The α9 Nicotinic Acetylcholine Receptor Shares Pharmacological Properties with Type A γ-Aminobutyric Acid, Glycine, and Type 3 Serotonin Receptors

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ABSTRACT

In the present study, we provide evidence that the α9 nicotinic acetylcholine receptor (nAChR) shares pharmacological properties with members of the Cys-loop family of receptors. Thus, the type A γ-aminobutyric acid receptor antagonist bicuculline, the glycine receptor antagonist strychnine, and the type 3 serotonin receptor antagonist ICS-205,930 block ACh-evoked currents in α9-injected Xenopus laevis oocytes with the following rank order of potency: strychnine > ICS-205,930 > bicuculline. Block by antagonists was reflected in an increase in the agonist maximal response or Hill coefficient, which suggests a competitive type of block. Moreover, whereas neither γ-aminobutyric acid nor glycine modified ACh-evoked currents, serotonin blocked responses to ACh in a concentration-dependent manner. The present results suggest that the α9 nAChR must conserve in its primary structure some residues responsible for ligand binding common to other Cys-loop receptors. In addition, it adds further evidence that the α9 nAChR and the cholinergic receptor present at the base of cochlear outer hair cells have similar pharmacological properties.

Nicotinic acetylcholine receptors (nAChRs) are complexes of protein subunits that coassemble to form an ion channel that is gated through the binding of the neurotransmitter acetylcholine (ACh) to its ligand-binding site (Changeux et al., 1987). 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 (α1)2β1γδ stoichiometry (Galzi et al., 1991). Ten genes that encode neuronal nAChR subunits have been identified in the vertebrate central or peripheral nervous system: α2 to α8, β2 to β4 (Sargent, 1993; McGehee and Role, 1995). In heterologous expression systems, the neuronal subunits α2, α3, α4, and α6 lead to the assembly of functional nAChR in combination with either β2 or β4. They 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 α7 and α8 subunits form part of a different group within the neuronal nAChR, because they can assemble into functional receptors in the absence of any other subunit and account for the α-bungarotoxin-binding sites in the central nervous system (Couturier et al., 1990; Gerzanich et al., 1994).

The cloning of the α9 subunit added a peculiar member to the family of nAChRs (Elgoyhen et al., 1994). It is a distant member of the family: whereas neuronal nAChR α subunits and the muscle α1 subunit share sequence homologies ranging from 48 to 70%, the sequence identity between α9 and all known nAChR subunits is less than 39%. When expressed in Xenopus laevis oocytes, α9 forms a homomeric receptor-channel complex that is activated by ACh but not by nicotinic; α9 also 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 α9 receptor are strikingly similar to those described for the cholinergic receptor that mediates synaptic transmission between efferent cholinergic fibers and cochlear outer hair cells (Housley and Ashmore, 1991; Fuchs and Murrow, 1992; Elgoyhen et al., 1994; Erostegui et al., 1994). Moreover, the α9 subunit gene exhibits a unique and restricted expression pattern. Whereas α9 message has not been found in the central nervous system, it is present in the cochlear and vestibular hair cells (Elgoyhen et al., 1994; Hiel et al., 1996; Morley et al., 1998). This has led to the proposal that the α9

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; Ach, acetylcholine; GABA, γ-aminobutyric acid; GABA_A, type A γ-aminobutyric acid receptor; 5-HT_3, type 3 serotonin receptor; BAPTA/AM, 2-bis(2-aminophenoxo)ethane-N,N,N',N''-tetraacetic acid/acetoxymethyl ester.
subunit is a component of the cholinergic receptor that is present at the base of the outer hair cells and therefore participates in the efferent modulation of the cochlear amplifier and the control of the dynamic range of hearing (Elgoyhen et al., 1994; Sewell, 1996).

The alkaloid strychnine, an established blocker of glycine-gated chloride channels, is one of the most potent antagonists described so far for both the recombinant α9 and the hair cell native receptors (Elgoyhen et al., 1994; Erostegui et al., 1994). Nicotinic AChRs as well as glycine receptors are members of a family of neurotransmitter-gated ion channels that also includes the type A γ-aminobutyric acid receptor (GABA<sub>α</sub>) and the type 3 serotonin receptor (5-HT<sub>3</sub>) (Karlin and Akabas, 1995). The subunits of these receptors have similar sequences and distributions of hydrophobic, membrane-spanning segments. Each subunit contains, in its ligand-binding, amino-terminal half, 2 cysteine residues separated by 13 other residues that are presumably disulfide-linked, thus giving this family the name of the Cys-loop receptors. Although at the level of detailed molecular mechanisms there do exist structural determinants that specify selectivity of ligand binding to each of these receptors, the potent strychnine block of the α9 nAChR indicates that some features are conserved between the α9 nACh and the glycine receptors.

The aim of the present work was to study, on the recombinant α9 receptor, the effect of selective drugs that interact with other members of the Cys-loop family. We report that the α9 nAChR shares several pharmacological properties with GABA<sub>α</sub>, 5-HT<sub>3</sub>, and glycine receptors.

### Experimental Procedures

**Expression in X. laevis Oocytes and Electrophysiological Procedures.** A full-length α9 rat cDNA constructed in the vector pGEMHE suitable for X. laevis oocyte expression studies was used as described previously (Elgoyhen et al., 1994). cRNA was synthesized using the mMessage mMachine T7 transcription kit (Ambion, Austin, TX), with plasmid linearized with NheI.

The isolation and maintenance of oocytes has been described previously (Boulter et al., 1987). Each oocyte was injected with 50 nl of RNase-free H2O containing 1 to 10 ng of cRNA. Electrophysiological recordings were performed 3 to 5 days after injection, under two-electrode voltage-clamp with either an Oocyte Clamp OC-725B amplifier (Warner Instruments, Hamden, CT) or a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). Both voltage and current electrodes were filled with 3 M KCl and had a resistance of ~1 MΩ. Unless otherwise stated, the holding potential was −50 mV. All records were digitized and stored on a PC-compatible computer. Data were analyzed using CLAMPFIT from the pCLAMP 6 software (Axon Instruments, Foster City, CA).

Oocytes were continuously superfused with frog saline (10 mM HEPES, pH 7.2, 115 mM NaCl, 1.8 mM CaCl<sub>2</sub>, and 2.5 mM KCl) at a rate of 10 ml/min. Drugs were applied along with the perfusion solution of the oocyte chamber. No responses were observed by the application of drugs to uninjected oocytes. Concentration-response curves were normalized to the maximal agonist response in each oocyte. For the inhibition curves, antagonists were coapplied with 10 μM ACh (EC<sub>50</sub>; Elgoyhen et al., 1994) and responses were referred to as a percentage of this value. Unless otherwise stated, data are presented as the mean ± S.E.M. of peak current responses of at least four oocytes per experiment. Curve fits and statistical analysis were performed on a PC. Agonist concentration-response curves were fitted with the equation \( I/I_{\text{max}} = A^+ + EC_{50}^{-n} \) in an iterated fashion,

**Fig. 1.** Effect of agonists of Cys-loop receptors on the α9 nAChR. Shown in A, B, and C are representative current responses to 10 μM ACh either alone or in the presence of GABA, glycine, or serotonin, respectively. D, inhibition curve performed by the coapplication of 10 μM ACh and increasing concentrations of serotonin. Only peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and S.E.M. of five experiments are shown.
where \( I \) is the peak inward current evoked by agonist at concentration \( A \), \( I_{\text{max}} \) is the maximal inward current evoked by a saturating concentration of agonist, \( EC_{50} \) is the concentration of agonist that induces half-maximal current response, and \( n \) is the Hill coefficient. An equation of the same form was used to analyze the concentration dependence of antagonist-induced blockade. 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 (\( n \)).

ACh EC\(_{50}\) displacements in the presence of antagonists were analyzed with a one-tailed Student’s \( t \) test. Multiple comparisons of IC\(_{50}\) values were performed with a one-way analysis of variance followed by Tukey’s test. A \( p \) value of < .05 was considered significant.

To preclude the interference of the endogenous oocyte Cl\(^-\) current, which is activated in response to the entrance of Ca\(^{2+}\) through the \( \alpha_9 \) receptor (Elgoyhen et al., 1994), a set of control experiments was performed in 1,2-bis(2-aminophenoxy)ethyl-N,N,N',N'-tetraacetate acid/acetoxymethyl ester (BAPTA/AM)-treated oocytes. Oocytes were incubated for 3 h in frog saline that contained 0.1 mM BAPTA/AM. This treatment has been shown previously to effectively chelate intracellular Ca\(^{2+}\) ions and, therefore, to impair the activation of the oocyte Cl\(^-\) current (Gerzanich et al., 1994). Under our conditions, the ability of BAPTA/AM to chelate intracellular Ca\(^{2+}\) was tested, eliciting Ca\(^{2+}\) entrance through voltage-dependent Ca\(^{2+}\) channels (de-polarizing voltage steps from \(-100 \text{ mV} \) to \(+20 \text{ mV} \)), as described by Boton et al. (1989). Transient outward currents disappeared after treatment with BAPTA/AM, even in frog saline solution containing 10 mM Ca\(^{2+}\). Another set of control experiments was done in frog saline solution containing 0.8 mM Ba\(^{2+}\) as the only divalent cation, because this ion does not activate the oocyte Cl\(^-\) current (Barish, 1983). Moreover, neither serotonin, bicuculline, strychnine, nor ICS-205,930 were able to block the Cl\(^-\) current in oocytes permeabilized with the ionophore A23187 and exposed to 1.8 mM Ca\(^{2+}\) (\( n = 3 \) per drug, data not shown), an experimental condition described previously by Boton et al. (1989).

Results

Interaction of GABAergic, Glycinergic, and Serotoninergic Drugs with the \( \alpha_9 \) nAChR. Voltage-clamped \( X. \) laevis oocytes injected with \( \alpha_9 \) cRNA responded to ACh with a fast peak current that rapidly decayed to a plateau level. Fig. 1 shows representative traces in the presence of 10 \( \mu \text{M} \) ACh, a concentration previously shown to correspond to the EC\(_{50}\) of the agonist (Elgoyhen et al., 1994). As expected for a nAChR, neither GABA, glycine, nor serotonin evoked inward currents in \( \alpha_9 \)-injected oocytes (Fig. 1). Moreover, neither GABA nor glycine modified responses to ACh, and traces obtained in the presence of these drugs did not differ from the control traces (Fig. 1, A and B). However, ACh-evoked currents were reduced by serotonin. As shown in Fig. 1, C and D, serotonin blocked both peak and plateau re-

![Fig. 2](https://molpharm.aspetjournals.org/atm/1994/264/issue/article-fig2.jpg)

**Fig. 2.** Effect of antagonists of Cys-loop receptors on the \( \alpha_9 \) nAChR. A, representative traces to 10 \( \mu \text{M} \) ACh either alone or in the presence of increasing concentrations of bicuculline, strychnine or ICS-205,930. B, inhibition curves performed by the coapplication of 10 \( \mu \text{M} \) ACh and increasing concentrations of antagonists. Only peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and S.E.M of three to five experiments per group are shown.
sponses to ACh in a concentration-dependent manner with an IC_{50} of 251 ± 30 μM.

Shown in Fig. 2 are the effects of antagonists of different members of the Cys-loop family of receptors on the α9 nAChR. As indicated in Fig. 2A, both peak and plateau responses to 10 μM ACh were reduced in the presence of the GABA_A antagonist bicuculline, the glycinegic antagonist strychnine, and the 5-HT_3 antagonist ICS-205,930. In all cases, the effect was concentration-dependent, with a rank order of potency of strychnine (IC_{50} 17.8 ± 0.9 nM, n = 4) > ICS-205,930 (IC_{50} 166 ± 6 nM, n = 3) > bicuculline (IC_{50} 768 ± 40 nM, n = 5). Block by these antagonists was reversible, because initial control responses to ACh were recovered after washes of the oocytes with frog saline (not shown).

**Mechanism of Block.** Serotonin interacts with the binding site of 5-HT_3 receptors and gates channel opening (Maricq et al., 1991). On the other hand, bicuculline, ICS-205,930, and strychnine are known to interact with GABA_A, 5-HT_3, and the glycine receptor-binding sites, respectively, and to block agonist-evoked responses in a competitive manner (Akaïke et al., 1987; Maricq et al., 1991; Schmieden et al., 1992). To further characterize the mechanism underlying the blocking effects on the α9 nAChR, block by drugs was studied at increasing concentrations of the agonist. The concentrations of antagonists tested were the ones that corresponded to the IC_{50} values derived from Figs. 1D and 2B. As shown in Fig. 3, 1 μM bicuculline, 20 nM strychnine, and 300 μM serotonin produced a parallel rightward shift of ACh-evoked currents. A significant increase of the ACh EC_{50} values was observed, with no changes in agonist maximal responses and Hill coefficients (Table 1), which suggests a competitive type of block.

**Block of the α9 nAChR in BAPTA/AM-Treated Oocytes.** In α9-injected oocytes, part of the ACh-evoked response is carried by a Ca^{2+}-activated Cl⁻ current (Elgoyhen et al., 1994). To analyze whether the effect described is a direct block on the α9 receptor or not a nonspecific block of the oocyte Cl⁻ channel, the effect of drugs was studied in oocytes that had been treated with the fast Ca^{2+} chelator BAPTA/AM. The effectiveness of the treatment with BAPTA/AM was assessed as described in Experimental Procedures. Antagonists were applied at plateau responses achieved with two different ACh concentrations: a low, nonsaturating one (10 μM) and a saturating maximal concentration (300 μM) (Fig. 4). Responses to 10 μM ACh were blocked 82 ± 5% (n = 3), 40 ± 7% (n = 3), and 51 ± 8% (n = 3) in the presence of 1 μM bicuculline, 20 nM strychnine, and 300 μM serotonin, respectively. The blocking effect was drastically reduced or abolished when the ACh concentration was raised to 300 μM. This result suggests again that the block by the drugs tested is competitive and that the observed effect is a direct block on the α9 receptor.

**Discussion**

The present study contributes to the pharmacological characterization of the newly cloned α9 nAChR and indicates that this receptor shares striking properties with other members of the Cys-loop family of receptors. Thus, the recombinant α9 nAChR is blocked by GABA_A, 5-HT_3, and glycine receptor antagonists. The IC_{50} values found for bicuculline and strychnine block of α9, 0.8 μM and 0.02 μM, respectively, are similar to those reported for GABA_A (0.9 μM; Sigel et al., 1992) and glycine receptors (0.05 μM; Schmieden et al., 1992) expressed in X. laevis oocytes. In addition, the nanomolar potency of ICS-205,930 to block ACh-evoked currents in α9-injected oocytes is in the same order of magnitude as that required for both recombinant (Maricq et al., 1991) and native 5-HT_3 receptors present in the guinea pig submucosal plexus and rabbit heart (Vanner and Suprenant, 1990; Turconi et al., 1991). More-

![Fig. 3. Displacements of ACh concentration-response curves.](image-url)
over, among all of the nicotinic antagonists tested on α9-injected oocytes, only α-bungarotoxin and κ-bungarotoxin have high blocking potencies that are comparable to those of strychnine and ICS-205,930 (Elgoyhen et al., 1994; Johnson et al., 1995). Nicotinic drugs such as d-tubocurarine, mecamylamine, and dihydro-β-erythroidine have IC50 values in the micromolar range (Elgoyhen et al., 1994; unpublished observations). Taken together, these results indicate that, based on its pharmacological properties, the α9 subunit is an unusual member of the nAChR family. It is activated by ACh (although not by nicotine; Elgoyhen et al., 1994); therefore, it should be considered to be in the cholinergic family of ionotropic receptors. However, the profile of block by antagonists does not allow the inclusion of the α9 subunit in any specific Cys-loop subfamily of receptors. The present observations are in accordance with the finding that the comparison of sequence similarities and gene structure indicates that α9 is the most distant member within the nAChR family. Some amino acid residues that are conserved along all members of the gene family have a nonconservative substitution in the α9 primary structure, which might contribute to the unique properties of this receptor (Elgoyhen et al., 1994).

The blockage of the α9 nAChR by serotonin resembles what has been described for other nAChRs. The function of native and recombinant nAChRs can be modified by this neurotransmitter (García-Colunga and Miledi, 1995; Palma et al., 1996). Thus, α7 nAChRs expressed in X. laevis oocytes are blocked by micromolar concentrations of serotonin (Palma et al., 1996). In contrast to that found for the α9 nAChR, the block of the α7 receptor is noncompetitive, which suggests different underlying modes of action. Sensitivity to bicuculline has been reported for nAChRs present in isolated pig pituitary intermediate lobe cells and cultured embryonic rat skeletal muscle (Zhang and Feltz, 1991; Liu et al., 1994). However, the IC50 values reported in those preparations are 1 to 2 log units higher than those found for bicuculline block of both α9 and GABA_A (present results; Sigel et al., 1992). Moreover, although α7 nAChRs are also blocked by strychnine (Gerzanich et al., 1994), the potency of this antagonist on α7 receptors is 2 orders of magnitude lower than that reported for both α9 (present observations) and glycine receptors (Schmieden et al., 1992). Therefore, our results indicate that among the nAChR gene family, it is only α9 that most closely resembles other members of the Cys-loop family.

Members of the Cys-loop family of receptors include both cationic, 5-HT3, and nACh, as well as anionic, glycine, and GABA_A receptors. They all have a high degree of amino acid sequence similarity and some highly characteristic sequence motifs, both in the binding, extracellular, amino-terminal domain, and in the four hydrophobic, putative transmembrane regions (Karlin and Akabas, 1995). They all share a common evolutionary ancestor, and within the cationic branch, the homo-oligomeric receptors are probably the most primitive of all receptors because they conserve the closest similarity with the hypothetical ancestor (Le Novere and Changeux, 1995; Ortells and Lunt, 1995). When expressed in X. laevis oocytes, the α9 nAChR forms homo-oligomeric receptors, as well as continuing to conserve pharmacological properties typical of each of the subfamilies, which suggests that it is a primitive member of the Cys-loop family and that it had a very early evolutionary split. In support of this

![Fig. 4](https://molpharm.aspetjournals.org/)

**Fig. 4.** Inhibition of ACh-evoked currents in BAPTA/AM-treated oocytes. BAPTA/AM-treated oocytes were incubated with the ester for 3 h before experiments and oocytes were voltage-clamped at –70 mV. Shown are representative traces of three experiments per group obtained at two different ACh concentrations: 10 μM in A and 300 μM in B. Either bicuculline, strychnine, or serotonin were applied at steady-state responses to ACh.
hypothesis is the observation that nAChRs in organisms that appeared before mammals in evolution, such as the nematode *Ascaris suum*, the marine snail *Aplysia* sp. and the insect *Schistocerca* sp., are sensitive to both strychnine and bicuculline block (Ono and Salvaterra, 1981; Marshall et al., 1990; Walker et al., 1992).

The simplest interpretation of the competitive type of block as suggested here for bicuculline, strychnine, and serotonin on the α9 nAChR is that these compounds share at least part of the binding pocket with the agonist, in such a way that occupancy of the site is mutually exclusive. Within the Cys-loop family of receptors, the amino-terminal extracellular domain is known to form the binding site. In the most thoroughly characterized member of this class of receptors, the nAChR, several residues in the extracellular domain of the α subunit have been identified as forming part of the agonist- and antagonist-binding sites using photoaffinity labeling and site-directed mutagenesis (Galzi et al., 1991; Karlin and Akabas, 1995). The fact that the α9 nAChR shares pharmacological properties with GABA<sub>α</sub>, 5-HT<sub>3</sub>, and strychnine receptors indicates that it must conserve in its primary structure some residues that are common to each of these receptors and that are responsible for agonist and antagonist binding. Asp-148, Tyr-161, and Tyr-202, known as determinants in the strychnine-binding site of the glycine receptor, are conserved in the α9 nAChR (Vandenberg et al., 1992a, 1993; Elgoyhen et al., 1994). Thus, the binding sites for antagonists on the glycine receptor and the α9 nAChR would be conserved and would form a similar tertiary structure, leading to a common mechanism of antagonism in these receptors. Glycine and GABA are simple molecules, and the number of specific interactions that they can achieve with their respective receptors is limited. Removal of one such interaction would be expected to result in a dramatic reduction in the affinity of agonists, but not antagonists, for the receptor (Vandenbergh et al., 1992b).

This might explain the fact that although both bicuculline and strychnine block the α9 nAChR, neither GABA nor glycine modify ACh-evoked currents or elicit responses in α9-injected oocytes. Thr-204, shown to be important for glycine-binding but not strychnine-binding to its receptor, is not conserved in the α9 nAChR (Vandenbergh et al., 1992b; Elgoyhen et al., 1994).

Although not typical of what has been described for an nAChR, the bicuculline and strychnine block of the recombinant α9 nAChR resembles what has been shown for the native cholinergic receptor present in outer hair cells. Thus, nanomolar concentrations of strychnine and micromolar concentrations of bicuculline block ACh-evoked currents in both isolated guinea pig outer hair cells (Erostegui et al., 1994) and α9 injected oocytes. As suggested previously (Elgoyhen et al., 1994), these findings add further data to support the hypothesis that the α9 nAChR is a component of the cholinergic receptor present at the base of the outer hair cells, responsible for the efferent modulation of the cochlear amplifier.

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**Fig. 5.** Inhibition of ACh-evoked currents in Ba<sup>2+</sup> frog saline. Oocytes were voltage-clamped at −90 mV. Shown are representative traces of three to four experiments per group obtained at two different ACh concentrations: 10 μM in A and 300 μM in B. Either bicuculline, strychnine, or serotonin were applied at steady-state responses to ACh.
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