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Direct Interaction of Serotonin Type 3 Receptor Ligands with Recombinant and Native $\alpha9\alpha10$-Containing Nicotinic Cholinergic Receptors

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ABSTRACT

In the present work, we characterized the effects of serotonin type 3 receptor ligands on recombinant and native $\alpha9\alpha10$-containing nicotinic acetylcholine receptors (nAChRs). Our results indicate that the recombinant $\alpha9\alpha10$ nAChR shares striking pharmacological properties with 5-HT_3 ligand-gated ion channels. Thus, 5-HT_3 receptor antagonists block ACh-evoked currents in $\alpha9\alpha10$-injected *Xenopus laevis* oocytes with a rank order of potency of tropisetron (IC_{50}, 70.1 $\pm$ 0.9 nM) > ondansetron (IC_{50}, 0.6 $\pm$ 0.1 μM) = MDL 72222 (IC_{50}, 0.7 $\pm$ 0.1 μM). Although serotonin does not elicit responses in $\alpha9\alpha10$-injected oocytes, it blocks recombinant $\alpha9\alpha10$ receptors in a noncompetitive and voltage-dependent manner (IC_{50}, 5.4 $\pm$ 0.6 μM). On the other hand, we demonstrate an in vivo correlate of these properties of the recombinant receptor, with those of the $\alpha9\alpha10$-containing nAChR of frog saccular hair cells. The possibility that the biogenic amine serotonin might act as a neuromodulator of the cholinergic efferent transmission in the vestibular apparatus and in the organ of Corti is discussed.

Nicotinic acetylcholine receptors (nAChRs) are complexes of protein subunits that coassemble to form an ion channel gated through the binding of the neurotransmitter ACh to its ligand-binding site (Karlin, 2002). A diversity of subunits have been cloned in recent years. The nAChR at the neuromuscular junction mediates fast synaptic transmission and is thought to have a $(\alpha1)_2\beta1\gamma\delta$ stoichiometry (Galzi et al., 1991). Ten genes that encode neuronal nAChR subunits have been identified in the vertebrate central or peripheral nervous system: $\alpha2$–$\alpha8$ and $\beta2$–$\beta4$ (Le Novére and Changeux, 1999). In heterologous expression systems, the neuronal nAChRs may assemble from single α-subunits, from multiple α- and β-subunits, and heteromeric nAChRs formed via pair-wise combinations of $\alpha2$, $\alpha3$, $\alpha4$, or $\alpha6$ with either the $\beta2$ or $\beta4$ subunits (for references, see Elgoyhen et al., 2001). Neuronal nAChRs preserve the structural motif of muscle nAChR, with a pentameric structure that includes two α and three β subunits (Anand et al., 1991; Cooper et al., 1991).

The cloning of the $\alpha9$ and $\alpha10$ subunits added two peculiar members to the family of nAChRs (Elgoyhen et al., 1994, 2001). They are distant members of the family; whereas neuronal nAChR α subunits and the muscle $\alpha1$ subunit share sequence homologies ranging from 48 to 70%, the sequence identity between $\alpha9$ and all known nAChR subunits is less than 40%. When expressed in *Xenopus laevis* oocytes, $\alpha9$ and $\alpha10$ subunits form a heteromeric receptor-channel complex that is activated by ACh but not by nicotine and displays a very distinct pharmacological profile that falls into neither the nicotinic nor the muscarinic subdivision of the pharmacological classification scheme of cholinergic receptors. However, the properties of the recombinant $\alpha9\alpha10$ receptor are strikingly similar to those described for the cholinergic receptor that mediates synaptic transmission between efferent cholinergic fibers and cochlear outer hair cells (Fuchs, 1996; Elgoyhen et al., 2001). Moreover, the $\alpha9$ and $\alpha10$ gene subunits exhibit a unique and restricted expression pattern. Whereas $\alpha9$ and $\alpha10$ message has not been found in the central nervous system, it is present in the cochlear and

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; 5-HT, 5-hydroxytryptamine; BAPTA-AM, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; MDL 72222, 3-tropanyl-3,5-dichlorobenzoate; I-V, current-voltage; 5HTQ, trimethyl serotonin; ICS 205,930, tropisetron.

vestibular hair cells (Elgoyhen et al., 1994, 2001; Hiel et al., 1996; Morley et al., 1998). This has led to the proposal that efferent modulation of cochlear and vestibular hair cell function occurs, at least in part, via heteromeric nAChRs assembled from both α9 and α10 subunits (Elgoyhen et al., 2001).

Nicotinic AChRs are members of a family of neurotransmitter-gated ion channels that also includes, $GABA_A$, $GABA_C$, glycine, 5-HT_3 and some invertebrate anionic glutamate receptors (Le Novére and Changeux, 1999). The subunits of these receptors have similar sequences and distributions of hydrophobic, membrane-spanning segments. Each subunit contains in its ligand-binding N-terminal half two cysteine residues separated by 13 other residues that are presumably disulfide-linked, thus giving this family the name of the Cys-loop receptors. We have demonstrated (Rothlin et al., 1999) that homomeric α9 nAChRs share several pharmacological properties with $GABA_A$, glycine, and 5-HT_3 receptors. The aim of the present work was to perform an extensive characterization of the effects of 5-HT_3 receptor ligands on recombinant and native α9α10-containing nicotinic cholinergic receptors. Our results indicate that the recombinant α9α10 nAChR shares striking pharmacological properties with type 3 serotonin ligand-gated ion channels. Moreover, it demonstrates an in vivo correlate of these properties of the recombinant receptor, with those of the α9α10-containing nAChR of frog saccular hair cells.

Materials and Methods

Expression of Recombinant Receptors in *X. laevis* Oocytes. For expression studies, α4, α7, α9, α10, and β2 rat nAChR subunits were subcloned into a modified pGEMHE vector (Liman et al., 1992). Capped cRNAs were in vitro transcribed from linearized plasmid DNA templates using the mMessage mMachine T7 transcription kit (Ambion Corporation, Austin, TX). The maintenance of *X. laevis* and the preparation and cRNA injection of stage V and VI oocytes have been described in detail elsewhere (Katz et al., 2000). Typically, oocytes were injected with 50 nl of RNase-free water containing 0.01 to 1.0 ng of cRNA (at a 1:1 molar ratio when pair-wise combined) and maintained in Barth's solution at 17°C.

Electrophysiological recordings were performed 2 to 6 days after cRNA injection under two-electrode voltage clamp with a Geneclamp 500 amplifier (Axon Instruments Corp., Union City, CA). Both voltage and current electrodes were filled with 3 M KCl and had resistances of ~1 to 2 MΩ. Data acquisition was performed using a Digidata 1200 and the pClamp 7.0 software (Axon Instruments). Data were analyzed using Clampfit from the pClamp 6.1 software. During electrophysiological recordings, oocytes were continuously superfused (~10 ml/min) with normal frog saline composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES buffer, pH 7.2. Unless otherwise indicated, the membrane potential was clamped to −70 mV. Drugs were applied in the perfusion solution of the oocyte chamber. To minimize activation of the endogenous Ca^{2+}-sensitive chloride current (Elgoyhen et al., 2001), all experiments were performed in oocytes incubated with the Ca^{2+} chelator BAPTA-AM (100 μM) for 3 to 4 h before electrophysiological recordings.

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 before the addition of 10 μM ACh and then were coapplied with this agonist. Responses were referred to as a percentage of the response to ACh. The mean and S.E.M. of peak current responses are represented. Agonist concentration-response curves were iteratively fitted with the equation: $I/I_{max} = A^{nH}/(A^{nH} + EC_{50}{}^{nH})$, where I is the peak inward current evoked by 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_H is the Hill coefficient. An equation of the same form was used to analyze the concentration dependence of antagonist-induced blockage. The parameters derived were the concentration of antagonist producing a 50% block of the control response to ACh (IC_{50}) and the associated interaction coefficient (n_H). Current-voltage relationships were obtained by applying 2-s voltage ramps from −120 to +50 mV, 10 s after the peak response to 10 μM ACh from a holding potential (V_{hold}) of −70 mV. Leakage correction was performed by digital subtraction of the current-voltage (I-V) curve obtained by the same voltage ramp protocol before the application of ACh. Generation of voltage protocols and data acquisition were performed using a Digidata 1200 and the pClamp 6.1 or 7.0 software (Axon Instruments). Data were analyzed using Clampfit from the pClamp 6.1 software.

Statistical significance was evaluated by the Student's t test (two-tailed, unpaired samples). Multiple comparisons of IC_{50} values were performed with a one-way analysis of variance followed by Tukey's test. $p < 0.05$ was considered significant.

Isolation and Patch Clamp Recording from Saccular Hair Cells. Frog saccular hair cells were isolated enzymatically. The dissociation protocol, optimized for the purpose of observing reliable ACh responses, was described previously (Holt et al., 2001). Briefly, leopard frogs (*Rana pipiens*) were chilled, pithed,[1] and decapitated. Each side of the head was placed into a perilymph-like standard external solution composed of 105 mM NaCl, 2.5 mM KCl, 0.81 mM $MgCl_2$, 1.8 mM $CaCl_2$, 3.4 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 1 mM ascorbate, 4 mM glucose, and 5 mM pyruvate. The otic capsule from each side was opened to gain access into the inner ear. The saccule was dissected, and the macula was excised from the saccule. The macula was then incubated in 0.05% trypsin in Hanks' balanced salt solution (Invitrogen, Carlsbad, CA) for 8 to 10 min, after 30 s of rinsing in a low-calcium dissociation solution (105 mM NaCl, 2.5 mM KCl, 0.81 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3.4 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 2.5 mM Na_2HPO4, 1 mM ascorbate, 4 mM glucose, and 5 mM pyruvate), which contained 10% fetal calf serum (Invitrogen). It was then washed in dissociation solution containing 500 μg/ml bovine serum albumin for 5 to 10 min. The macula was then transferred to the recording chamber, previously filled with external solution, and hair cells were gently separated from the epithelium with the help of a thin, hook-shaped glass wisp. The hair cells settled to the base of the recording chamber and adhered firmly to the bottom, which was precoated with 2 mg/ml concanavalin A. The bath was perfused with external solution at a rate of 1 ml/min. All solutions had an osmolality of 220 mOsm and a pH of 7.2.

The recording chamber was placed on the stage of an inverted microscope (IMT-2; Olympus, Tokyo, Japan) equipped with a 40× objective with modulation-contrast optics. Continuous gravity perfusion (1 ml/min) was used for exchange of recording solutions. The drugs were applied at a rate of ~ 3.6 μl/s using a gravity-driven microperfusion pipette placed ~300 to 600 μm from the cell of interest. Experiments were performed at room temperature (20–22°C). Currents and voltages were recorded with the perforated patch-clamp technique. The antibiotic amphotericin B (0.6 mg/ml) was added to the internal solution, containing 75 mM KCl, 2 mM $MgCl_2$, 30 mM K_2SO_4, 6 mM glucose, and 10 mM HEPES. Borosilicate glass pipettes were pulled from 1.5 mm o.d. × 0.75 mm i.d. capillary tubing (Longreach Scientific Resources, Orr's Island, ME) with a Flaming/Brown micropipette puller (model P-97; Sutter Instruments, Novato, CA) and heat-polished (Narashige Scientific Instrument La, Tokyo, Japan). The tip potential between the internal solution of the pipette and the bath was nulled before seal formation. The junction potential

[1] A pithed frog has had its central nervous system destroyed (its spinal cord has been severed). It is dead, but some of its organs continue to function for a brief period.

was calculated to be approximately −7 mV. The zero current potential was −48 ± 7 mV and ranged from −37 to −68 mV; the series resistance was 16 ± 4 MΩ and was partially compensated (80%) during voltage-clamp recordings using the compensation circuitry of the amplifier. Average cell capacitance was 15 ± 3 pF. Series resistance and cell capacitance compensations were updated continuously throughout all recordings.

The Axopatch 1D patch-clamp amplifier (Axon Instruments) was used for all voltage-clamp experiments in recording from saccular hair cells. Saccular hair cells were stepped from −60 mV (holding potential) to −10 mV during the voltage-clamp protocol. This protocol was used because −10 mV coincides with the peak of ACh-induced calcium activated outward K^+ currents, as determined from previously reported I-V relationships (Erostegui et al., 1994). Voltage-clamp data were sampled at 50- to 300-μs intervals. Records were low-pass filtered at 5 to 10 kHz with a four-pole Bessel filter. Stimuli were generated and data were sampled with a 12-bit digital-to-analog and analog-to-digital converter (DigiData 1200 series interface; Axon Instruments) and controlled by the data acquisition software package pClamp7 (Axon Instruments). Voltage-clamp data were stored digitally and later analyzed offline using the pClamp6 Clampfit (Axon Instruments), Excel 97 (Microsoft Corp., Redmond, WA), and Origin (ver. 5; OriginLab Corp., Northampton, MA) programs. Dose-response curves were normalized to the maximal agonist response in each hair cell. For the inhibition dose-response curves, cells were superfused with compounds for 1.5 to 2.5 min before coinjection with 20 μM ACh (~EC_{50}), and responses were calculated as a percentage of the response elicited by the agonist.

Materials. ACh chloride or iodide was bought from Sigma Chemical Co. (St. Louis, MO). Serotonin hydrochloride, 1-(*m*-chlorophenyl)-biguanide hydrochloride, trimethyl serotonin iodide (5HTQ), 3-tropanyl-indole-3-carboxylate hydrochloride (tropisetron; ICS 205,930), and 3-tropanyl-3,5-dichlorobenzoate (MDL 72222) were obtained from RBI/Sigma (Natick, MA). Ondansetron hydrochloride was kindly donated by Raffo Laboratories (Buenos Aires, Argentina). Drugs were dissolved in distilled water as 10 mM stocks and stored aliquoted at −20°C. BAPTA-AM (Molecular Probes, Eugene, OR) was stored at −20°C as aliquots of a 100 mM solution in dimethyl sulfoxide, thawed, and diluted 1000-fold into saline solution shortly before incubation of the oocytes.

All experimental protocols were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/guide/guidex.htm).

Results

Effect of Serotonin on ACh-Evoked Currents through Recombinant α9α10 nAChRs. Figure 1A shows representative responses to 10 μM ACh of *X. laevis* oocytes injected with α9 and α10 cRNAs. As expected for a nAChR, serotonin did not evoke currents through the α9α10 receptor. However, currents elicited by ACh were blocked by serotonin in a concentration-dependent manner (Fig. 1, A and B) with an IC_{50} of 5.4 ± 0.6 μM (mean ± S.E.M.; n = 6). Block by serotonin was reversible, because initial control responses to ACh were recovered after washes of the oocytes with frog saline (data not shown).

To further characterize the mechanism underlying the blocking action of serotonin on the α9α10 receptor, the effect of this drug was analyzed at increasing concentrations of ACh (V_{hold}, −70 mV). As shown in Fig. 2A, 10 μM serotonin produced a significant reduction of the agonist maximal response (percentage of maximal response, 58.1 ± 5.4, n = 4) and no change in the EC_{50} value (7.9 ± 1.0 and 11.6 ± 5.3 μM in the absence and presence of serotonin, respectively). This result is compatible with a noncompetitive mechanism of block, because the effect of serotonin is reversible. Moreover, as shown in the representative I-V curves of Fig. 2B, block by serotonin of ACh-evoked responses was voltage-dependent. Thus, whereas responses were not modified at positive depolarized potentials, they were diminished at negative hyperpolarized membrane holding potentials (I/I_{max}, 39 ± 4.7 and 20.1 ± 0.4% of control values at −90 mV for 10 and 30 μM serotonin, respectively, $p < 0.01$, n = 3).

Effect of Serotonin Type 3 Receptor Agonists on the Recombinant α9α10 nAChR. To analyze whether the effect observed with serotonin could be extended to other serotoninergic agonists, the effect of 5HTQ and 1-(*m*-chlorophenyl)-biguanide, selective for 5-HT$_3$ receptors, was studied on α9α10-injected *X. laevis* oocytes. Contrary to that observed with serotonin, both 5-HT$_3$ agonists elicited inward currents through the α9α10 receptor (Fig. 3A). However, both compounds behaved as weak partial agonists, because maximal responses were only 10.8 ± 0.8% (n = 3) for 5HTQ and 3.1 ± 0.7% (n = 3) for 1-(*m*-chlorophenyl)-biguanide of the maximum obtained with ACh (Fig. 3B).

The effect of increasing concentrations of 1-(*m*-chlorophenyl)-biguanide was further analyzed at a fixed 10 μM ACh concentration (V_{hold}, −70 mV). As shown in Fig. 4, A and B, responses to ACh were potentiated by low concentrations of 1-(*m*-chlorophenyl)-biguanide (3–10 μM) that did not elicit currents per se, whereas inhibition of ACh-evoked currents predominated at higher concentrations of the compound. Potentiation reached 43.8 ± 5.7% (n = 4) at 3 μM 1-(*m*-chlorophenyl)-biguanide and 21 ± 5.5% (n = 6) at 10 μM. When analyzed at different holding potentials, both potentiation by 10 μM 1-(*m*-chlorophenyl)-biguanide as well as block by 100 μM 1-(*m*-chlorophenyl)-biguanide resulted in voltage independence (Fig. 4C). Thus, potentiation of responses at −90 mV (32 ± 13%), did not significantly differ from that at +40 mV (51 ± 15%; n = 3), and inhibition of responses at −90 mV (65 ± 6%) did not differ from that at +40 mV (61 ± 4%; n = 4). However, if the blocking effect of 1-(*m*-chlorophenyl)-biguanide starts at concentrations lower than 3 to 10 μM, both potentiation and blockage will overlap at 10 μM 1-(*m*-chloro-

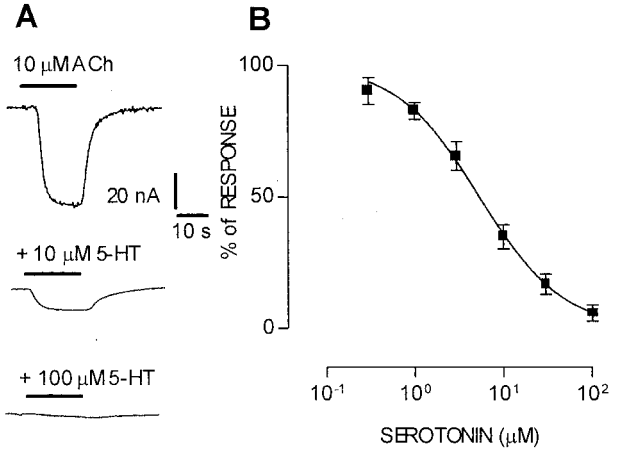

Fig. 1. Effect of serotonin on ACh-evoked currents through recombinant α9α10 nAChRs. A, representative traces to ACh either alone or in the presence of serotonin. B, inhibition curve performed by the coapplication of 10 μM ACh and increasing concentrations of serotonin. Oocytes were incubated with each concentration of serotonin for 2 min before the addition of ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and S.E.M. of six experiments are shown.

phenyl)-biguanide. Therefore, the shape of the I-V curves at low concentrations of 1-(*m*-chlorophenyl)-biguanide might not solely reflect the potentiating action of the drug. The mechanism underlying potentiation of ACh responses in the presence of 1-(*m*-chlorophenyl)-biguanide was not further analyzed. As shown in Fig. 4D, block of ACh-evoked responses in the presence of 100 μM 1-(*m*-chlorophenyl)-biguanide was noncompetitive, because although the effect was reversible, block was not surmounted at high concentrations of ACh (V_{hold}, −70 mV). Thus, 100 μM 1-(*m*-chlorophenyl)-biguanide produced a significant reduction of the agonist maximal response (percentage of maximal response, 32.6 ± 2.9, n = 10)

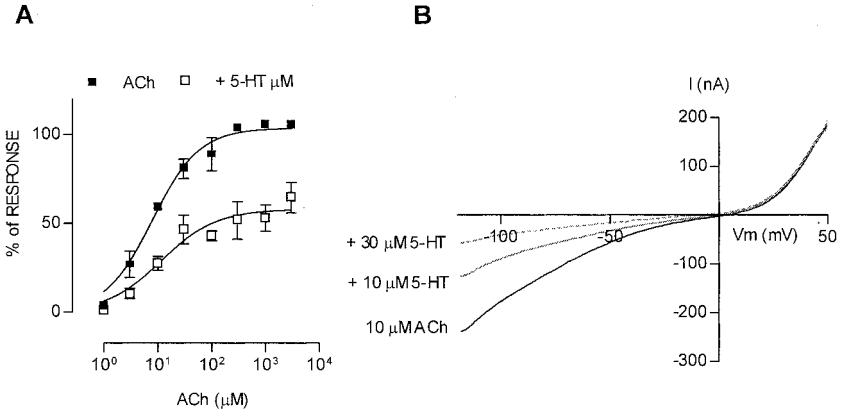

Fig. 2. Mechanism of blockage by serotonin of ACh-evoked responses. A, concentration-response curves to ACh performed either alone or in the presence of 10 μM serotonin. Peak current values were normalized and referred to the maximal peak response to ACh. The mean and S.E.M. of three to four experiments per group are shown. B, representative I-V curves obtained upon application of 2-s voltage ramps (−120 to +50 mV) 10 s after the peak response to 10 μM ACh from a holding potential (V_{hold}) of −70 mV, either alone or in the presence of serotonin (n = 3 for each curve).

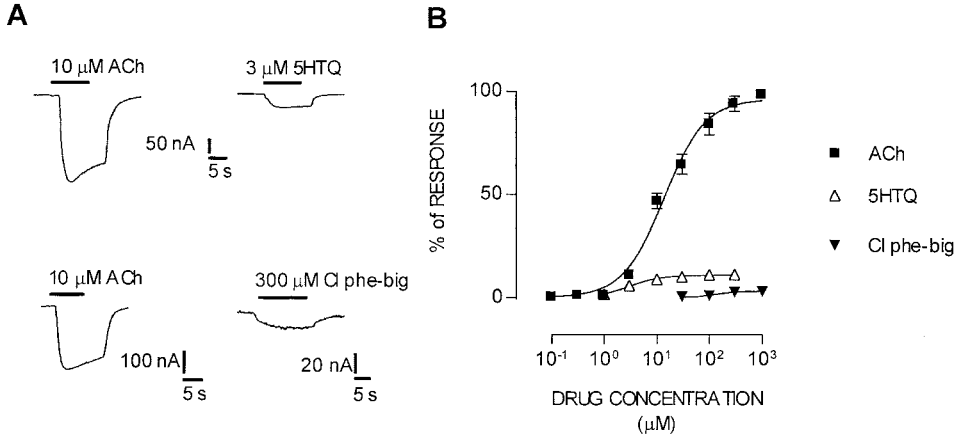

Fig. 3. Effect of serotonin type 3 receptor agonists on the recombinant $\alpha 9\alpha 10$ nAChR. A, representative current responses to 5-HT_3 receptor agonists. B, concentration-response curves to ACh, 5HTQ, and 1-(*m*-chlorophenyl)-biguanide. Peak current values were normalized and referred to the maximal peak response to ACh. The mean and S.E.M. of three experiments per group are shown.

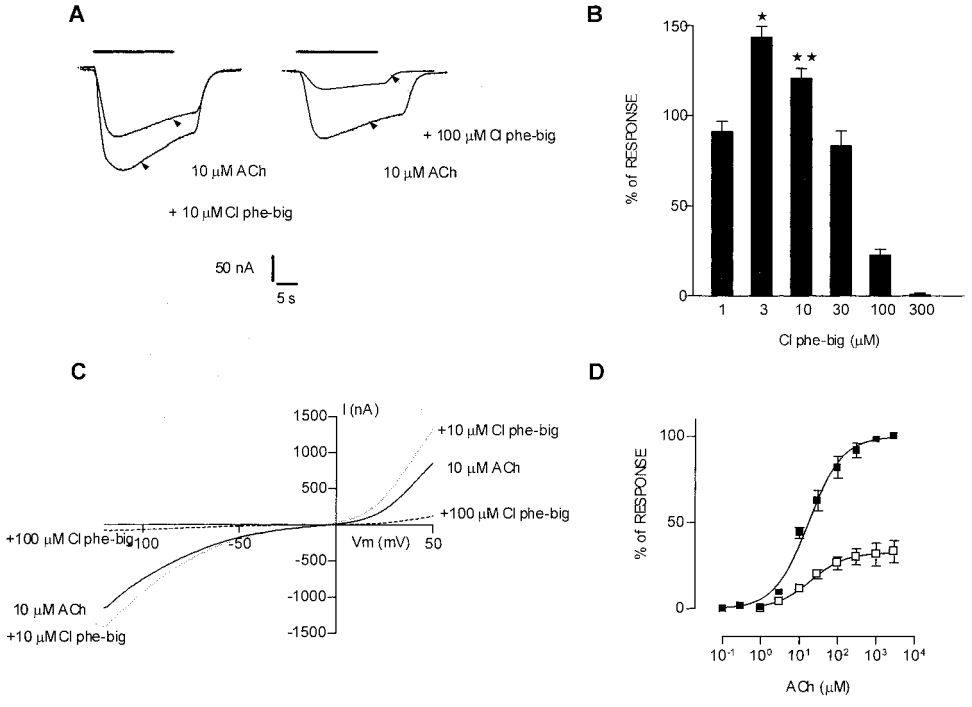

Fig. 4. Effect of 1-(*m*-chlorophenyl)-biguanide on ACh-evoked currents. A, representative traces to ACh either alone or in the presence of 10 μM or 100 μM 1-(*m*-chlorophenyl)-biguanide. Oocytes were preincubated for 2 min with 1-(*m*-chlorophenyl)-biguanide before the addition of 10 μM ACh. B, responses to 10 μM ACh at different concentrations of 1-(*m*-chlorophenyl)-biguanide (n = 4–6); *, $p < 0.05$; **, $p < 0.01$, Tukey's test. C, representative I-V curves obtained upon application of 2-s voltage ramps (−120 to +50 mV) 10 s after the peak response to 10 μM ACh from a holding potential (V_{hold}) of −70 mV, either alone or in the presence of 1-(*m*-chlorophenyl)-biguanide (n = 3 for each curve). D, concentration-response curves to ACh performed either alone or in the presence of 100 μM 1-(*m*-chlorophenyl)-biguanide. Peak current values were normalized and referred to the maximal peak response to ACh. The mean and S.E.M. of 10 experiments per group are shown.

and no change in the EC_{50} value [17.1 ± 1.0 and 19.8 ± 8.9 μM in the absence and presence of 1-(*m*-chlorophenyl)-biguanide, respectively].

Effect of Serotonin on ACh-Evoked Currents in Isolated Saccular Hair Cells. The effect of serotonin on a native hair cell cholinergic receptor preparation was analyzed on isolated frog vestibular hair cells. It has been described previously that hyperpolarization of the frog saccular hair cells in response to the exogenous application of ACh is caused by the activation of α9-containing nicotinic cholinergic receptors and the subsequent activation of the small-conductance, Ca^{2+}-activated (SK) potassium current (Athas et al., 1997; Lioudyno et al., 2000; Holt et al., 2001). The α9-containing nAChR in frog saccular hair cells is pharmacologically indistinguishable from the α9α10 nAChR of the cochlear hair cells and the recombinant α9α10 nAChR, suggesting that both subunits are involved in the ACh-evoked response (Fuchs, 1996; Elgoyhen et al., 2001; Holt et al., 2001). As shown in the representative traces of Fig. 5A, the application of ACh to isolated saccular hair cells typically produces an outward current. The concentration of ACh that produces a half-maximal current response (EC_{50}) has been reported previously to be 18.4 μM (Holt et al., 2001).

The addition of the neurotransmitter serotonin to voltage-clamped saccular hair cells did not elicit a detectable response. However, the coapplication of serotonin and 20 μM ACh blocked the ACh-evoked currents in a concentration-dependent manner (Fig. 5, A and B). The concentration of serotonin required to block 50% of the response to ACh (7.5 ± 6.9 μM, $n = 4$) was in the same order of magnitude as that derived from Fig. 1B for recombinant α9α10 receptors.

Effect of Serotonin Type 3 Receptor Antagonists on ACh-Evoked Currents through Recombinant α9α10 nAChRs. Tropisetron, an established blocker of 5-HT$_3$ receptors (Vanner and Sruprenant, 1990; Maricq et al., 1991), is one of the most potent antagonists described so far for the α9α10 nAChR (Fig. 6) (Elgoyhen et al., 2001). High sensitivity of α9α10 to 5-HT$_3$ antagonists seems to be a pharmacological property of this receptor and is not a peculiarity restricted to tropisetron. Thus, ACh-evoked currents in α9α10-injected oocytes were also blocked by the 5-HT$_3$ antagonists ondansetron and MDL 72222 (Fig. 6, A and B). In all cases, the effect was concentration-dependent, with a rank order of potency of tropisetron (IC_{50}, 70.1 ± 0.9 nM, $n = 8$) > ondansetron (IC_{50}, 0.6 ± 0.1 μM, $n = 6$) = MDL 72222 (IC_{50}, 0.7 ± 0.1 μM, $n = 4$). Block by antagonists was reversible, because initial control responses to ACh were recovered after washes of the oocytes with frog saline (not shown). Moreover, antagonists did not elicit per se responses in oocytes expressing α9α10 receptors. On the other hand, ketanserin (3 μM), an antagonist of 5-HT2A and 5-HT2C metabotropic serotonin receptors, did not significantly block ACh-evoked currents ($n = 3$ oocytes).

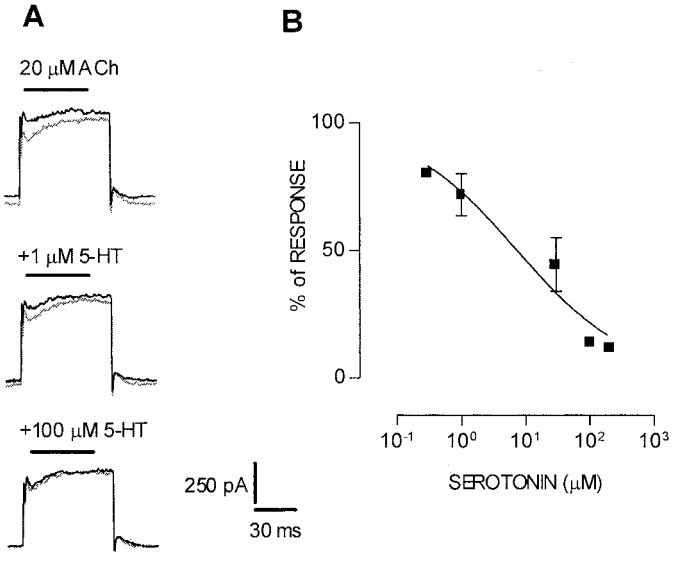

Fig. 5. Effect of serotonin on ACh-evoked currents in isolated saccular hair cells. A, representative traces of ACh-evoked currents recorded from isolated frog saccular hair cells either alone or in the presence of serotonin (black traces). The membrane holding potential was stepped from −60 to −10 mV during the voltage-clamp protocol (gray, control traces). B, inhibition curve performed by the coapplication of 20 μM ACh and increasing concentrations of serotonin. Cells were incubated with each concentration of serotonin for 2 min before the addition of ACh. Current values are plotted, expressed as the percentage of the control current evoked by ACh. The data shown are the mean ± S.E.M. of four experiments.

Tropisetron is known to interact with 5-HT$_3$ receptor-binding sites and to block agonist-evoked responses in a competitive manner (Maricq et al., 1991). To further characterize the mechanism underlying the blocking effect of this compound on the α9α10 nAChR, block by tropisetron was analyzed at increasing concentrations of ACh. As shown in Fig. 6C, tropisetron produced an almost parallel rightward shift of ACh-evoked currents. A significant increase in the ACh EC_{50} from 13.6 ± 2.9 ($n = 10$) to 31.1 ± 3.5 μM ($n = 10$; $p < 0.001$) was observed, with no changes in agonist maximal responses. This result is compatible with a competitive type of block. However, an increase in the Hill coefficient from 1.1 ± 0.1 to 1.8 ± 0.1 in the presence of tropisetron was observed ($p < 0.001$), suggesting that the underlying inhibitory mechanism could be more complex than just a competitive block. Schild plots were not constructed because high concentrations of ACh produced an increased desensitization of the receptor, resulting in nonreproducible responses.

Effect of Serotonin Type 3 Receptor Antagonists on ACh-Evoked Currents in Isolated Saccular Hair Cells. The native vestibular and cochlear hair cell cholinergic receptor and the recombinant α9α10 nAChR share similar pharmacological properties (for references, see Elgoyhen et al., 2001). To analyze whether this also holds true in the case of 5-HT$_3$ receptor antagonists, the effect of these compounds was studied on a native hair cell cholinergic receptor preparation, such as the isolated frog saccular hair cells.

Figure 7A shows representative traces to 20 μM ACh and block of these responses in the presence of either tropisetron, ondansetron, or MDL 72222. As shown previously for the α9α10 nAChR, block by antagonists was concentration-dependent (Fig. 7 B), with a rank order of potency of tropisetron (IC_{50}, 0.33 ± 0.01 μM, $n = 4$) > ondansetron (IC_{50}, 0.64 ± 0.14 μM, $n = 4$) = MDL 72222 (IC_{50}, 0.8 ± 0.2 μM, $n = 4$).

Effect of Tropisetron on α4β2 and α7 nAChRs. To analyze whether the block by tropisetron is selective for the α9α10 nAChR or is a general feature common to other nAChRs, the effect of this compound was studied on recombinant α4β2 and α7 nAChRs. Although α4β2 nAChRs were blocked by tropisetron (Fig. 8A), the IC_{50} value (5.9 ± 0.6 μM, $n = 5$) derived from the concentration-response curves, was 2 orders of magnitude higher than that required to block α9α10

nAChRs. On the other hand, tropisetron elicited inward currents in α7-injected oocytes, albeit with a maximal response that reached only 15.4 ± 1.1% of the maximum obtained with nicotine (Fig. 8B).

Discussion

The present study contributes to the pharmacological characterization of the α9α10 nAChR and indicates that this receptor shares striking properties with type 3 serotonin ligand-gated ion channels. Moreover, it demonstrates an in vivo correlate of these properties of the recombinant receptor, with those of the α9α10-containing nAChR of frog saccular hair cells. Finally, it poses the possibility that the biogenic amine serotonin might act as a neuromodulator of the cholinergic efferent transmission in the vestibular apparatus and in the organ of Corti.

The IC_{50} values found for tropisetron, ondansetron, and

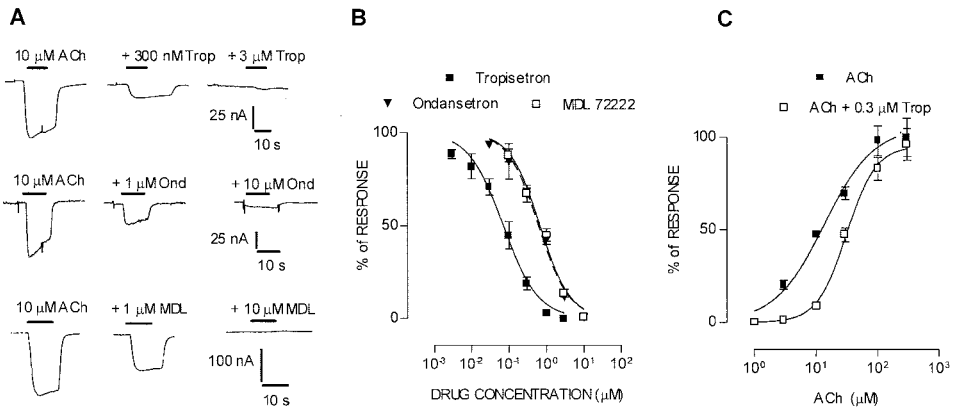

Fig. 6. Effect of serotonin type 3 receptor antagonists on ACh-evoked currents through recombinant α9α10 nAChRs. A, representative traces to 10 μM ACh, either alone or in the presence of tropisetron, ondansetron, or MDL 72222. B, inhibition curves performed by the coapplication of 10 μM ACh and increasing concentrations of the antagonists. Oocytes were incubated with each concentration of antagonists for 2 min before the addition of ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. C, concentration-response curves to ACh performed either alone or in the presence of 0.3 μM tropisetron. Peak current values were normalized and referred to the maximal peak response to ACh. The mean and S.E.M. of four to ten experiments per group are shown in B and C.

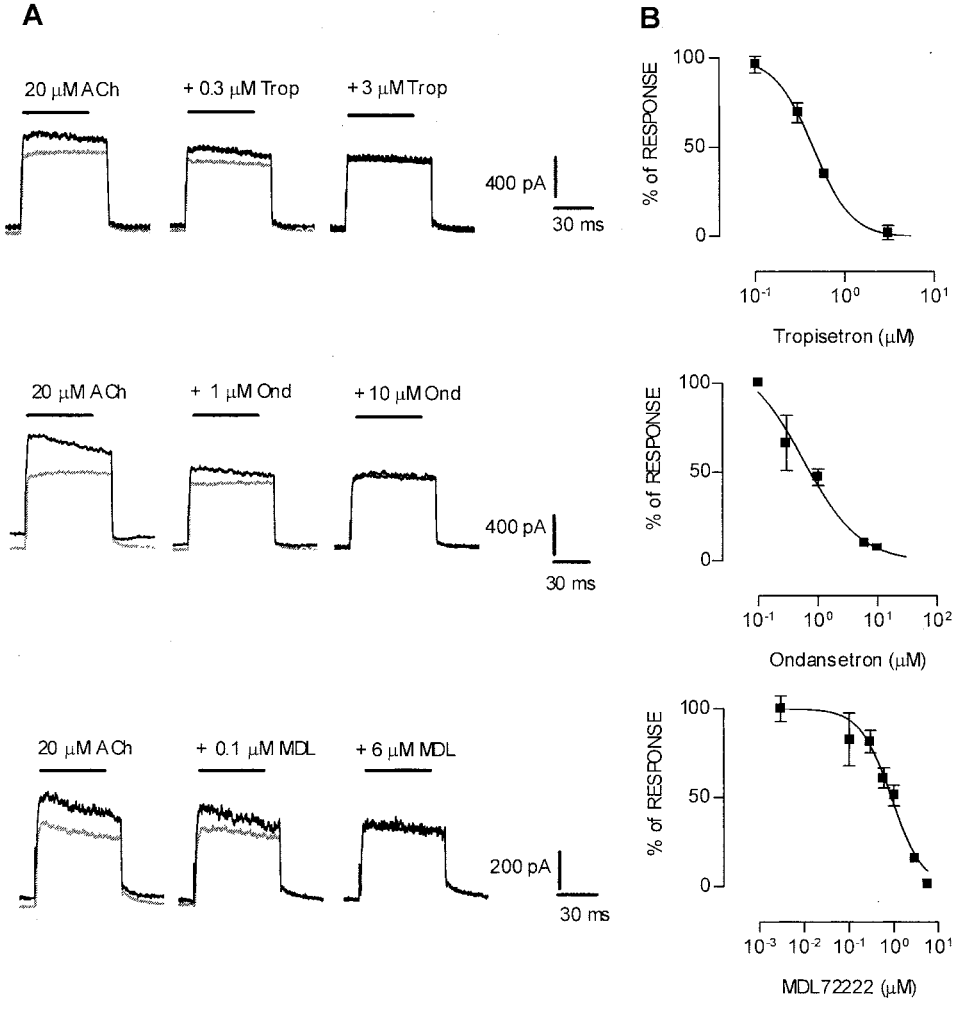

Fig. 7. Effect of serotonin type 3 receptor antagonists on ACh-evoked currents in isolated saccular hair cells. A, representative traces to 20 μM ACh, either alone or in the presence of tropisetron, ondansetron, or MDL 72222 (black traces). The cells were held at −60 mV and the command potential was stepped from −60 to −10 mV during the voltage-clamp protocol (gray, control traces). B, inhibition curves performed by the coapplication of 20 μM ACh and increasing concentrations of the antagonists. Cells were incubated with each concentration of antagonist for 2 min before the addition of ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean ± S.E.M of four to five experiments per antagonist concentration are shown.

MDL 72222 to block ACh-evoked currents on the $\alpha9\alpha10$ and on the native hair cell receptor are in the same order of magnitude as those required for both recombinant (Maricq et al., 1991; Dubin et al., 1999) and native 5-HT$_3$ receptors present in the guinea pig submucosal plexus and the rabbit heart (Vanner and Sruprenant, 1990; Turconi et al., 1991). Moreover, among all the compounds tested on the $\alpha9\alpha10$ nAChR (Elgoyhen et al., 2001; Sgard et al., 2002), the 5-HT$_3$ antagonist tropisetron/as well as the glycinergic antagonist strychnine and the nicotinic antagonist α-bungarotoxin, have the highest blocking potencies. Thus, the heteromeric $\alpha9\alpha10$ receptor has peculiar pharmacological properties within the nAChR family, and resembles what has been described previously for the homomeric $\alpha9$ recombinant receptor, which preserves pharmacological properties that are characteristic of other members of the Cys-loop family of ligand-gated ion channels (Rothlin et al., 1999). Moreover, this finding is in accordance with the fact that $\alpha9$ shares with $\alpha10$ the highest amino acid sequence identity, being more distantly related to other members of the nAChR family (Elgoyhen et al., 2001).

The binding of 5-HT$_3$ antagonists such as tropisetron to 5-HT$_3$ receptors is known to require several tryptophan residues in the extracellular amino terminus, probably involved in cation-π interactions with the positive amine in the tropane of these antagonists (Venkataraman et al., 1999; Yan et al., 1999; Spier and Lummis, 2000). Interestingly, in the $\alpha9$ and $\alpha10$ nAChR subunits, most residues aligned with the critical tryptophans are conserved. Thus the high-affinity site of interaction of tropisetron with the $\alpha9\alpha10$ nAChR could be extracellular and resemble its site on the 5-HT$_3$ receptor. This is supported by the finding that blockage of the $\alpha9\alpha10$ receptor by tropisetron is surmounted at high concentrations of ACh, compatible with a competitive type of block. Competitive antagonists of members of the Cys-loop family of ligand-gated ion channels are most probably binding with an extracellular site of the receptor (Karlin, 2002). Moreover, tropisetron turns to a high-affinity agonist of an $\alpha9\alpha10$ receptor bearing a leucine-to-threonine mutation at position L9′ within transmembrane region II (P. V. Plazas, E. Katz, and A. B. Elgoyhen, unpublished observations), further reinforcing the hypothesis of an extracellular amino-terminal binding site for this compound.

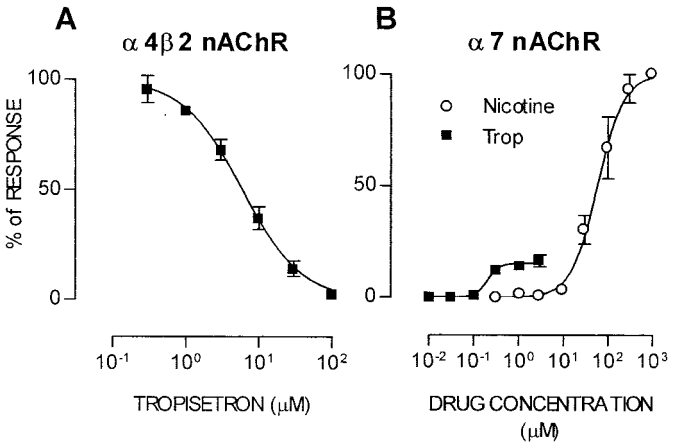

Fig. 8. Effect of tropisetron on $\alpha4\beta2$ and $\alpha7$ nAChRs. A, inhibition curve performed by the coapplication of 10 μM ACh and increasing concentrations of tropisetron to $\alpha4\beta2$-injected oocytes. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. B, concentration-response curves to tropisetron and nicotine in $\alpha7$-injected oocytes. Peak current values were normalized and referred to the maximal peak response to nicotine. The mean and S.E.M. of three to five experiments per group are shown.

Within the nAChR family, high-affinity (nanomolar) block by the 5HT$_3$ antagonist tropisetron seems to be a distinctive feature of $\alpha9$ (Rothlin et al., 1999) and $\alpha9\alpha10$ receptors. As derived from Fig. 8, micromolar concentrations of this antagonist are required to block $\alpha4\beta2$ receptors expressed in *X. laevis* oocytes. Moreover, affinity constants for tropisetron on $\alpha4\beta2$ and $\alpha1\beta1\gamma\delta$ derived from binding assays are all in the micromolar range (Macor et al., 2001). On the other hand, although tropisetron binds with high affinity to $\alpha7$ nAChRs (Macor et al., 2001), it behaves as a weak partial agonist of this receptor subtype (Fig. 8) (Macor et al., 2001).

From a pharmacological standpoint, ondansetron and tropisetron are widely prescribed drugs for chemotherapy-induced emesis (Hesketh, 2000). A myriad of signaling pathways lead from the periphery to the emetic center, including inputs from the vestibular apparatus, of special importance during motion sickness (Pasricha, 2001). Because of its high affinity for the $\alpha9\alpha10$ receptor, tropisetron will block the $\alpha9\alpha10$-containing vestibular cholinergic receptor at the concentrations used in therapeutics. This effect might (or might not) contribute to its antiemetic action, a question that will only be elucidated when a better understanding of the function of the efferent system to the vestibular apparatus is gained.

The observation that $\alpha9\alpha10$ and the native saccular hair cell receptors were blocked by serotonin at low micromolar concentrations and by an apparent noncompetitive mechanism was unexpected. Block of the closely related homomeric $\alpha9$ receptor by serotonin seems to be competitive and requires high micromolar to millimolar concentrations (IC_{50}, 251 μM; Rothlin et al., 1999), thus suggesting that serotonin probably binds to different sites within the $\alpha9$ and the $\alpha9\alpha10$ receptor proteins. Moreover, the fact that block of ACh-evoked currents through $\alpha9\alpha10$ was dependent upon variations of the membrane holding potential, being evident only at hyperpolarized potentials, might suggest that serotonin is acting at a site within the channel lumen. An interaction of serotonin with the ion channel lumen has been reported for other nAChRs (Arias, 1998). However, although serotonin also blocks neuronal $\alpha7$, $\alpha3\beta4$, $\alpha4\beta4$, $\alpha2\beta4$, and $\alpha3\beta2$ (Nakazawa et al., 1995; Palma et al., 1996), the apparent affinity for these receptor subtypes is 1 or 2 orders of magnitude lower than that reported here for the $\alpha9\alpha10$ nAChR. Therefore, within the nAChR family of receptors, the antagonist specificity of serotonin is highest for the $\alpha9\alpha10$ receptor.

Serotonin differs from other 5-HT$_3$ receptor agonists such as 5HTQ and 1-(*m*-chlorophenyl)-biguanide in that the latter behave as weak partial agonists of the recombinant $\alpha9\alpha10$ receptor. However, as observed in Fig. 4, the effect of 1-(*m*-chlorophenyl)-biguanide is more complex than just a partial agonism, because both potentiation and inhibition of ACh-evoked responses were observed, probably indicating more than one site of interaction of the compound with the receptor. As reported for the effect of atropine on $\alpha4\beta4$ nicotinic receptors and curare-like drugs on neuronal nicotinic receptors containing the $\beta4$ subunit, where both potentiation and blockage of ACh-evoked responses are observed (Cachelin and Rust, 1994; Zwart and Vivjerberg, 1997), potentiation might result from the interaction with the agonist binding

site of the receptor, where simultaneous occupation of the two binding sites, one agonist recognition site with ACh and the other agonist recognition site with 1-(*m*-chlorophenyl)-biguanide, leads to subsequent ion channel activation. On the other hand, blockage was noncompetitive and independent of the membrane holding potential, thus suggesting an interaction with a regulatory site independent of the ion channel pore. As for all experiments in which more than one receptor subunit is expressed, one cannot preclude the possibility of assembly of different receptors subtypes with different stoichiometries to account for the different effects of 1-(*m*-chlorophenyl)-biguanide. Further studies that go beyond the scope of the present work will be required to fully explain the complex effects of 1-(*m*-chlorophenyl)-biguanide on the $\alpha9\alpha10$ receptor.

Does the interaction of serotonin with the $\alpha9\alpha10$ recombinant and the saccular hair cell cholinergic receptor underlie a physiological implication? The function of a serotonergic innervation to the inner ear is unknown. Intracochlear injection of serotonin reduces the compound action potential of the auditory nerve (Bobbin and Thompson, 1978). Recent evidence suggests the presence of serotonin and its metabolite 5-hydroxyindole-3-acetic acid in the mammalian cochlea and vestibule (Gil-Loyzaga et al., 1997a,b, 2000) and subunit A of 5-HT_3 receptors has been identified by in situ hybridization in the cochlear and vestibular sensory epithelium of the developing rat (Johnson and Heinemann, 1995). In the case of the organ of Corti, serotonergic fibers reach the cochlea accompanying the olivocochlear efferent system. At the periphery, these fibers form glomerulus-like structures within the inner spiral bundle and could directly contact inner hair cells. They reach the Corti's tunnel and then follow a spiral distribution, branching at least on the first row of outer hair cells (Gil-Loyzaga et al., 2000). The fact that serotonergic fibers are present at the base of the outer hair cells, together with the finding that low micromolar concentrations of serotonin block ACh-evoked currents through the $\alpha9\alpha10$ receptor, opens the possibility that serotonin might function as a neuromodulator of the efferent cholinergic innervation to the inner ear.

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