were filled with an ATP-PSR to reduce the rate of rundown of type IA currents (Alkondon et al., 1994). The ATP-PSR was prepared daily from a stock solution of 60 mM CsCl, 60 mM CsF, 10 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10 mM HEPES and 2 mM MgCl₂ by addition of 5 mM ATP (Tris salt), 20 mM phosphocreatine (di-Tris salt), 50 U/ml creatine phosphokinase and a small amount (~5 μl) of 1 N CsOH (to adjust the solution to pH 7.3 and the concentration of Cs⁺ to 155 mM). The final osmolality was 340 mOsm. In other studies, the standard solution inside the pipette consisted of 80 mM CsCl, 80 mM CsF, 10 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid and 10 mM HEPES, and the pH of the solution was adjusted with CsOH to pH 7.3 (the final concentration of Cs⁺ was 187 mM, and the osmolality was 340 mOsm). Studies of type IA current that used the standard solution in the pipette were begun 20 min after establishment of the whole-cell patch, when the rundown of type IA currents is considerably smaller than immediately after patch formation, and the studies were completed within 10 min (Alkondon and Albuquerque, 1993). The standard pipette solution was also used in studies of the slowly decaying type II and type III nicotinic currents (Alkondon and Albuquerque, 1993) and of non-nicotinic currents that do not run down within the time of the experiments.

**Drug applications.** External solutions with ACh or other agonists, with or without amantadine, were applied to neurons using the U-tube system (Albuquerque et al., 1991). A U-shaped tube, which had a 250- to 400-μm pore at the apex, was positioned ~50 μm above the neuronal soma. A drug solution, which was selected by a manual switch valve, was delivered to one end of the U-tube and was withdrawn by suction through a polyethylene tube at the opposite end. A small amount of bath solution was also removed to prevent leakage of drug solution out of the U-tube. When a solenoid valve was closed briefly (~2 sec) by an electric pulse, a rapid flow of drug solution was forced out through the pore, completely displacing the bath solution surrounding the neuronal soma and dendrites.

Two protocols were used to apply amantadine hydrochloride (1–500 μM) to the neurons. In one procedure, a neuron was superfused for several minutes with an external bath solution containing amantadine, and the agonist solution delivered from the U-tube always contained the same concentration of the drug. In the other procedure, amantadine was applied to the target neuron only briefly (~2 sec) when a mixture of agonist and drug were delivered through the U-tube system.

Amantadine · HCl, ACh · HCl, NMDA, glycine · HCl, kainic acid, GABA, tetrodotoxin, atropine sulfate, ATP (Tris salt), phosphocreatine (di-Tris salt) and creatine phosphokinase (type I) were obtained from Sigma Chemical Co. (St. Louis, MO). DHβE · HBr was a

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Data analysis. The peak amplitude and the decay-time constants of the whole-cell currents were determined using the program pCLAMP. The IC$_{50}$ and Hill coefficient ($n_H$) values were determined with the program Sigma Plot by the best fit of the data to the equation:

$$I = I_{max} \left( 1 - \frac{[\text{amantadine}]^{n_H}}{[\text{amantadine}]^{n_H} + IC_{50}} \right)$$

where $I$, $I_{max}$, [amantadine], IC$_{50}$, and $n_H$ are the peak whole-cell current at the test concentration of amantadine, the maximum peak current in the absence of amantadine, the concentration of amantadine applied, the calculated concentration of amantadine that would reduce the current by 50% and the Hill coefficient, respectively. Values are expressed as the mean ± S.E. A P value of 0.05 in the Students’ t test was taken to be statistically significant.

Results

Brief applications of ACh (0.5–1 sec, 1 mM) to cultured hippocampal neurons evoked one of three types of nicotinic current. Each current was classified according to its kinetic and pharmacological characteristics, in accordance with the previous description; type IA current decays rapidly and is blocked by the competitive antagonist MLA (1 nM), and the slower decaying type II and type III currents are blocked by DHβE (100 nM) and mecamylamine (1 μM), respectively (Alkondon and Albuquerque, 1993, 1995; Albuquerque et al., 1995a). A current that is a combination of type IA and type II currents, called type IB current, could also be recorded from some neurons (Alkondon and Albuquerque, 1993, 1995).

Effects of amantadine on type IA nicotinic current.

Type IA currents were isolated by using an external solution containing DHβE (100 nM) to block any type II current. Under this condition, rapidly decaying type IA currents were found in 66 of 70 neurons tested in this phase of the study. To prevent rundown of the type IA currents during long protocol procedures, patch pipettes were filled with ATP-RS.

After the type IA current evoked by ACh (0.3 mM) was recorded several times to ensure a stable response, amantadine (1–30 μM) was applied to each neuron for 10 min and was found to reduce the peak amplitude of this current in a concentration-dependent manner (fig. 1A). The amplitude of the small, slowly decaying component recorded in some of the type IA currents was also decreased by amantadine (fig. 1B). Nine neurons exhibiting a type IA current in response to ACh were treated in this manner with one or more concentrations of amantadine for 10 min each, and the results were combined by expressing the peak amplitude of each current evoked by ACh plus amantadine as a percentage of the peak amplitude before exposure to the drug. The inhibition of the type IA current by amantadine was concentration dependent, with potency reflected by the IC$_{50}$ of 6.5 μM and the $n_H$ of 0.96 (fig. 1C).

To determine the mechanism by which amantadine inhibits type IA current, several concentrations of ACh (0.05, 0.1, 0.3, 1 or 3 mM) were applied at 2-min intervals before and after each neuron was superfused for 10 min with an external solution containing the drug (10 μM). As expected, the peak amplitude of type IA currents increased with increasing ACh concentration (fig. 2A). The peak current amplitude elicited by each concentration of ACh was considerably smaller during exposure of the neuron to amantadine (fig. 2A, center). For each neuron, the peak current amplitudes were normalized relative to the response to 3 mM ACh in the absence of amantadine. Linear regression analysis of the normalized responses in a double-reciprocal plot revealed that the maximum response to ACh in the presence of amantadine was reduced to 53% of control (fig. 2B). The apparent affinity of the receptor for ACh was, however, unchanged by treatment with the drug (fig. 2B). Superfusion of the neurons with amantadine-free external solution reversed the inhibitory effect of amantadine (fig. 2A); the small differences in amplitude noted during the recovery phase could be accounted for by the expected rundown of control responses over the 20-min interval. These results indicated that amantadine does not compete for the ACh binding site of the α7 nAChR that subserves the type IA current in hippocampal neurons, suggesting that the drug acts at an allosteric site on the receptor. Also, the impairment of mACHR function is reversible.

The effect of membrane potential on the inhibition caused by amantadine was studied by recording type IA currents at negative and positive potentials before and after superfusion of the neuron with amantadine-containing external solution.
from the U-tube and through the bath. Pulses of ACh (1 mM, 0.5 sec) were initially applied to five neurons at holding potentials from $-120$ to $60$ mV in 20-mV steps. The peak current-voltage relationships of the type IA currents in the control condition revealed a significant inward rectification (fig. 3B), which could be accounted for by the Mg$^{2+}$ in the ATP-RS solution (Alkondon et al., 1994; Bonfante-Cabarcas et al., 1996). Ten minutes after bath superfusion of the neurons with amantadine (50 µM), pulses of ACh plus the drug (50 µM) were applied while the neurons were held at the same range of potentials (fig. 3A). Amantadine reduced the normalized peak amplitudes at negative potentials. However, the peak amplitude of the type IA current at positive potentials was decreased to a lesser degree by amantadine, such that the current-voltage relationship was linear from $-120$ to $60$ mV (fig. 3B). Thus, rectification was apparently reduced. For each holding potential, the means of the ratios of the peak amplitudes in the presence of amantadine to the amplitudes under the control condition were plotted (fig. 3C).

The ratio was small (~0.2) at negative potentials and increased to up to 0.4 with depolarization to 50 mV.

The protocol used for figure 3 was repeated with a lower concentration of amantadine (30 µM), again applied to the neurons via bath and U-tube perfusion, and a lower concentration of ACh (0.3 mM). Under these conditions, as at higher concentrations, amantadine reduced the peak amplitude of the type IA current (fig. 4, A and B) ($n=7$ neurons). The current-voltage relationship appeared to confirm the loss of rectification (fig. 4B), and the ratio of peak amplitude in the presence of amantadine to the corresponding amplitude under the control condition was small (~0.2) at negative potentials (fig. 4C). At 40 mV, 30 µM amantadine was somewhat less effective at reducing the peak amplitude of the current elicited by 0.3 mM ACh (~40% inhibition) (fig. 4C) than was 50 µM amantadine at reducing the peak amplitude of the current evoked by 1 mM ACh (60% inhibition) (fig. 3C). This suggests a lack of competition between ACh and amantadine, because the amount of inhibition was reduced when the drug

**Fig. 2.** Noncompetitive blockade by amantadine of type IA current. A, type IA currents were evoked by 1-sec pulses of ACh at various concentrations (left). Then amantadine was applied via bath and U-tube for 10 min before various test concentrations of ACh were reapplied (center). Finally, the preparation was washed with drug-free solution for 10 min and the test concentrations of ACh were reapplied (right). All traces were obtained from a single neuron held at $-60$ mV. B, a series of experiments was summarized by normalizing the amplitude of each response to the peak current amplitude elicited by ACh (3 mM) in the absence of amantadine in the same neuron. The data (mean ± S.E.) from three to eight neurons are shown in a double-reciprocal plot. Linear regressions of each group of data gave the affinity for ACh as 0.28 mM in the control condition and 0.27 mM in the amantadine (10 µM)-treated condition.

**Fig. 3.** Voltage-dependent inhibition by amantadine (50 µM), applied via bath and U-tube, of type IA current evoked by ACh (1 mM). A, pulses of ACh (1 mM, 0.5 sec) were first applied while holding potential was increased in 20-mV steps from $-120$ to 60 mV; sample current traces are shown for three holding potentials (left). Ten minutes after changing to the bath solution containing amantadine (50 µM), pulses of ACh plus amantadine (50 µM) were applied at the same potentials (right). All traces shown were obtained from one neuron. B, the peak amplitude measured under all conditions was normalized to the peak amplitude at $-120$ mV induced by ACh in the absence of amantadine. The plotted data represent the mean ± S.E. from experiments in five neurons. The current-voltage relationships below 0 mV were used to fit the lines. C, the relationship of the ratio of the amplitude in the presence of amantadine (50 µM) to the corresponding amplitude in control solution vs. holding potential is shown.
Six neurons were found to comprise a large (1993). Therefore, the decay phases of control type IA currents (fig. 5A) (Alkondon and Albuquerque, 1993). The type IA currents elicited by ACh (0.3 mM) were each fitted by a double-exponential desensitizing component and a small (20%), slow-desensitizing component (fig. 5A) (Alkondon and Albuquerque, 1993). Therefore, the decay phases of control type IA currents (ACh, 0.3 mM) were each fitted by a double-exponential function. The two decay-time constants (τ values) at each holding potential averaged in the range of 24 to 33 msec and 254 to 426 msec and were unaffected by changes in voltage (−120 to −20 mV) under the control condition (fig. 5B). For comparison, the current traces obtained at −60 and −100 mV in the presence of amantadine (30 μM) were scaled to match the peak amplitude of the corresponding control traces (fig. 5A). Analysis revealed that the drug had reduced the decay phase of the type IA current to a single-exponential function with a τ of 31 to 36 msec, which was voltage insensitive (fig. 5B). Furthermore, the decay rate of ACh-evoked type IA currents in the presence of amantadine was similar to the fast decay rate of the currents under the control condition.

The effect of pre-exposure to amantadine on resting nAChRs was compared with the effect of short-pulse exposure of neurons to amantadine. Because these experiments used only short-pulse applications of amantadine coapplied with ACh (1 mM) and could be performed in <10 min, a time frame with little rundown of current amplitude without the use of ATP-RS, the internal pipette solution contained CsF and was nominally Mg2+-free. Under this condition, the inhibitory effect of amantadine on the type IA current was concentration dependent, with an IC50 of 130 ± 10 μM and a nH of 1.1 ± 0.1 (n = 6) (fig. 6). Whereas the application of 10 μM amantadine via U-tube alone decreased the peak amplitude of the type IA current elicited by ACh (1 mM) only to 94 ± 3% of control (fig. 6), the application of 10 μM amantadine via bath and U-tube reduced the current amplitude to 39 ± 8% of control (n = 7) (fig. 1B), and the difference was highly significant.

**Effects of amantadine on type II nicotinic currents.** Some hippocampal neurons respond to ACh application with the slowly decaying type II nicotinic current, which is sensitive to blockade by DHβE (Alkondon and Albuquerque, 1993). Because type II nicotinic current is not subject to rundown, these currents were elicited from six neurons by using patch pipettes filled with nominally Mg2+-free internal solution including CsF. In contrast to type IA currents, which showed inward rectification with Mg2+-containing ATP-RS (figs. 3B and 4B) but not with the CsF-based, nominally Mg2+-free internal solution (fig. 7A) (Alkondon and Albuquerque, 1993; Alkondon et al., 1994), the type II current showed inward rectification at positive potentials with internal solutions with or without Mg2+ (fig. 7B) (Alkondon and Albuquerque, 1993; Alkondon et al., 1994).

Four neurons that responded to pulses of ACh (1 mM, 2 sec) delivered through the U-tube with type II currents were subjected to coapplication of amantadine (10 μM) with ACh. Amantadine tended to decrease the peak amplitude of the type II current (fig. 8), lowering the average peak amplitude to 90 ± 8% of control (n = 4). The decay phase of each current was fitted to a single-exponential decay function with a baseline offset, revealing that coapplication of 10 μM amantadine decreased the decay-time constants to 77 ± 10% of their control values. Whereas under the control condition the current at the end of the ACh pulse (1 mM, 2 sec) was 59 ± 6% of peak amplitude, with coapplication of amantadine and agonist the current amplitude decayed to 25 ± 9% of the peak amplitude. Thus, amantadine (10 μM) had greater effects on
the decay phase and on the amplitude of the steady-state current than on the peak amplitude of the type II current.

Because of the small number of neurons expressing receptors for type II current, it was not possible to perform complete dose-response or time-dependent studies. Coapplication of 500 μM amantadine with ACh reduced the peak response to 50% of control in one neuron. Exposure of another neuron for 10 min to 10 μM amantadine also decreased the peak amplitude to about 50% of control. Thus, the effect of amantadine on type II currents appeared to be concentration and time dependent.

Comparison of the effects of short applications of amantadine on three types of neuronal nAChR currents. The effects of a high concentration of amantadine (500 μM) on type IA, type II and type III nicotinic currents of hippocampal neurons are compared in figure 9. Each neuron was held at −60 mV, a nicotinic current was evoked by a pulse of ACh (1 mM, 0.5–1 sec) and 1 or 2 min later a pulse of ACh (1 mM) plus amantadine (500 μM) was applied to the neuron. Only one neuron displaying type II current in the course of these experiments was treated with this concentration of the drug, and the frequency of occurrence of type III current was <1%. Amantadine (500 μM) decreased the peak amplitude of the type IA current to 20 ± 3% of control (n = 6 neurons), that of the type II current to 50% of control (n = 1) and that of the type III current to 15% of control (n = 1) (fig. 9). Normal responses of each type were recovered 2 to 5 min after exposure of the neurons to amantadine-free external solution (fig. 9). Thus, amantadine reversibly inhibited each of the three types of nicotinic current, and the greatest effects on the peak amplitude were on type IA and type III currents. Amantadine also markedly shortened the early decay phase.
Our study revealed that amantadine is a potent inhibitor of nAChR function in hippocampal neurons. Exposure of the neurons to amantadine for several minutes inhibited type IA currents, which are mediated by α7-bathing nAChRs. The effect of amantadine on the α7 nAChR was noncompetitive with respect to the agonist, and the IC_{50} (6.5 μM) (fig. 1) for inhibition of the type IA current was very close to the concentrations of amantadine found in the striatal dialysate of rats whose behavior was altered by treatment with amantadine and to the concentrations found in serum and cerebrospinal fluid of patients who had received amantadine therapy for Parkinson’s disease (Kornhuber et al., 1995). Thus, inhibition of neuronal nAChRs can be expected to occur during therapy for Parkinson’s disease. Using a different experimental protocol, amantadine was coapplied with ACh for only a few seconds, the drug was less potent in reducing the amplitude of the type IA, α7 nAChR-mediated current (IC_{50} = 130 μM). Short application also had weak effects on the peak amplitudes of type II, α4β2 nAChR-mediated current and type III, α3β4 nAChR-mediated current, although this treatment accelerated the initial decay and reduced the amplitude of the later, slowly decaying component of the α-BGT-insensitive type II and type III currents.

Mechanism of type IA nicotinic current blockade by amantadine. The double-reciprocal plots of the amplitudes of type IA currents obtained from cultured hippocampal neurons stimulated by ACh in the presence and absence of amantadine showed that the maximal response to ACh was decreased but the affinity of the nAChR for ACh was not affected by amantadine. This result indicates that amantadine does not compete with ACh for binding to the nAChR that mediates the type IA current. The mechanism of action of amantadine on this neuronal nAChR, composed of α7 subunits (Lindstrom, 1995; Palma et al., 1996), is apparently similar to that on the muscle-type nAChR, where amantadine (100 μM) did not affect the binding of [3H]ACh or α-BGT to the muscle-type nAChR of Torpedo electric organs (Tsai et al., 1978).

The reduction of the peak amplitude of type IA current by amantadine was voltage dependent, and the drug also shortened the decay phase of the type IA current. One way to determine whether an open-channel blocking action of amantadine contributes to the inhibition of the type IA current, in the manner that it contributes to the inhibition of EPCs (Albuquerque et al., 1978; Tsai et al., 1978; Dumbill and Albuquerque, 1987), would be to determine whether amantadine shortens the open time of the α7 nAChR channel. This approach, however, would be very difficult because the 73-pS channels, the most common conductance state subserving type IA current, have a very brief open time and a fast rate of desensitization under control conditions (Castro and Albuquerque, 1993) and other subconductance states of the α7 nAChR, whose open times are longer, occur infrequently (Pereira et al., 1993; Castro and Albuquerque, 1993). The physiological process accounting for the decay of the type IA current, i.e., desensitization, is determined by the rate of receptor inactivation (Castro and Albuquerque, 1993) and is voltage independent under the control condition (fig. 5) (Alkondon and Albuquerque, 1993). Amantadine did not shorten the initial rate of decay of the type IA current, irre-
perspective of voltage, but the drug eliminated the later slow-decay phase of the current (fig. 5). Thus, this effect of amantadine on the type IA current is apparently caused not by open-channel blockade but by an increase in the extent of desensitization, suggesting that amantadine decreases the rate of nAChR reactivation. Amantadine applied through the U-tube alone was relatively weak at inhibiting the peak amplitude of the type IA current ($IC_{50} = 130 \mu M$). An explanation for this finding is that amantadine also has a rapid action as an open-channel blocker of the nAChR, as well as of the NMDA receptor (fig. 10). Different pharmacodynamics and pharmacokinetics of the actions of amantadine at two different sites on the nAChR for type IA current can explain the findings that low micromolar concentrations of the drug act slowly and noncompetitively, with respect to ACh, on the closed-channel conformation and that the drug rapidly blocks open channels at high micromolar concentrations.

The possibility that the more potent, slower, noncompetitive action of amantadine on the nAChR may occur at the allosteric site for agonists should be considered. Amantadine activates the muscle-type nAChR (Dumbill and Albuquerque, 1987), and such activation was not blocked by $\alpha$-BGT (E. F. R. Pereira and E. X. Albuquerque, unpublished observations), indicating that amantadine activates the nAChR by an action on the second agonist site (Shaw et al., 1985; Pereira et al., 1993; Schrattenholz et al., 1993). In the absence of endogenous agonist, such a weak allosteric agonist action by amantadine could cooperate with a slowed rate of reactivation of receptor in the presence of amantadine (suggested above) to favor the desensitized state of the nAChR, accounting for the more potent depression of peak amplitude elicited by ACh when amantadine is applied to the neuron for a longer time, i.e., in the bath.

**Comparison of the effects of amantadine on neuronal and muscle-type nAChRs.** Although amantadine could be applied to relatively fewer neurons showing the type II and type III currents, compared with many neurons showing type IA currents, it was clear that the drug inhibited the

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**Fig. 9.** Inhibitory effects of amantadine, applied via U-tube, on three types of neuronal nicotinic currents. Upper, the three traces were obtained by application to a single neuron, via a U-tube, of a 0.5-sec pulse of ACh (1 mM), followed 1 min later by a pulse of ACh plus amantadine (500 $\mu M$) and 1 min thereafter by a pulse of ACh (1 mM) alone. The response to ACh (1 mM) was recovered at 2 min (not shown). Center, type II current was obtained in another neuron using a 1-sec pulse of ACh without amantadine and 2 min later a pulse of ACh with amantadine. The current was recovered at 2 min after the application of amantadine. Lower, type III current was obtained from another neuron. The 0.5-sec pulse of ACh plus amantadine was applied to the neuron 10 min after the control, and the response to ACh was recovered 5 min after the application of amantadine. All traces were obtained at $-60$ mV holding potential.

**Fig. 10.** Relative sensitivity of ligand-gated ion channels to amantadine. At least five independent experiments were performed with each agonist, and membrane potentials were held at $-60$ mV. Each bar represents the mean ± S.E of the amplitude in the presence of amantadine as the percentage of control amplitude. The data for ACh-evoked currents are summarized here. The agonists kainate, GABA, glycine and NMDA plus glycine were applied as 2-sec pulses to different neurons; 10 min after changing to the bath solution containing amantadine (10 $\mu M$), a 2-sec pulse of the same agonist plus amantadine (10 $\mu M$) was applied. Amantadine (10 $\mu M$), in the bath and in the U-tube, had a significant effect on the type IA current induced by ACh. For the type IA currents, the effects of amantadine (10–100 $\mu M$) applied via a U-tube alone were less than that of amantadine (100 $\mu M$) applied via bath and U-tube. For the current evoked by NMDA plus glycine, the effect of amantadine (100 $\mu M$) applied via a U-tube alone was the same as that of amantadine (100 $\mu M$) applied via bath and U-tube.
three neuronal types of nicotinic currents with different potencies. The inhibitory potency of amantadine when drug treatment was limited to coapplication with the agonist, i.e., via U-tube only, appeared to be greater against the peak amplitude of the type IA and type III currents than against the peak amplitude of the type II current. On the other hand, amantadine shortened the decay phase of type II and III currents substantially, but shortening of the decay phase of the already rapidly desensitizing type IA current evoked by ACh (1 mM) was less apparent. The kinetic changes in the type II and type III currents suggest that amantadine may act on these nAChRs as an open-channel blocker.

Amantadine also decreases the response of muscle-type nAChRs in frog sartorius, rat soleus and rat diaphragm muscles (Tsai et al., 1978). The peak amplitude of the EPC of frog sartorius muscle at −90 mV is reduced in a concentration-dependent manner by amantadine, with an IC50 of 64 μM (Warnick et al., 1982). Furthermore, the binding of [3H]perhydrohistrionicotoxin to Torpedo electric organ membranes is inhibited by amantadine (K i = 60 μM) (Tsai et al., 1978). Thus, amantadine is 10-fold less potent against EPCs of α2βδ nAChRs in muscle than against the type IA current of α7 nAChRs in the hippocampal neurons (IC50 = 6.5 μM). The mechanisms of actions of amantadine on these α-BGT-sensitive receptors also differ. The effects of amantadine on EPCs, i.e., nonlinearity in the peak amplitude-voltage relationship and reversal of the voltage dependence of decay rate with prolongation at depolarized potentials (Warnick et al., 1982), suggest that ion-channel blockade of muscle-type nAChRs occurs at hyperpolarized potentials. The greater potency of amantadine against the type IA nicotinic current than against the EPC appears to arise from the strong action on a closed-channel state of the α7 nAChR at hyperpolarized potentials. To a lesser extent, a weaker action on the open state of the neuronal nAChRs may explain inhibition of type IA nicotinic currents at depolarized potentials, and perhaps also the type II and type III currents.

In addition to the inhibitory effects of amantadine thus far discussed, amantadine has been shown to augment the response mediated by nAChR under a different condition. In the rat phrenic nerve-diaphragm preparation, muscle twitch was potentiated for up to 45 min after the start of application of 150 μM amantadine, and only later was the response inhibited (Tsai et al., 1978). The possibility that amantadine may be able to potentiate nicotinic responses in brain tissue is currently being studied.

Effects of amantadine on other ligand-gated ion channels in hippocampal neurons. In comparison with its effects on nAChRs in the cultured hippocampal neurons, amantadine at 100 μM was a weak inhibitor (60% of control current) of the NMDA-induced currents, and amantadine at 10 μM was ineffective against NMDA-, kainate-, GABA- or glycine-induced currents. Thus, the lower concentration of amantadine selectively inhibited the nicotinic currents, leaving other ligand-gated channels of hippocampal neurons relatively unaffected.

Therapeutic mechanisms of amantadine. The pathology of Parkinson’s disease has often been attributed to an imbalance of dopaminergic and cholinergic transmission. Recent studies of the caudate nucleus in vitro have shown mechanisms by which amantadine may increase the dopamine/ACh ratio in treatment of Parkinson’s disease. Aman-
amantadine combines relative selectivity against nAChRs with clinical usefulness and proven safety.

Acknowledgments

The authors are grateful to Barbara Marrow and Mabel Zelle for expert technical assistance.

References


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