

Memantine Inhibits Efferent Cholinergic Transmission in the Cochlea by Blocking Nicotinic Acetylcholine Receptors of Outer Hair Cells

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ABSTRACT

Memantine is a blocker of Ca^{2+} -permeable glutamate and nicotinic acetylcholine receptors (nAChR). We investigated the action of memantine on cholinergic synaptic transmission at cochlear outer hair cells (OHCs). At this inhibitory synapse, hyperpolarization of the postsynaptic cell results from opening of SK-type Ca^{2+} -activated K^+ channels via a highly Ca^{2+} -permeable nAChR containing the $\alpha 9$ subunit. We show that inhibitory postsynaptic currents recorded from OHCs were reversibly blocked by memantine with an IC_{50} value of $16 \mu\text{M}$. RT-PCR revealed that a newly cloned nAChR subunit, $\alpha 10$, is expressed in OHCs. In contrast to homomeric expression, co-expression of $\alpha 9$ and $\alpha 10$ subunits in *Xenopus laevis* oocytes resulted in robust acetylcholine-induced currents, indicating that the OHC nAChR may be an $\alpha 9/\alpha 10$ heteromer. Accord-

ingly, nAChR currents evoked by application of the ligand to OHCs and currents through $\alpha 9/\alpha 10$ were blocked by memantine with a similar IC_{50} value of about $1 \mu\text{M}$. Memantine block of $\alpha 9/\alpha 10$ was moderately voltage dependent. The lower efficacy of memantine for inhibition of inhibitory postsynaptic currents (IPSCs) most probably results from a blocking rate that is slow with respect to the short open time of the receptor channels during an IPSC. Thus, synaptic transmission in OHCs is inhibited by memantine block of Ca^{2+} influx through nAChRs. Importantly, prolonged receptor activation and consequently massive Ca^{2+} influx, as might occur under pathological conditions, is blocked at low micromolar concentrations, whereas the fast IPSCs initiated by short receptor activation are only blocked at concentrations above $10 \mu\text{M}$.

The adamantane derivative 3,5-dimethyl-1-adamantanamine (memantine) is a well established blocker of ligand-gated ion channels permeable for Ca^{2+} such as the NMDA-type glutamate receptor (Chen et al., 1992; Parsons et al., 1993; Bresink et al., 1996) or nicotinic acetylcholine receptors (Buisson and Bertrand, 1998). In either case, memantine acts as an open channel blocker, that enters the channel pore and sterically occludes the ion pathway (Chen et al., 1992; Buisson and Bertrand, 1998). For NMDA-receptors, this pore-block strongly depends on the transmembrane voltage, with highest efficacy at hyperpolarized potentials (Bresink et al., 1996); for nAChRs, the voltage-dependence is less pronounced (Buisson and Bertrand, 1998). Therapeutically, memantine is used to prevent excitotoxic neuronal cell death associated with neurodegeneration and stroke and is induced by mas-

sive influx of Ca^{2+} through overactivated NMDA receptors (Chen et al., 1992; Parsons et al., 1999).

Cochlear outer hair cells (OHCs) are the central element of the active mechanical amplification mechanism that is crucial for the exquisite sensitivity and frequency-resolving capacity of the mammalian hearing organ (Dallos, 1992; Dallos and Evans, 1995). Mechanistically, this amplification is based on the ability of OHCs to alter their cell length in response to changes in membrane potential, a process that works at frequencies of up to at least 70 kHz (Brownell et al., 1985; Gale and Ashmore, 1997; Frank et al., 1999). Cochlear amplification is controlled by the cholinergic medial olivocochlear system that synapses onto OHCs (reviewed by Guinan, 1996). This inhibitory synapse uses an excitatory nAChR to supply the postsynaptic OHC with Ca^{2+} that initiates an inhibitory hyperpolarization by opening Ca^{2+} -activated SK2 channels (Fig. 1A) (Fuchs and Murrow, 1992; Blanchet et al., 1996; Evans, 1996; Oliver et al., 2000).

The OHC nAChR contains the $\alpha 9$ subunit (Elgoyhen et al.,

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ABBREVIATIONS: memantine, 3,5-dimethyl-1-adamantanamine; NMDA, N-methyl-D-aspartate; OHC, outer hair cell; nAChR, nicotinic acetylcholine receptor; BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; IPSC, inhibitory postsynaptic current; ACh, acetylcholine; RT-PCR, reverse transcriptase-polymerase chain reaction.

1994; Glowatzki et al., 1995; Vetter et al., 1999) and exhibits a high permeability for Ca^{2+} (Jagger et al., 2000; Katz et al., 2000). This high permeability allows for significant Ca^{2+} influx into the OHC that may lead to globally increased cytoplasmic $[\text{Ca}^{2+}]$ (Doi and Ohmori, 1993). In analogy to excitotoxic processes in CNS neurons, abnormally high Ca^{2+} levels may initiate or support degradation of OHC; loss of functional OHCs is well known to be a major cause of sensorineural hearing loss induced by various harmful stimuli including noise or ototoxic agents (Cody and Russell, 1985; Patuzzi et al., 1989). So far, it has been shown that Ca^{2+} entering through the nicotinic receptor can trigger structural alterations of OHCs, resulting in a decreased axial stiffness (Dallos et al., 1997; Sridhar et al., 1997), and elevated intracellular Ca^{2+} has been implicated in the impairment of OHC function after acoustic overstimulation (Fridberger et al., 1998). Here we test the effect of memantine on efferent synaptic transmission and Ca^{2+} -influx in OHCs and investi-

gate block of the nAChR as the mechanism underlying the observed inhibitory effects.

Materials and Methods

Patch-Clamp Recordings on Outer Hair Cells. The apical turn of the organ of Corti was dissected from cochleae of three- to six-week-old Wistar rats as described previously (Oliver et al., 2000). The preparation was performed in a solution containing 144 mM NaCl, 5.8 mM KCl, 0.1 mM CaCl_2 , 2.1 mM MgCl_2 , 10 mM HEPES, 0.7 mM Na_2HPO_4 , and 5.6 mM glucose, pH adjusted to 7.3 with NaOH. For recordings, OHCs located between half and one turn from the apex of the cochlea were chosen. If necessary, supporting cells were removed with gentle suction from a cleaning pipette carefully avoiding mechanical disturbance of the efferent nerve terminals.

Whole-cell, patch-clamp recordings were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at room temperature (22–25°C). Electrodes were pulled from quartz glass, had resistances of 2–3 M Ω and were filled with intracellular solution: 135 mM KCl, 3.5 mM MgCl_2 , 0.1 mM CaCl_2 , 5 mM EGTA, 5 mM HEPES, and 2.5 mM Na_2ATP . For one series of experiments, an intracellular solution containing the Ca^{2+} -chelator BAPTA was used: 120 mM KCl, 3.5 mM MgCl_2 , 10 mM BAPTA, 5 mM HEPES, and 2.5 mM Na_2ATP . The pH of both solutions was adjusted to pH 7.3 with KOH. Membrane potential was corrected for the electrode junction potential (–4 mV). Whole-cell series resistance ranged from 4 to 9 M Ω and was not compensated. Currents were filtered at 1 kHz and sampled at 5 kHz.

The specimen was continuously superfused with extracellular solution (144 mM NaCl, 5.8 mM KCl, 2 mM CaCl_2 , 0.9 mM MgCl_2 , 10 mM HEPES, 0.7 mM Na_2HPO_4 , and 5.6 mM glucose, pH adjusted to 7.3 with NaOH). Chemicals as well as depolarizing solutions were applied via a glass capillary (diameter approximately 80 μm) positioned close to the organ of Corti. For the depolarizing external solution, KCl was substituted for an equal amount of NaCl to result in $[\text{K}^+]_{\text{ex}}$ of 47 mM. Memantine and acetylcholine (both from Sigma, St. Louis, MO) were added to the extracellular solution from aqueous stock solutions. To block the large OHC resting K^+ current, $I_{\text{K},\text{n}}$, 10 μM linopirdine (Sigma/RBI, Natick, MA) or 1–5 μM XE991 (obtained from DuPont Pharmaceuticals, Wilmington, DE) was added to the standard extracellular medium from stock solutions made with dimethyl sulfoxide (final concentration $\leq 0.1\%$) (Housley and Ashmore, 1992; Marcotti and Kros, 1999). XE991 is a M -current blocker (Wang et al., 1998) that inhibits $I_{\text{K},\text{n}}$ at submicromolar concentrations in a poorly reversible manner (D.O., unpublished observations).

Postsynaptic responses evoked by K^+ depolarization were quantified as the mean response over a 10-s period; current was baseline-subtracted, averaging started 5 s after switching to high K^+ solution. Evaluation with this method included all postsynaptic events and yielded stable values for a given cell despite some variation in response time course between individual K^+ applications.

Electrophysiology on Heterologously Expressed Channels. AChR subunits and SK2 channels were heterologously expressed in *Xenopus laevis* oocytes. Oocytes were surgically removed from adult females and dissected manually. Four to 5 days before electrophysiological recordings, Dumont stage VI oocytes were injected with about 50 ng RNA. For coexpression experiments, the total amount of RNA was kept constant and the different RNAs were coinjected in equal concentrations.

Two-electrode voltage-clamp measurements were performed with a TurboTec 01C amplifier (npi, Tamm, Germany), using microelectrodes of 0.1 to 0.5 M Ω filled with 3 M KCl. Extracellular medium was CaNFR, containing 115 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , and 10 mM HEPES, pH adjusted to 7.3 with NaOH. For experiments in the absence of extracellular Ca^{2+} , the extracellular solution was MgNFR (115 mM NaCl, 2.5 mM KCl, 2 mM MgCl_2 , and 10 mM HEPES, pH adjusted to 7.3 with NaOH). ACh and memantine were added from stock solutions and applied through an application sys-

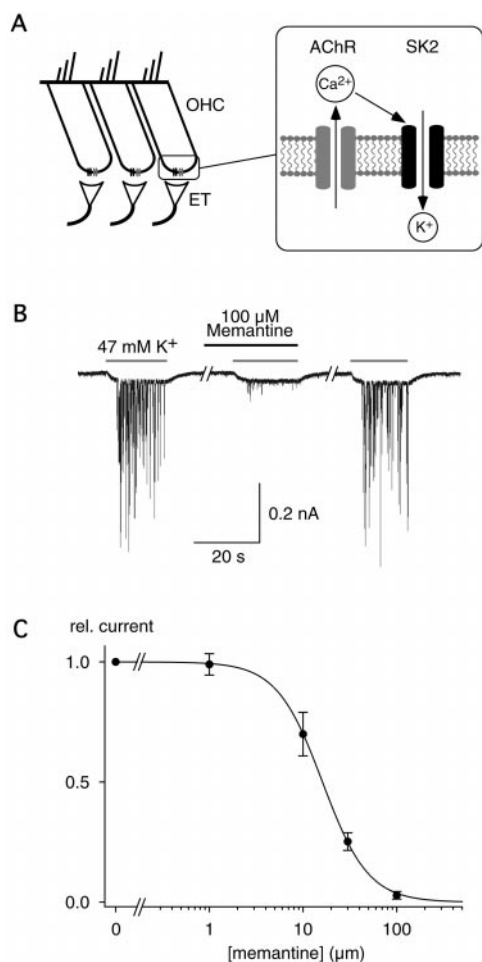


Fig. 1. Memantine blocks IPSCs in OHCs. **A**, diagram illustrating efferent innervation of OHCs and postsynaptic Ca^{2+} -signaling that generates fast IPSCs carried by SK2 channels (ET, efferent synaptic terminals). **B**, whole-cell voltage-clamp recording from an OHC at -64 mV. Increase of the extracellular K^+ concentration induced IPSCs, which were reversibly inhibited by 100 μM memantine. The baseline shift induced by high K^+ is caused by an unblocked fraction of the hair cell's resting K^+ conductance. **C**, dose-response curve for inhibition of IPSCs by memantine. Postsynaptic currents were normalized to the response in the absence of memantine (see *Materials and Methods*). Line indicates the best fit of the Hill equation to data obtained from seven OHCs ($n_{\text{H}} = 1.8$; $\text{IC}_{50} = 16.1$ μM).

tem, that allowed solution exchange with a time constant of ≈ 1 s. Currents were filtered at 100 Hz and sampled at a frequency of 1 kHz.

Dose-inhibition relations obtained from electrophysiological experiments were fitted with the empirical Hill equation, $I_{\text{norm}} = 1 / [1 + (c / IC_{50})^{n_H}]$, where I_{norm} is the normalized current, c is the blocker concentration, IC_{50} is the half-inhibitory concentration, and n_H is the Hill coefficient.

Data analysis and fitting was performed with IgorPro (WaveMetrics, Lake Oswego, OR) on a Macintosh PowerPC. Unless stated otherwise, data are presented as mean \pm S.D.

Molecular Biology. The coding region of the rat $\alpha 10$ gene (GenBank accession no. AF196344) was amplified from rat brain cDNA by PCR using 5'- and 3'- adapter-primers containing suitable restriction sites (GAGACCCGGGAGCTCCACC, ATGGGGACAAGGAGC-CACCTACC, and GAGTCTAGATTACAGGGCTTGCACCAG-TACAATG). The amplified fragments were subcloned into the *X. laevis* oocyte expression vector pGEM-HE (gift of Dr. J. Tytgat), yielding pGEM-HE-nAChR- $\alpha 10$, verified by sequencing. Capped mRNAs for $\alpha 9$, $\alpha 10$, and SK2 were synthesized in vitro using the mMESSAGE mACHINE kit (Ambion, Austin, TX). To detect $\alpha 10$ and $\alpha 9$ transcripts, PCR was performed using reverse transcribed RNA isolated from either OHCs (containing some Deiters cells) or supporting cells (Deiters and Hensen's cells) as template. Cells were collected from rat organs of Corti using suction glass micropipettes (diameter 10 μm). RNA was prepared from ~ 100 cells of each fraction using the QIAGEN RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. The oligonucleotides used as primers in the PCR reactions were chosen to span an intron in the human $\alpha 9$ and $\alpha 10$ genes to allow differentiation between products originating from cDNA and products originating from contaminating genomic DNA ($\alpha 9$ sense, CGTCCATATCGTTCCTCGTCCG; $\alpha 9$ antisense, TGGTAAGGGCTGTGGAGGCAGTGA; $\alpha 10$ sense, GCAGC-CTACGTGTGCAACCTCCTGC; and $\alpha 10$ antisense, AGGTGTC-CCAGCAGGAGAACCCGAG). For each PCR reaction, RNA corresponding to ~ 3 cells was used as template; the number of cycles was 40 for amplification of $\alpha 9$ and $\alpha 10$, and 30 for GAPDH used as a control.

Results

IPSCs were recorded from whole-cell voltage-clamped OHCs in acutely isolated organs of Corti during depolarization of the efferent presynaptic terminal with elevated extracellular $[K^+]$. As shown previously, these IPSCs are K^+ -currents through SK2 channels activated by brief elevation of subsynaptic $[Ca^{2+}]$ (Oliver et al., 2000). Ca^{2+} -transients are generated by the opening of postsynaptic nAChRs in response to presynaptically released ACh (Glowatzki and Fuchs, 2000; Oliver et al., 2000). In the presence of memantine, IPSCs were reversibly reduced in a concentration dependent manner (Fig. 1B). Data obtained from seven OHCs yielded a half-blocking concentration for memantine of 16.1 μM (Fig. 1C). Based on the known effect of memantine on ligand-gated ion channels, it seemed most likely that inhibition of the complex IPSCs resulted from block of Ca^{2+} -entry via the nAChR. We therefore aimed at testing the action of memantine on the OHC nAChR expressed heterologously in *X. laevis* oocytes. However, application of 100 μM ACh to oocytes injected with $\alpha 9$ -specific cRNA yielded very small currents (9.3 ± 5.0 nA at -80 mV; Fig. 2A), consistent with previous reports (Elgoyhen et al., 1994; Katz et al., 2000). No currents exceeding background levels were observed with the rat homolog of the $\alpha 10$ subunit, a member of the nAChR family recently identified by Boulter and colleagues (GenBank accession no. AF196344; Fig. 2, A and D). As shown in Fig. 2B by RT-PCR on OHCs isolated from the rat organ of Corti (see *Materials and Methods*), $\alpha 10$ mRNA is indeed present in these sensory cells, although it was not detected in the supporting cell fraction containing Hensen and Deiters cells. In a control experiment with RNA from OHCs that was not reverse transcribed, PCR amplified a fragment of ~ 900 bp (Fig. 2B, lane 4) which most likely resulted from contamination with genomic DNA as the length of this fragment is in good agreement with the sequence defined by the primer pair

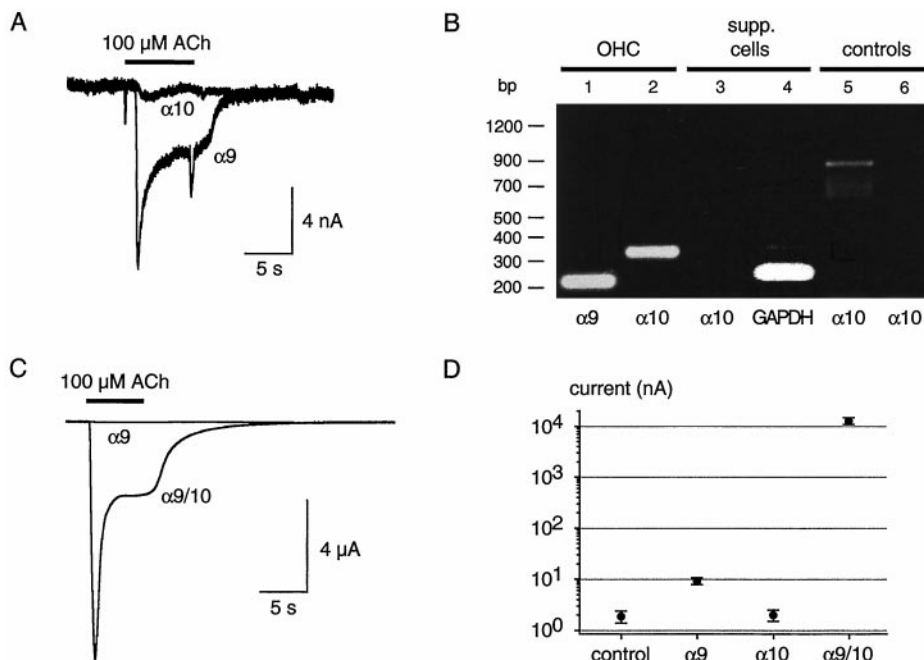


Fig. 2. $\alpha 9$ and $\alpha 10$ nAChR subunits are expressed in OHCs and coassemble to functional channels. A, ACh (100 μM) induced inward currents in oocytes injected with RNA coding for $\alpha 9$ (lower trace) but not in oocytes injected with RNA coding for $\alpha 10$ (upper trace). Holding potential was -80 mV. Fast downward spikes in the traces are artifacts resulting from switching between solutions. B, detection of $\alpha 10$ and $\alpha 9$ mRNA in OHCs by RT-PCR. Fragments of the expected length were amplified for $\alpha 10$ and $\alpha 9$ from OHCs (lanes 1 and 2) but not from supporting cells (lane 3, data for $\alpha 9$ not shown). Controls were GAPDH amplified from supporting cells (lane 4), OHC-RNA without RT added (lane 5), and water (lane 6) as a template for PCR. C, same experiment as in A, but for coexpression of both subunits. Note the different current scaling. The recording from A ($\alpha 9$) was added for comparison. D, current amplitudes from experiments as in A and C summarized for oocytes injected with RNA coding for a nonconducting SK2 channel mutant (control), $\alpha 9$, $\alpha 10$, and $\alpha 9/\alpha 10$ (values are mean \pm standard error of 5, 13, 13, and 18 oocytes, respectively).

in the human genome (bacterial artificial chromosome from chromosome 11; GenBank accession no. AC060812).

When both $\alpha 9$ and $\alpha 10$ were coexpressed in oocytes, large inward currents with peak amplitudes of up to $-35 \mu\text{A}$ (at -80 mV) were recorded upon application of ACh (Fig. 2C, D). Similar to $\alpha 9$ -mediated currents, the time-course was characterized by an initial transient declining to a smaller plateau of variable amplitude with respect to the peak current. The increase in current amplitude of more than 3 orders of magnitude (compared with homomeric $\alpha 9$ expression) together with the coexpression of $\alpha 9$ and $\alpha 10$ in OHCs suggest that heteromultimerization of both subunits is essential to give fully functional receptor channels. Moreover, the absence of any other of the known nAChR subunits (Morley et al., 1998) strongly suggests that the OHC nAChR is a heteromer composed of $\alpha 9$ and $\alpha 10$ subunits.

A characteristic feature of homomeric $\alpha 9$ channels is their exceptionally high Ca^{2+} -permeability (Jagger et al., 2000; Katz et al., 2000). This Ca^{2+} -permeability is thought to be essential for the OHC nAChR, since it allows for a Ca^{2+} influx sufficiently high to effectively activate SK-type potassium channels. However, high endogenous expression levels of Ca^{2+} -activated Cl^- -channels characterize the oocyte expression system (Stühmer and Parekh, 1995). Therefore, opening of Ca^{2+} -permeable channels in an external medium containing Ca^{2+} leads to coactivation of a Cl^- conductance. When external Ca^{2+} was substituted for Mg^{2+} , currents induced by ACh application onto $\alpha 9/\alpha 10$ heteromeric channels were reduced by a factor of roughly 10 (Fig. 3A). Thus, a large fraction of the ACh-induced current measured in CaNFR was attributable to opening of Ca^{2+} -activated Cl^- -currents. This was also supported by the reversal potential of the current in CaNFR of about -25 mV (data not shown), close to the

estimated Cl^- equilibrium potential in *X. laevis* oocytes (Stühmer and Parekh, 1995). Consequently, $\alpha 9/\alpha 10$ heteromeric channels had a significant Ca^{2+} -permeability, similar to what is known from homomeric $\alpha 9$ receptors. Application of ACh in the absence of extracellular Ca^{2+} allowed recording of $\alpha 9/\alpha 10$ currents in isolation. Heteromeric channels yielded currents that were 100-fold larger than currents recorded from $\alpha 9$ channels under the same conditions, confirming the large gain of receptor conductance by coexpression that was observed in the presence of external Ca^{2+} (Fig. 3, B and C). In the absence of Ca^{2+} , $\alpha 9/\alpha 10$ also showed consistent kinetics characterized by slow desensitization on a time scale of seconds. Desensitization was not observed with $\alpha 9$ channels within the limits of the speed of solution exchange (Fig. 3B).

The action of memantine was tested on the isolated $\alpha 9/\alpha 10$ current in MgNFR. Memantine blocked this current in a completely reversible manner with an IC_{50} value of $1.6 \mu\text{M}$ and a Hill coefficient of 1.2 (Fig. 3, D and E). Channel block by memantine has been analyzed most extensively at NMDA-type glutamate receptors (Chen et al., 1992; Bresink et al., 1996), where block of the open pore is strongly voltage-dependent. To address the issue of blocking mechanism and voltage dependence at $\alpha 9/\alpha 10$ channels, current-voltage relations were determined from voltage ramps in the absence and presence of memantine (Fig. 3F). In either case, current-voltage curves were highly nonlinear and showed considerable rectification at negative and positive potentials. The reversal potential was $-6.4 \pm 1.0 \text{ mV}$ ($n = 3$), consistent with a nonspecific cation channel. As shown in Fig. 3F, memantine block was observed over the whole voltage range tested. It increased by a factor of 1.6 from $+50 \text{ mV}$ to -80 mV and thus exhibited only mild voltage-dependence. We also measured the impact of memantine on the chloride current, ac-

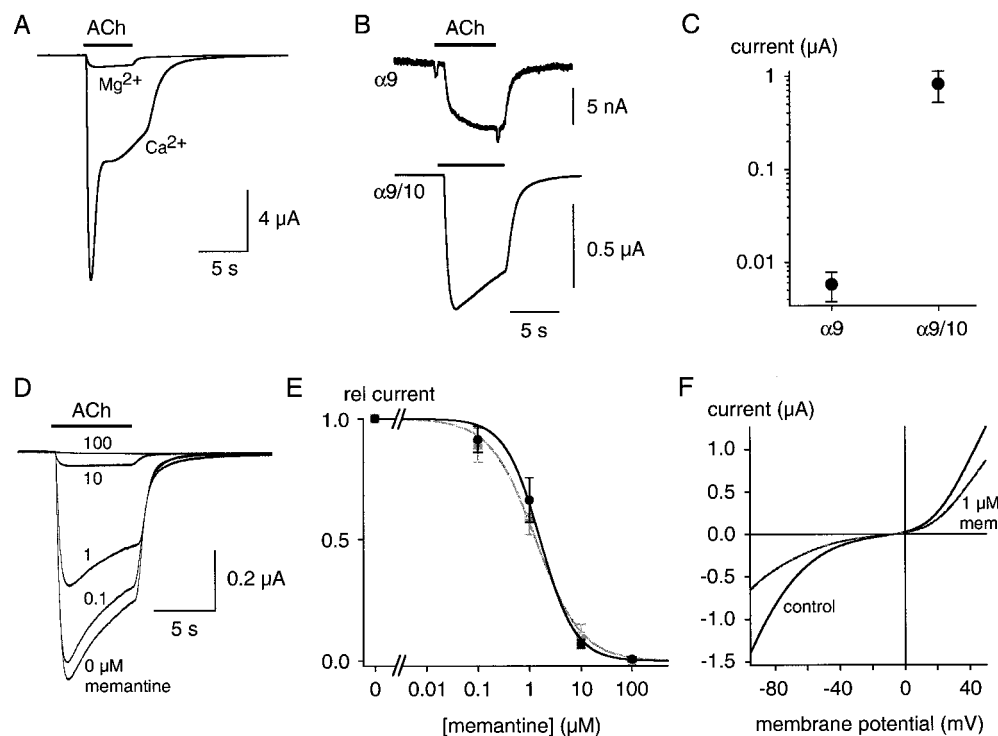


Fig. 3. Memantine blocks heteromeric $\alpha 9/\alpha 10$ receptors with mild voltage dependence. A, currents evoked by activation of $\alpha 9/\alpha 10$ nAChRs are dependent on external Ca^{2+} . Traces show subsequent applications of $100 \mu\text{M}$ ACh to the same oocyte in CaNFR (Ca^{2+}) and MgNFR (Mg^{2+}) at -80 mV . B, currents from homomeric $\alpha 9$ (upper trace) and heteromeric $\alpha 9/\alpha 10$ nAChRs (lower trace), recorded by application of $100 \mu\text{M}$ ACh in nominally Ca^{2+} -free external solution (MgNFR; -80 mV). C, current amplitudes from oocytes expressing $\alpha 9$ and $\alpha 9/\alpha 10$, recorded as in Fig. 3B (mean \pm S.E. from 12 and 15 oocytes, respectively). D, ACh-induced ($100 \mu\text{M}$) currents recorded from $\alpha 9/\alpha 10$ nAChRs in the presence of the memantine concentrations indicated. E, memantine dose-inhibition curves of $\alpha 9/\alpha 10$ nAChR. Peak currents were recorded as in D and normalized to the current amplitude after washout of the blocker. Continuous lines show fits of the Hill equation to data from 7 oocytes measured in MgNFR (solid) and CaNFR (gray), yielding IC_{50} values of 1.6 and $1.3 \mu\text{M}$ and n_H of 1.2 and 1.0 , respectively. F, current-voltage relations of $\alpha 9/\alpha 10$ nAChR were recorded in the absence (black) and presence (gray trace) of $1 \mu\text{M}$ memantine in response to 2-s voltage ramps from -100 to $+50 \text{ mV}$. Currents were recorded in MgNFR and were leak-corrected by subtracting the response to the same voltage ramp preceding the application of $100 \mu\text{M}$ ACh.

tivated secondarily in the presence of external Ca^{2+} . Memantine inhibited the peak chloride current with an efficiency not significantly different from block of the isolated $\alpha 9/\alpha 10$ current (Fig. 3E).

In hair cells, Ca^{2+} influx via nAChRs activates SK2 channels to give rise to IPSCs. Figure 4 shows, that this activation cascade may be reconstituted in *X. laevis* oocytes by coexpression of the $\alpha 9/\alpha 10$ nAChR with SK2 channels. In oocytes expressing both channel species, application of ACh evoked a biphasic response at -70 mV. An initial inward current carried mainly by chloride (see above) was followed by an outward current due to the activation of SK2 channels (Fig. 4A). With the Cl^- driving force largely abolished and an increased driving force for K^+ at a membrane potential of -30 mV, ACh induced a monophasic potassium outward current, similar to the response of isolated OHCs to ACh application (Blanchet et al., 1996; Evans, 1996). However, in

coinjected oocytes, SK channel activation occurred considerably more slowly than in OHCs ($20\text{--}80\%$ rise time of 1.8 ± 0.2 s, $n = 3$). Memantine block of the SK2 current showed a dose-response relation that was characterized by an IC_{50} value of $0.7 \mu\text{M}$ and a Hill coefficient of 1.1 (Fig. 4, B and C), very similar to the values obtained for the memantine block of $\alpha 9/\alpha 10$ receptors (see Fig. 3E).

However, these values were considerably different from those obtained for memantine-induced inhibition of IPSCs in OHCs (Fig. 1C). Thus, inhibition of IPSCs required 10-fold higher concentrations of memantine than inhibition of SK2 currents in the oocyte system (Fig. 4C). This difference might suggest that the nAChR underlying the generation of IPSCs is less susceptible to memantine block than $\alpha 9/\alpha 10$ and may thus be indicative for a different subunit composition of OHC nAChRs. To test this possibility, we examined the effect of memantine on the nAChR of OHCs directly. AChR currents can be measured in isolation by uncoupling SK channel activation from Ca^{2+} influx via the nAChR with the fast Ca^{2+} -chelator BAPTA (Fuchs and Murrow, 1992; Blanchet et al., 1996). Application of ACh to OHCs dialyzed with 10 mM BAPTA from the recording pipette induced inward currents of 110 ± 39 pA at -94 mV ($n = 4$). These currents were blocked by memantine with an IC_{50} value of $1.1 \mu\text{M}$ and a Hill coefficient of 0.9 (Fig. 5A, B). This affinity is in close

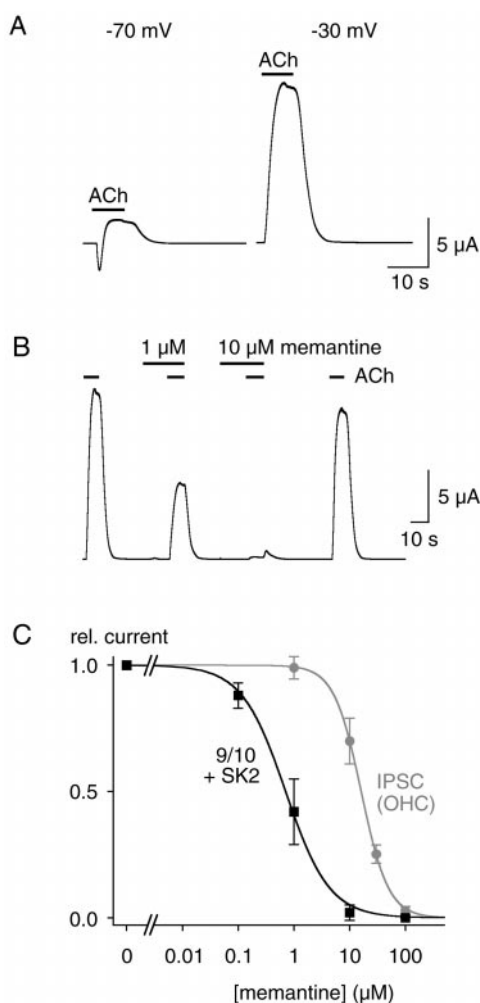


Fig. 4. Activation of SK2 channels by coexpressed $\alpha 9/\alpha 10$ receptors is blocked by memantine. A, application of $100 \mu\text{M}$ ACh for the time indicated to an oocyte coexpressing $\alpha 9$, $\alpha 10$, and SK2. Traces are subsequent recordings from the same oocyte at the holding potentials indicated. B, K^+ current induced by ACh ($100 \mu\text{M}$) in an oocyte as in A was reversibly blocked by memantine at the concentrations indicated. Holding potential was -30 mV. C, memantine dose-response curve for inhibition of the nAChR-induced SK2 current ($n = 5$ oocytes). Peak outward currents were recorded at -30 mV and normalized to the values preceding memantine application. Continuous line shows fit of the Hill equation ($\text{IC}_{50} = 0.7 \mu\text{M}$; $n_H = 1.1$). Inhibition curve of IPSCs by memantine (●) is replotted from Fig. 1C.

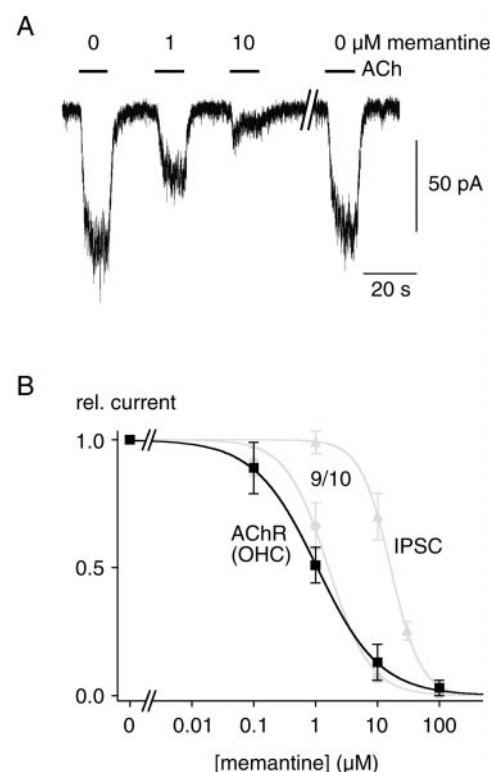


Fig. 5. Steady-state block of nAChR currents in OHCs by memantine. A, currents through nAChRs were recorded from an OHC at a holding potential of -94 mV. Inward currents induced by application of $200 \mu\text{M}$ ACh were reversibly blocked by coapplication of memantine as indicated by horizontal bars. The intracellular solution contained 10 mM BAPTA to prevent activation of SK currents. B, dose-inhibition relation for memantine block of nAChR currents (■), recorded from four OHCs as in A. Steady-state amplitudes were normalized to the value recorded in the absence of memantine. The fit to the Hill equation (continuous line) yielded $\text{IC}_{50} = 1.1 \mu\text{M}$ and $n_H = 0.9$. Dose-inhibition curves for memantine block of $\alpha 9/\alpha 10$ currents in oocytes (○) and of IPSCs (△) are replotted for better comparison.

agreement with the values obtained for the block of $\alpha 9/\alpha 10$ receptors and strongly suggests that the observed differences in blocking potency of memantine are not caused by a different receptor; instead, they may result from the specific mechanism of coupling between nAChRs and SK channels in OHCs.

Discussion

We have examined the action of memantine, a pore blocker of ligand gated ion channels, on inhibitory synaptic input to cochlear OHCs. It is shown that memantine abolishes IPSCs in the micromolar range by blocking Ca^{2+} entry via the OHC nAChR. To be able to analyze this block at the molecular level, we aimed to reconstitute the nAChR in a heterologous expression system.

Identity of the nAChR of OHCs. The nAChR of OHCs has been shown to contain the $\alpha 9$ subunit by a variety of methods including *in situ* hybridization (Elgoyhen et al., 1994; Morley et al., 1998), single-cell RT-PCR (Glowatzki et al., 1995), transgenic expression of green fluorescent protein controlled by the $\alpha 9$ promoter (Zuo et al., 1999), and inactivation of the $\alpha 9$ gene (Vetter et al., 1999). Homomeric $\alpha 9$ receptors, however, yield remarkably small currents when heterologously expressed in *X. laevis* oocytes (Figs. 2A and 3B; see also Elgoyhen et al., 1994), raising the possibility that an additional subunit is needed to yield the fully functional OHC receptor. However, OHCs lack any other known nAChR subtypes (Morley et al., 1998).

A GenBank search yielded a new subunit of the nAChR family (GenBank accession no. AF196344, submitted by J Boulter) designated $\alpha 10$. Therefore, we tested this subunit as a candidate subunit for the OHC receptor. RT-PCR using cDNA from OHCs as the template revealed that $\alpha 10$ is indeed expressed in these cells. Moreover, the robust currents measured in oocytes injected with RNAs coding for both $\alpha 9$ and $\alpha 10$ contrast with the lack of functional expression of homomeric $\alpha 10$ receptors and the weak expression of $\alpha 9$ receptors, indicating that both subunits assemble to functional heteromeric nAChRs. This conclusion is supported by the changed kinetics of the $\alpha 9/\alpha 10$ current compared with the current mediated by $\alpha 9$ alone. Taken together, these findings strongly suggest that both subunits form heteromeric $\alpha 9/\alpha 10$ receptors in OHCs.

The properties of $\alpha 9/\alpha 10$ receptors reported here, namely their sensitivity for memantine and their significant permeability for Ca^{2+} , are in agreement with those of the OHC receptor. In particular, coexpression experiments showed that these receptors are able to effectively activate SK2 K^+ -channels, their primary function in OHCs.

Memantine Block of $\alpha 9/\alpha 10$ Receptors. Heteromeric $\alpha 9/\alpha 10$ channels are reversibly blocked by memantine. This adamantane derivative is a well-characterized open-channel blocker of NMDA-type glutamate receptors with an IC_{50} value of around $1 \mu\text{M}$ (at -70 mV) and also blocks neuronal $\alpha 4/\beta 2$ nAChRs with an IC_{50} value of $7 \mu\text{M}$ (at -100 mV) (Chen et al., 1992; Parsons et al., 1993; Buisson and Bertrand, 1998). Thus, the affinity for memantine of $\alpha 9/\alpha 10$ receptors ($\approx 1 \mu\text{M}$, see Figs. 3E and 5B) is considerably higher than of neuronal nAChRs and equals the affinity of NMDA receptors.

For NMDA receptors, however, this high affinity is observed only at hyperpolarized potentials because of the pro-

nounced voltage dependence of the block [electrical distance (δ) of about 1; Bresink et al., 1996]. In contrast, block of $\alpha 9/\alpha 10$ receptors was only weakly voltage-dependent; i.e., the channels exhibit high affinity block over the entire voltage range tested (-100 to $+50 \text{ mV}$) with only a moderate increase of blocking efficacy at negative voltages. The pore blocking observed with Mg^{2+} parallels this differential blocking by memantine. Although Mg^{2+} completely occludes NMDA receptors under physiological conditions for extracellular Mg^{2+} and membrane potential (Mayer et al., 1984; Burnashev et al., 1992), $\alpha 9/\alpha 10$ receptors are virtually left unchanged by Mg^{2+} as indicated by a lack of current decrease at negative potentials (Fig. 3F). Accordingly, amplitude or time-course of IPSCs in OHCs are not altered by removal of extracellular Mg^{2+} (D.O., unpublished observation).

The sensitivity of OHC IPSCs for inhibition by memantine was an order of magnitude lower than the sensitivity of the receptor itself (Figs. 1C and 5B). This divergence may be explained by considering the blocking mechanism of memantine. As an open channel blocker, it will enter and occlude the pore only after the channel is opened by the ligand. AChRs underlying IPSCs open for some 20 ms, and complete activation of SK2 channels occurs within 10 ms after onset of the nAChR current (Oliver et al., 2000). Thus, memantine must occlude the channel within milliseconds to effectively block IPSCs. Consequently, blocking efficacy will be determined more by the on-rate of the pore block than by the equilibrium affinity of the blocker that was measured with steady-state nAChR currents. Because the blocking time constant decreases with the blocker concentration, efficacy of inhibition of fast IPSCs will increase with concentration of memantine. Blocking time constants on the order of seconds, as suggested by fast application of memantine at half-blocking concentration to open $\alpha 4/\beta 2$ nAChRs (see Fig. 6B in Buisson and Bertrand, 1998), indicate that blocking speed may indeed be limiting for the inhibition of fast IPSCs. In accordance with this consideration, the increased Hill coefficient of 2 with respect to $n_{\text{H}} \approx 1$ in all steady-state measurements suggests that inhibition of IPSCs is not determined by a simple steady-state pore block.

Action of Memantine on Cochlear Efferent Transmission. The block of OHC synaptic transmission by memantine establishes an impact on cochlear physiology by a drug that is used therapeutically (Parsons et al., 1999). The olivocochlear system acts on the cochlea by reducing its response to acoustic stimulation on two time scales. A "fast effect" caused by the quick opening of Ca^{2+} -activated K^+ channels by the nAChRs is followed by a slower effect, which is thought to involve second-messenger systems (Sridhar et al., 1995, 1997). Because the most obvious effect of memantine is the block of fast IPSCs, it can be expected to block the fast efferent effect, based on SK2 channel opening. Moreover, because this inhibition is caused by abolishment of Ca^{2+} -influx via the OHC nAChR, the second, slower efferent effect that is thought to be triggered by the Ca^{2+} influx in the same synaptic events (Sridhar et al., 1997) is also expected to be blocked by memantine.

Memantine is used therapeutically to prevent excitotoxic neuronal cell death triggered primarily by massive Ca^{2+} influx via overstimulated NMDA-type glutamate receptors (Chen et al., 1992; Parsons et al., 1999). Loss or damage of

OHCs in the cochlea is well known to be a major cause of sensorineural hearing loss (Cody and Russell, 1985; Patuzzi et al., 1989). The mechanisms of OHC degeneration are not fully understood, but in analogy to neurons it may be hypothesized that Ca^{2+} -influx might contribute to this process. The main sources for Ca^{2+} -influx into OHCs are mechanoelectrical transducer channels and P2X purinoceptors at the apex and nAChRs at the basal pole. Intracellular rise in $[\text{Ca}^{2+}]$ is thought to be highly localized to the respective entry site under physiological conditions (Lenzi and Roberts, 1994; Oliver et al., 2000). However, acoustic overstimulation has been shown to result in globally increased cytoplasmic Ca^{2+} levels of OHCs that coincide with an impairment of cochlear function (Fridberger et al., 1998). Moreover, Ca^{2+} signals induced by synaptic activity can trigger second-messenger mediated processes that may involve secondary release of Ca^{2+} from internal stores (Sridhar et al., 1995; Dallos et al., 1997; Sridhar et al., 1997). These processes, which probably underlie the slow efferent effect, are thought to act protectively under physiological conditions (Sridhar et al., 1995). However, if Ca^{2+} influx could reach a toxic scale [e.g., during a tonic ACh release from efferent terminals due to K^{+} -depolarization as thought to occur in Ménière's disease (Dohlman, 1980)], block of nAChRs by memantine might exert a protective effect. It is important to note, that such a prolonged Ca^{2+} -influx is blocked by memantine with a 10-fold higher sensitivity than the fast physiological IPSCs, as outlined above.

In conclusion, memantine is well suited to impair the efferent cochlear physiology as well as to prevent pathological massive Ca^{2+} influx at doses that are used to affect its primary therapeutic target, the NMDA receptor. While this article was being considered for publication, an article by Elgoyhen et al. (2001) appeared in print that supports the conclusion of the OHC nAChR being composed of at least $\alpha 9$ and $\alpha 10$ subunits.

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