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Inhibition of the α9α10 nicotinic cholinergic receptor by neramexane, an open channel blocker of *N*-methyl-D-aspartate receptors

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Abstract

In this study we report the effects of neramexane, a novel amino-alkyl-cyclohexane derivative that is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, on recombinant rat α9α10 nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. We compared its effects with those of memantine, a well-studied pore blocker of NMDA receptors, currently used in therapeutics for the treatment of Alzheimer's disease. Our results indicate that both compounds block acetylcholine-evoked responses at micromolar concentrations with a rank order of potency of neramexane > memantine, $P<0.05$. Block by neramexane of acetylcholine responses was not overcome at high concentrations of the agonist, indicative of a non-competitive inhibition. The lack of interaction of neramexane with the ligand binding domain was confirmed by radioligand binding experiments in transfected tsA201 cells. Moreover, block did not involve an increase in desensitization kinetics, it was independent of the resting potential of the membrane at low concentrations of neramexane and slightly voltage-dependent at concentrations higher than 1 μM. Finally, clinically-relevant concentrations of neramexane blocked native α9α10-containing nicotinic acetylcholine receptors of rat inner hair cells, thus demonstrating a possible *in vivo* relevance in potentially unexplored therapeutic areas.

Keywords: Acetylcholine; Neramexane; *N*-methyl-D-aspartate receptor antagonist; Nicotinic acetylcholine receptors; Hair cells

1. Introduction

Nicotinic cholinergic receptors belong to the four-transmembrane superfamily of ligand-gated ion channels that also includes γ-aminobutyric acid types A and C, glycine, 5-hydroxytryptamine-3 and some invertebrate anionic glutamate receptors (Le Novere et al., 2002). A variety of nicotinic acetylcholine receptor subunits have been identified. Neuronal nicotinic acetylcholine receptors are assembled from at least ten subunits (α2–α8; β2–β4), which give rise to a plethora of receptors that differ with respect to their pharmacology, biophysics and localization (Karlin, 2002). In the central nervous system, the α4β2 and α7 receptors are the most abundant nicotinic acetylcholine receptors subtypes. The α9 and α10 nicotinic acetylcholine receptor subunits are the latest that have been cloned (Elgoyhen et al., 1994, 2001). They are distant members of the family and form a distinct phylogenetic early divergent subfamily (Elgoyhen et al., 1994, 2001; Le Novere et al., 2002). Heteromeric receptors assembled from α9 and α10 subunits exhibit a peculiar mixed nicotinic–muscarinic pharmacological profile, which is distinct from that of other nicotinic acetylcholine receptors. In addition, these subunits exhibit a restricted expression pattern, which includes the cochlear hair cells (Elgoyhen et al., 1994, 2001; Sgard et al., 2002). Several studies have shown that a α9α10-

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containing nicotinic acetylcholine receptor mediates efferent olivocochlear effects on hair cells, one of the few verified examples of postsynaptic function for a non-muscle nicotinic acetylcholine receptor (Elgoyhen et al., 1994, 2001; Gomez-Casati et al., 2005).

Nicotinic cholinergic receptors are affected by a variety of non-cholinergic drugs with biological and/or clinical relevance at concentrations at which they act on their classical targets (Connolly et al., 1992; Fryer and Lukas, 1999; Herrero et al., 1999; Houlihan et al., 2000; Lopez et al., 1993; Rana et al., 1993). In particular, the adamantane derivative 3,5-dimethyl-1-adamantanamine (memantine) is a well-established blocker of ligand-gated ion channels permeable to Ca^{2+} such as the NMDA type glutamate receptor (Bresink et al., 1996; Chen et al., 1992; Parsons et al., 1993) or nicotinic acetylcholine receptors including α9α10 (Buisson and Bertrand, 1998; Oliver et al., 2001). In either case, memantine acts as an open channel blocker, it enters the channel pore and sterically occludes the ion pathway (Buisson and Bertrand, 1998; Chen et al., 1992). For NMDA receptors, this pore block strongly depends on the transmembrane voltage, with highest efficacy at hyperpolarized potentials (Bresink et al., 1996); for nicotinic acetylcholine receptors, the voltage-dependence is less pronounced (Buisson and Bertrand, 1998).

Neramexane, a novel amino-alkyl-cyclohexane derivative, is also a non-competitive NMDA receptor antagonist, and is presently in phase II clinical trials for various indications including Alzheimer's disease and chronic pain (Danysz et al., 2002). The aim of the present study was to analyze the effect of this compound on α9α10 nicotinic acetylcholine receptors and to compare its potency with that of memantine. Our results indicate that as reported for memantine (Oliver et al., 2001) clinically-relevant concentrations of neramexane block recombinant α9α10 nicotinic acetylcholine receptors in a voltage-independent non-competitive manner, thus demonstrating possible *in vivo* relevance in potentially unexplored therapeutic areas via blockade of α9α10-containing nicotinic acetylcholine receptors of cochlear hair cells.

2. Materials and methods

2.1. Expression of recombinant receptors in Xenopus laevis oocytes

Capped cRNAs were *in vitro*-transcribed from linearized rat plasmid DNA templates using the mMessage mMachine Transcription Kit (Ambion Corporation, Austin, TX). The maintenance of *X. laevis*, as well as the preparation and cRNA injection of stages V and VI oocytes has been described in detail elsewhere (Katz et al., 2000). Typically, oocytes were injected with 50 nl of RNase-free water containing 0.1–1.0 ng of cRNAs (at a 1:1 molar), and maintained in Barth's solution at 17 °C.

Electrophysiological recordings were performed 2–6 days after cRNA injection under two-electrode voltage-clamp with an OC-725B oocyte clamp (Warner Instruments, Wamden, CT). Both voltage and current electrodes were filled with 3 M KCl, and had resistances of ~1–2 MΩ. Data were digitized and stored on a PC computer. Data were analyzed using Clampfit from the pClamp 6 software (Axon Instruments Corp., Union City, CA). During electrophysiological recordings, oocytes were continuously superfused (~10 ml/min) with normal frog saline (comprised of, in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, and 10 HEPES buffer, pH 7.2), and voltage-clamped at −70 mV. Experiments were performed in oocytes incubated with the Ca^{2+} chelator 1,2-bis (2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM, 100 μM) for 3–4 h prior to electrophysiological recordings. Drugs were applied in the perfusion solution of the oocyte chamber.

Current–voltage relationships were obtained by applying 2-s voltage ramps from −120 to +50 mV, 5 s after the peak response to 10 μM acetylcholine, from a holding potential (V_{hold}) of −70 mV. Leakage correction was performed by digital subtraction of the current–voltage curve obtained by the same voltage ramp protocol performed prior to the application of acetylcholine.

Concentration–response curves were normalized to the maximal agonist response in each oocyte. For the inhibition curves, antagonists were added to the perfusion solution for 2 min prior to the addition of 10 μM acetylcholine and then were coapplied with this agonist. Responses were referred to as a percentage of the response to acetylcholine. The mean and standard error of the mean of peak current responses are represented. Agonist concentration–response curves were iteratively fitted with the equation:

$$I/I_{max} = A^n/(A^n + EC_{50}^n)$$

where I is the peak inward current evoked by the agonist at concentration A; I_{max} is current evoked by the concentration of agonist eliciting a maximal response; EC_{50} is the concentration of agonist inducing half-maximal current response and n is the Hill coefficient. An equation of the same form was used to analyze the concentration dependency of antagonist-induced blockage. The parameters derived were the concentration of antagonist producing a 50% block of the control response to acetylcholine (IC_{50}) and the associated interaction coefficient (n). For constructing the inhibition curves the average peak amplitude of three control responses just before the exposure to the drug was used to normalize the amplitude of each test response in the presence of the drug.

2.2. Recordings from inner hair cells

Apical turns of the organ of Corti were excised from Sprague-Dawley rats at postnatal ages 8 to 10 days. Cochlear preparations were mounted under an Axioskope microscope (Zeiss, Oberkochem, Germany), and viewed with differential interference contrast (DIC) using a 40× water immersion objective and a camera with contrast enhancement (Hamamatsu C2400-07, Hamamatsu City, Japan). Methods to record from inner hair cells were essentially as described (Gomez-Casati et al., 2005). Briefly, inner hair cells were identified visually, by the size of their capacitance (7 to 12 pF) and by their characteristic voltage-dependent Na^+ and K^+ currents,

including at older ages a fast-activating K^+-conductance. Some cells were removed to access inner hair cells, but mostly the pipette moved through the tissue using positive fluid flow to clear the tip. The extracellular solution was as follows (in mM): 155 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 5.6 D-glucose, and 10 Hepes buffer; pH 7.4. The pipette solution was (in mM): 150 KCl, 3.5 $MgCl_2$, 0.1 $CaCl_2$, 10 BAPTA, 5 HEPES buffer, 2.5 Na_2ATP, pH 7.2. Glass pipettes (1.2 mm i.d.) had resistances of 7–10 MΩ. Cells were held at a holding potential of −90 mV. Postsynaptic currents due to the spontaneous release of acetylcholine from efferent synaptic terminals contacting inner hair cells are occasionally observed. Therefore, in order to study the effect of neramexane on synaptic currents in these cells, transmitter release from efferent endings was accelerated by depolarization using 40 mM external potassium saline. In this case, we wanted to evaluate the effects of neramexane on the physiological response evoked by acetylcholine which involves the activation of the α9α10 nicotinic acetylcholine receptors and the subsequent activation of small-conductance, calcium-activated potassium channels due to Ca^{2+} entry through the nicotinic acetylcholine receptors (Fuchs and Murrow, 1992; Oliver et al., 2001). Therefore, the pipette solution did not contain BAPTA and it was composed of (in mM): 150 KCl, 3.5 $MgCl_2$, 0.1 $CaCl_2$, 5 ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 HEPES buffer, 2.5 Na_2ATP, pH 7.2. Solutions containing 60 μM acetylcholine (the EC_{50} in this preparation) or elevated potassium (40 mM K^+) and neramexane were applied by a gravity-fed multi-channel glass pipette (~150 μm tip diameter) positioned about 300 μm from the recorded inner hair cell. The extracellular solution containing the drugs was similar to that described above, except that Mg^{2+} was omitted, and the Ca^{2+} concentration was lowered to 0.5 mM to optimize the experimental conditions for measuring currents flowing through the α9α10 receptors (Katz et al., 2004). To minimize the contribution of small-conductance calcium-activated potassium channels, 1 nM apamin, a specific channel blocker, was added to the external working solutions, except in the cases in which synaptic currents were measured. Currents in inner hair cells were recorded in the whole-cell patch-clamp mode using an Axopatch 200B amplifier, low-pass filtered at 2–10 kHz and digitized at 5–20 kHz with a Digidata 1200 board (Axon Instruments, Union City, CA). Recordings were made at room temperature (22–25 °C). Voltages were not corrected for the voltage drop across the uncompensated series resistance. For inhibition curves, cells were incubated with the antagonist for 1 min prior to the addition of acetylcholine.

Synaptic currents were analyzed with Minianalysis (Synaptosoft, Jaejin Software, Leonia, NJ) and were identified using a search routine for event detection and confirmed by eye. Rise times were measured between 10 and 90% of the total amplitude and current decay time courses were fit with a monoexponential.

2.3. Mammalian cell culture and transfection

Mammalian cell line, tsA201, derived from the human embryonic kidney HEK293 cell line, was cultured in Dulbecco's modified Eagle's medium (Gibco Invitrogen, Paisley, UK) containing 2 mM L-Glutamax™ (Gibco Invitrogen, Paisley, UK) plus 10% heat-inactivated fetal calf serum (Sigma, Poole, UK), with penicillin (100 U/ml) and streptomycin (100 μg/ml) and were maintained in a humidified incubator containing 5% CO_2 at 37 °C. Cells were cotransfected with pRK5-$\alpha9^{(L209)}$/$5HT_{3A}$ and pRK5-$\alpha10^{(L206)}$/$5HT_{3A}$ (Baker et al., 2004) using Effectene™ transfection reagent (Qiagen, Crawley, UK) according to the manufacturer's instructions. Cells were transfected overnight and assayed for expression approximately 40–48 h after transfection.

2.4. Radioligand binding

Binding studies with [^{3}H]methyllycaconitine ([^{3}H]MLA; American Radiolabeled Chemicals Inc., St. Louis, MO; specific activity, 3.7×10^{12} Bq/mmol) to cell membrane preparations were performed, essentially as described previously (Baker et al., 2004). Membranes (typically 120–200 μg of protein) were incubated with radioligand for 180 min at 4 °C in a total volume of 150 μl or 200 μl, in the presence of protease inhibitors: leupeptin (2 μg/ml), apoprotinin (2 μg/ml) and pepstatin (1 μg/ml). Radioligand binding was assayed by filtration onto Whatman GF/B filters (presoaked in 0.5% polyethylenimine), followed by rapid washing with cold 10 mM phosphate buffer using a Brandel cell harvester. Bound radioligand was quantified by scintillation counting. Curves for equilibrium binding were analyzed using non-linear least-squares fitting (Prism 4; GraphPad Software Inc., San Diego, CA). IC_{50} values were converted to K_i values using the equation: $K_i = IC_{50}/1+([L]/K_d)$, in which L is the free concentration of [^{3}H]MLA used in the assay and K_d is the dissociation constant for binding of [^{3}H]MLA.

2.5. Statistical analysis

The statistical significance of differences between mean values was assessed by Student's *t*-test (two-tailed, unpaired samples). Values of $P<0.05$ were considered significant.

2.6. Materials

Acetylcholine chloride was bought from Sigma Chemical Co. (St. Louis, MO). Memantine chloride and neramexane mesylate were a gift from Merz Pharmaceuticals GmbH, Franfurt, Germany.

All experimental protocols were carried out in accordance with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 80-23) revised 1978.

3. Results

3.1. Effects of neramexane and memantine on recombinant α9α10 receptors expressed in X. laevis oocytes

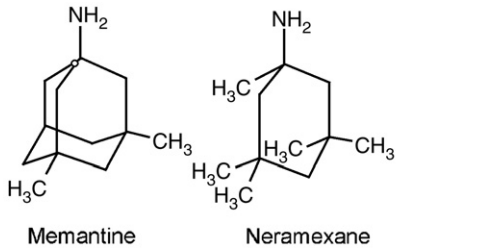
Fig. 1 indicates the chemical structures of the compounds used in the present study. Fig. 2A shows representative

responses to 10 μM acetylcholine (i.e. a concentration close to the one that produces a half-maximal response, EC_{50}, (Elgoyhen et al., 2001)) of *X. laevis* oocytes injected with α9 and α10 cRNAs, and block of these responses in the presence of either neramexane or memantine at a membrane potential of −70 mV. The amplitude of the acetylcholine responses was markedly reduced at micromolar concentrations of these compounds. In order to evaluate their blocking potency, inhibition curves were carried out (Fig. 2B). Currents evoked by acetylcholine were blocked in a concentration-dependent manner with a rank order of potency of neramexane > memantine, $P<0.05$. The IC_{50} and Hill coefficient values estimated from the inhibition curves were: 0.39±0.03 μM, 0.7±0.1, $n=6$ and 1.2±0.4 μM, 0.9±0.2, $n=6$, for neramexane and memantine, respectively. Blockage was reversible, because initial control responses to acetylcholine were recovered after washes of the oocytes with frog saline for 3 min. Moreover, neither neramexane nor memantine elicited responses *per se* in oocytes expressing α9α10 receptors. As the mechanism of action of memantine on α9α10 nicotinic acetylcholine receptors has been previously described (Oliver et al., 2001), further experiments were performed for neramexane.

3.2. Effects of neramexane on acetylcholine responses in rat inner hair cells

It is currently accepted that olivocochlear efferent innervation to developing cochlear inner hair cells is subserved by a nicotinic acetylcholine receptor composed from both α9 and α10 nicotinic subunits (Elgoyhen et al., 2001; Gomez-Casati et al., 2005). Current data support a model in which acetylcholine-gated depolarization is followed by activation of small-conductance, calcium-activated potassium channels and subsequent hair cell hyperpolarization (Fuchs and Murrow, 1992; Oliver et al., 2001). We therefore studied the effects of neramexane on acetylcholine responses (measured in isolation from the small-conductance Ca^{2+}-activated K^+ channels) in inner hair cells from acutely excised organs of Corti of postnatal 8–10 days rats, as a source of native receptors. As shown in Fig. 3, neramexane blocked acetylcholine-evoked responses in inner hair cells. The IC_{50} value obtained, 0.3±0.08 μM ($n=4$), was similar to that found for recombinant α9α10 receptors.

Acetylcholine mediated synaptic transmission is very different from experimental application of acetylcholine to oocytes or excised pieces of the organ of Corti. At synapses, transmitter is released into the synaptic cleft in very close proximity to postsynaptic receptors and reaches millimolar



Memantine 1-amino-3,5-Dimytheyl-adamantane
Neramexane 1-amino-1,3,3,5,5-Pentamethylmytheyl-cyclohexane

Fig. 1. Chemical structures of memantine and neramexane.

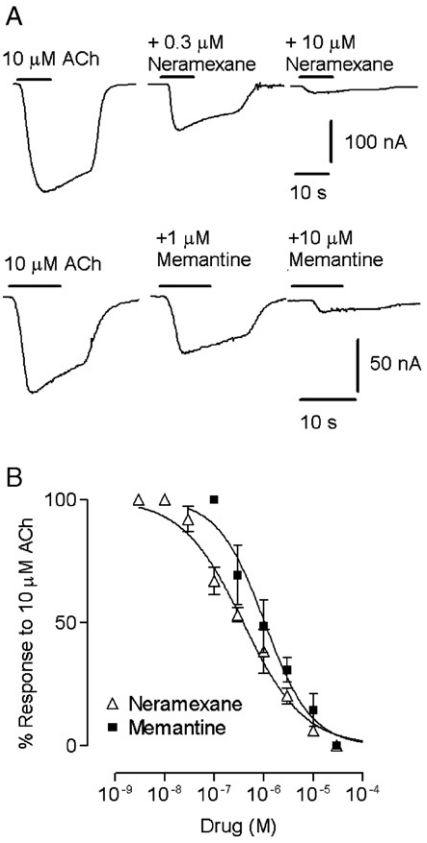


Fig. 2. Effects of neramexane and memantine on acetylcholine-evoked currents through recombinant α9α10 nicotinic acetylcholine receptors. A, Representative traces of the responses to 10 μM acetylcholine (ACh), either alone or in the presence of different concentrations of neramexane or memantine, in oocytes injected with α9 and α10 cRNAs. B, Inhibition curves performed by the coapplication of 10 μM acetylcholine and increasing concentrations of the compounds. Oocytes were incubated with each concentration of the antagonist for 2 min prior to the addition of acetylcholine. Peak current values are plotted, expressed as the percentage of the peak control current elicited by 10 μM acetylcholine. The mean and S.E.M. of six experiments per group are shown.

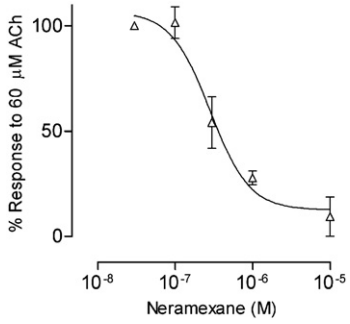


Fig. 3. Effects of neramexane on acetylcholine-evoked responses in rat inner hair cells. Inhibition curve performed by the coapplication of 60 μM acetylcholine and increasing concentrations of neramexane. Cells were incubated with each concentration of the antagonist for 1 min prior to the addition of acetylcholine. Peak current values are plotted, expressed as the percentage of the control current evoked by 60 μM acetylcholine. The mean and S.E.M. of four cells per point are shown.

concentrations sufficient to activate in the millisecond range receptors with a low affinity active state and a fast desensitization rate (Le Novere et al., 2002). Therefore the effect of neramexane in a more physiologically relevant model was studied by promoting synaptic acetylcholine release in isolated organs of Corti using high KCl concentrations. As shown in Fig. 4, 10 μM neramexane blocked responses to synaptically released acetylcholine, indicating that the compound is a valuable tool for inhibition of *in vivo* responses mediated by α9α10 nicotinic acetylcholine receptors. Fig. 4A shows K^+-evoked synaptic currents either in the absence or presence of neramexane. The observed block was rapidly reversed after neramexane was washed out of the preparation. Neramexane caused a reduction (Fig. 4B, $P<0.05$) in the amplitude of synaptic currents from 82.2±2.1 pA (652 events; 5 cells) to 55.3±2.1 pA (256 events; 5 cells,) with no changes in the frequency of the events (control: 2.5±0.4 Hz; neramexane: 1.6±0.4 Hz). Moreover, no changes either in the rise 10–90% (control: 17.12±1.40 ms, 108 events, 5 cells; neramexane: 15.40±1.50 ms, 71 events, 5 cells) or the decay time of synaptic currents (control: 39.84±1.6 ms, 108 events, 5 cells; neramexane: 43.27±2.3 ms, 71 events, 5 cells) were observed. These results confirm a postsynaptic effect of neramexane on the nicotinic acetylcholine receptors present in cochlear hair cells.

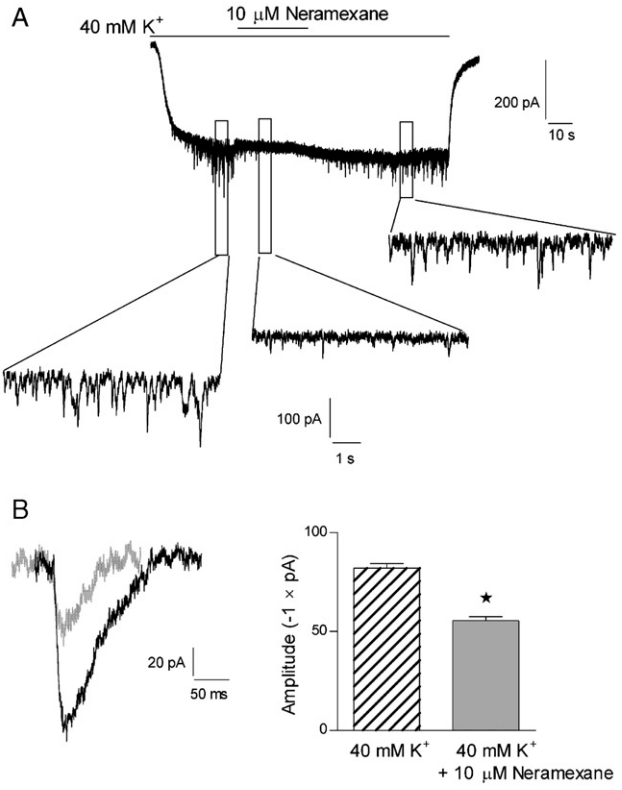


Fig. 4. Effect of neramexane on synaptic currents in inner hair cells. A, Representative traces of the effect of 10 μM neramexane on synaptic currents evoked by 40 mM KCl. The insets show the synaptic currents on an expanded time scale. B, Left, superimposed averages of synaptic currents evoked by 40 mM KCl either alone (black) or in the presence of 10 μM neramexane (grey). Right, bar diagram showing the effect of 10 μM neramexane on the amplitude of synaptic currents evoked by 40 mM KCl. The recordings are from five independent inner hair cells, and the numbers of analyzed events were 652 and 256, either in the absence or the presence of neramexane, respectively. The star denotes a significant difference, $P<0.05$.

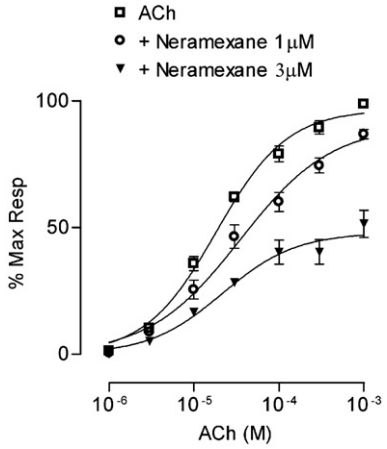


Fig. 5. Mechanism of block of acetylcholine-evoked currents by neramexane in oocytes expressing α9α10 receptors. Concentration–response curves to acetylcholine (ACh) performed either alone (□) or in the presence of 1 (○) or 3 μM (▼) neramexane. Peak current values were normalized and referred to the maximal peak response to acetylcholine. The mean and S.E.M. of 4 to 9 experiments per group are shown.

3.3. Underlying mechanism of block by neramexane

The mechanism underlying the blocking action of neramexane on the α9α10 receptor was further analyzed on recombinant receptors expressed in oocytes. Thus concentration–response curves to acetylcholine were performed in the absence or presence of the compound (Fig. 5, Table 1). At 1 μM, neramexane shifted the concentration–response curves to acetylcholine to higher concentrations (EC_{50} control: 18.3±0.6; EC_{50} neramexane: 35.1±4.6, $P<0.05$) without major changes in its maximal response, a behavior that is ascribed to a competitive antagonist. At higher concentrations of neramexane, however, the maximal acetylcholine-evoked response was reduced (Fig. 5; Table 1), with no changes in the EC_{50} values (control: 18.3±0.6; neramexane: 21.6±3.6). A shift to the right in the concentration–response curve, with concomitant insurmountable antagonistic effects at high concentrations of the agonist, is indicative of a non-competitive inhibition.

Competition radioligand binding was used to examine whether neramexane and memantine were able to displace

Table 1
Parameters derived from concentration–response curves performed in oocytes expressing α9α10 receptors, in the presence of neramexane

Neramexane (μM)	EC_{50} (μM)	Max. resp.[a] (%)	n_H[b]	n[c]
0	18.28±0.57	96.54±2.17	1.04±0.08	8
1	35.03±4.55[d]	90.36±4.64	0.81±0.10	3
3	21.56±3.59	47.94±3.38[d]	1.00±0.21	4

[a] % of the maximal response to acetylcholine.
[b] Hill coefficient.
[c] Number of experiments.
[d] $P<0.05$, with respect to the control in the absence of neramexane.

binding of the high affinity nicotinic antagonist methyllycaconitine (Fig. 6). Experiments were performed in transfected tsA201 cells using subunit chimeras ($\alpha9^{(L209)}/5HT_{3A}$ and $\alpha10^{(L206)}/5HT_{3A}$) in which the extracellular N-terminal domain of the α9 or α10 subunits were fused to the transmembrane and intracellular domains of the 5-hydroxytryptamine-3A subunit (Baker et al., 2004). These chimeric receptors express efficiently in transfected cells (compared to wild-type α9α10 receptors), binding experiments give a good specific to non-specific signal, and chimeric receptors retain the same pharmacological profile when compared to wild-type receptors expressed in *X. laevis* oocytes (Baker et al., 2004). As described previously, [^{3}H]methyllycaconitine binds with high affinity (K_d=7.5±1.2 nM) to cells coexpressing the α9 and α10 subunit chimeras (Baker et al., 2004). Complete displacement of bound [^{3}H]methyllycaconitine was observed for both neramexane and memantine, but only at very high concentrations of competing ligand (K_i=2.7±0.5 mM; n=5, for neramexane and K_i=0.8±0.2 mM; n=3, for memantine). In comparison to the IC_{50} values for the antagonist activity of neramexane and memantine derived from oocyte experiments (0.30±0.03 μM and 1.2±0.4 μM, respectively), the K_i values determined from competition-binding indicated a substantially lower affinity (~7000-fold and ~700-fold, respectively). Moreover, no displacement of [^{3}H]methyllycaconitine was observed at concentrations which gave full antagonist activity in oocytes. For comparison, competition binding was also performed with the nicotinic antagonist D-tubocurarine, which we have previously shown to be able to displace [^{3}H]methyllycaconitine binding in coexpressed $\alpha9^{(L209)}/5HT_{3A}+\alpha10^{(L206)}/5HT_{3A}$ nicotinic acetylcholine receptors with high affinity (0.29±0.09 μM; (Baker et al., 2004)). It would appear, therefore, that the antagonist activity of neramexane and memantine is not due to competitive binding with the agonist binding site of the receptor.

If the interaction of a compound with an ion channel requires the entrance into the transmembrane field, then depolarization should reduce inhibition. The simplest explanation for a voltage-dependent block is that the blocking molecule either has a binding site within the channel, partway across the electric field of the membrane (i.e. an open channel blocker) or that it docks within the channel vestibule impairing ion flow. Either mechanism would lead to a non-competitive mechanism of block (Arias et al., 2006). To assess the voltage-dependency of the block, 2-s voltage ramp protocols (−120 to +50 mV) were performed in the presence of 10 μM acetylcholine either alone or coapplied with increasing concentrations of neramexane. As shown in Fig. 7A, blockage produced by neramexane was observed both at hyperpolarized and depolarized potentials. As derived from Fig. 7B, which shows the normalized current amplitudes as a function of voltage, the block was independent of the membrane potential at all voltages tested at 0.3 μM. At 1 and 3 μM, block by neramexane exhibited a dependence upon voltage: 1 μM, I/I_{max}: 0.46±0.11 and 0.69±0.05, at −90 and +50 mV respectively, $P<0.05$, n=3; 3 μM, I/I_{max}: 0.35±0.09 and 0.58±0.04, at −90 and +50 mV respectively, $P<0.05$, n=5. However, as shown in Fig. 7B, an approximately e-fold difference in I/I_{max} was not achieved even every 160 mV, indicating only a slight dependence on membrane potential. These results indicate that neramexane could act as an open

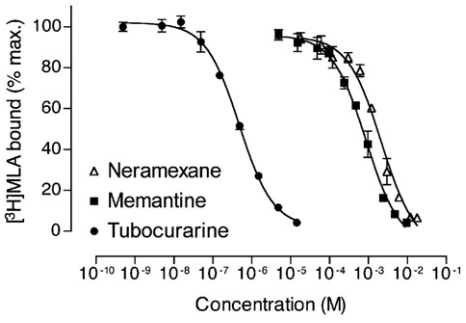


Fig. 6. Competition radioligand binding to transfected mammalian cells. Cultured mammalian tsA201 cells were cotransfected with subunit chimeras $\alpha9^{(L209)}/5HT_{3A}$ and $\alpha10^{(L206)}/5HT_{3A}$. Competition-binding data with neramexane (open triangles) and memantine (filled squares) is presented as a percentage of [^{3}H] methyllycaconitine (MLA) binding obtained in the absence of competing ligand. For comparison, displacement of [^{3}H]MLA binding with the higher affinity nicotinic antagonist d-tubocurarine is shown. Data are from a single experiment performed in triplicate (±S.E.M.), but are typical of three to five independent experiments.

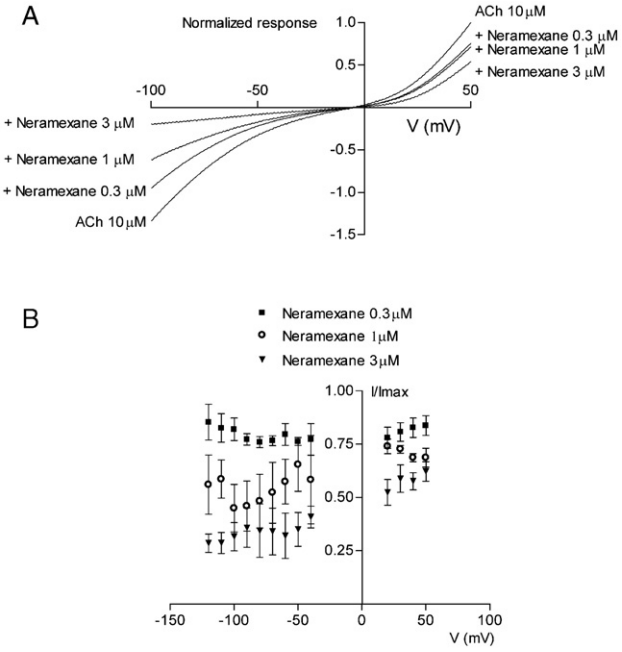


Fig. 7. Effect of neramexane at different holding potentials. A, Representative current–voltage curves performed in the presence of 10 μM acetylcholine (ACh), either alone or coapplied with 0.3, 1 or 3 μM neramexane, in response to 2-s voltage ramps from −120 to +50 mV, 5 s after the peak response to acetylcholine. Currents were leak-corrected by subtracting the response to the same voltage ramp performed prior to the application of 10 μM acetylcholine. B, Inhibition of the responses to 10 μM acetylcholine in the presence of neramexane at different holding potentials. Current amplitudes were obtained from current–voltage curves as those illustrated in A, and expressed as the percentage of the current amplitude obtained with acetylcholine alone at each holding potential. Mean and S.E.M. of 4–6 experiments per group are shown.

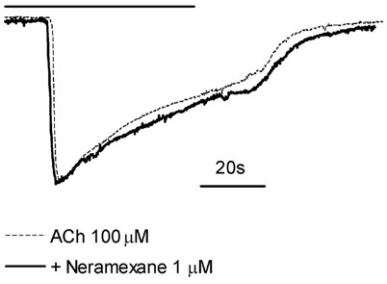


Fig. 8. Effect of neramexane on desensitization kinetics. Responses of α9α10 injected oocytes to 100 μM acetylcholine (ACh) either alone or in the presence of 1 μM neramexane. Responses were normalized to equivalent maximal responses in order to compare the traces. The figure is representative of $n=4$.

channel blocker of the α9α10 nicotinic acetylcholine receptor only at micromolar concentrations.

Finally, increased desensitization can also account for non-competitive antagonism of nicotinic acetylcholine receptors (Arias et al., 2006). As shown in Fig. 8, 1 μM neramexane did not change the desensitization kinetics of the responses to 100 μM acetylcholine (I_{20}/I_{max} control: 61.6±4.6; neramexane: 69.9±4.3, $n=5$), thus indicating that an increase in desensitization is not likely to be responsible for the non-competitive block of the α9α10 nicotinic acetylcholine receptors.

4. Discussion

In the present study we analyzed the effects of neramexane, a novel amino-alkyl-cyclohexane derivative that is a non-competitive NMDA receptor antagonist, on recombinant α9α10 nicotinic acetylcholine receptors expressed in *X. laevis* oocytes. We compared its effects with those of memantine, a well-studied pore blocker of NMDA receptors currently used in therapeutics for the treatment of Alzheimer's disease. We report that both compounds block acetylcholine-evoked responses in the low micromolar concentration range, neramexane being more potent than memantine. Moreover, in the case of neramexane we report a blocking action on native α9α10-containing receptors of rat cochlear inner hair cells.

4.1. Block of α9α10 nicotinic acetylcholine receptors by neramexane

Since neramexane was developed in a search for new NMDA receptor antagonists, based on the success of the use of memantine in therapeutics, we compared the effects of neramexane with those of memantine on rat recombinant α9α10 nicotinic acetylcholine receptors. Memantine, an adamantane derivative, is a well-established blocker of ligand-gated ion channels permeable to Ca^{2+}such as the NMDA type glutamate receptor (Bresink et al., 1996; Chen et al., 1992; Parsons et al., 1993). It has been shown to antagonize NMDA receptors expressed in heterologous systems with an IC_{50} of 1–3 μM (Bresink et al., 1996). Moreover, it also acts as an antagonist of human α4β2 and α7 nicotinic acetylcholine receptors (Buisson and Bertrand, 1998; Maskell et al., 2003), and rat α9α10 nicotinic acetylcholine receptors (Oliver et al., 2001). The present data indicates that the amino-alkyl-cyclohexane neramexane is, in addition to its well-characterized action as non-competitive NMDA receptor antagonist, a blocker of the α9α10 nicotinic acetylcholine receptors. Moreover, its blocking potency is higher than that of memantine. To our knowledge, this is the first time neramexane has been shown to interact with the α9α10 nicotinic acetylcholine receptor. This drug blocks both NMDA receptors with an IC_{50} value near 1 μM at −70 mV (Parsons et al., 1999), as well as $5HT_3$ receptors with an IC_{50} value near 2 μM at −70 mV (Rammes et al., 2001). Thus, the apparent affinity of neramexane on recombinant rat α9α10 receptors found in the present study is slightly higher than that reported for NMDA and $5HT_3$ receptors. Since neramexane blocked acetylcholine-evoked responses in inner hair cells with an IC_{50} value (0.3± 0.08 μM), similar to that found for recombinant α9α10 receptors, we could speculate a possible therapeutic relevance for the blocking effect of neramexane on this receptor subtype.

The fact that no effect of micromolar concentrations of neramexane was observed on ligand binding experiments, together with the fact that this compound reduced the maximal response to acetylcholine in concentration–response experiments, indicates that neramexane behaves as a non-competitive antagonist. Nicotinic acetylcholine receptors are pentameric proteins that belong to the Cys-loop receptor superfamily. Their essential mechanism of functioning is to couple neurotransmitter binding, which occurs at the extracellular domain, to the opening of the membrane-spanning cation channel. The function of these receptors can be modulated by structurally different non-competitive antagonists. Receptor modulation has proved to be highly complex for most non-competitive antagonists. Non-competitive antagonists may act by more than one mechanism and at distinct sites in the same receptor subtype. The binding site location for one particular molecule depends on the conformational state of the receptor. Defining the underlying mechanism of non-competitive blockers has become challenging in most cases. These may act at least by two different mechanisms: an allosteric and/or a steric mechanism (Arias et al., 2006).

Distinct allosteric mechanisms may account for non-competitive block of nicotinic acetylcholine receptors. For example, non-competitive antagonists may bind to the receptor and stabilize a non-conducting conformational state (e.g., resting or desensitized state), and/or increase the receptor desensitization rate (Arias et al., 2006). The fact that neramexane did not modify the rate of desensitization might indicate that this is not the underlying mechanism of block.

The simplest explanation representing a steric mechanism is that the antagonist molecule physically blocks the ion channel. The fact that block by low concentrations of neramexane was independent of the voltage, suggests an interaction of the drug with a site other than the ion channel pore. This differs from the mechanism proposed for the block by neramexane of NMDA receptors where it is only observed at hyperpolarized potentials because of its pronounced voltage-dependence (Parsons et al.,

1999). Block of nicotinic acetylcholine receptors by high concentrations of neramexane was significantly larger at hyperpolarized than at depolarized potentials. The simplest explanation for a voltage-dependent block could be described by the Woodhull model (Woodhull, 1973), where the blocking molecule either has a binding site within the channel, partway across the electric field of the membrane, or that it docks within the channel vestibule impairing ion flow. However, in the case of α9α10 nicotinic acetylcholine receptors, the percentage of blockage obtained at different membrane potential, shown in Fig. 7, did not give a good fit to the Woodhull model, since an approximately e-fold difference in I/I_{max} was not achieved even every 160 mV. These results suggest that blockage by neramexane was only slightly dependent on the membrane potential, and that additional mechanisms might be involved.

4.2. Clinical implications

Neramexane, a novel alkyl-cyclohexane derivative that has recently entered phase II of clinical trials, shows some similarity to memantine on its effect on NMDA type glutamate receptors, e.g. channel blocking kinetics, voltage dependency, and affinity (Danysz et al., 2002). Preclinical tests indicate particularly good activity in animal models of alcoholism (self-administration, withdrawal-induced audiogenic seizures, etc. (Bienkowski et al., 2001; Danysz et al., 2002; Kotlinska et al., 2004)) and pain (chronic pain, inhibition of tolerance to the analgesic effects of morphine (Malyshkin et al., 2005; Houghton et al., 2001)). Moreover, due to their open channel inhibitory effects at NMDA receptors, (Parsons et al., 1999), memantine and neramexane are thought to have neuroprotective (Liu et al., 2000; Wenk et al., 1998), analgesic (Houghton et al., 2001) and cognitive enhancement efficacy (Danysz and Parsons, 2003). The plasma concentrations achieved with memantine and neramexane at therapeutic doses is around 0.4–1 μM (Danysz et al., 1997; Hesselink et al., 1999; Parsons et al., 1999). Since these plasma levels are near the IC_{50} of neramexane and memantine on α9α10, it is possible that memantine or neramexane-based therapies lead to a reduction in the function of this nicotinic acetylcholine receptor.

Loss or damage of hair cells in the cochlea is well known to be the major cause of hearing loss (Patuzzi et al., 1989). In addition, most cases of tinnitus (a debilitating state due to ringing in the ear) follow on from insults to the inner ear such as loud sound trauma or the use of ototoxic antibiotics which leads to hair cell loss (Eggermont, 2005). Despite the significant unmet clinical need for a safe and effective drug to alleviate tinnitus, there currently is no FDA-approved drug on the market. The mechanisms of hair cell degeneration in general are not fully understood, but in analogy to neurons, it can be hypothesized that Ca^{2+} influx may contribute to this process. One such site of Ca^{2+} entry is the α9α10 nicotinic acetylcholine receptor present at the basal pole of outer hair cells (Elgoyhen et al., 2001). If Ca^{2+} influx could reach a toxic scale, block of α9α10 receptors might exert a protective effect. Therefore, the present results raise the possibility that neramexane may exert a biological and/or therapeutic action within the inner ear when used clinically.

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