Functional expression and properties of a nicotinic α9/5-HT3$_A$ chimeric receptor

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We describe the functional properties of a nicotinic α9/serotonin subtype 3A (5HT3$_A$) chimeric receptor expressed in *Xenopus laevis* oocytes. The chimera preserved ligand-binding properties of α9 and channel properties of 5HT3$_A$. Thus, it responded to acetylcholine in a concentration-dependent manner with an EC$_{50}$ of 70 μM but not to serotonin. It was blocked by methyllycaconitine, strychnine, atropine and nicotine, with the same rank order of potency as α9 receptors. The current–voltage relationship of currents through the α9/5HT3$_A$ chimera was similar to that of the 5HT3$_A$ receptors. These results are an evidence of functional coupling between the ligand-binding and the channel domains of the chimeric receptor. *NeuroReport* 14:1931–1934 © 2003 Lippincott Williams & Wilkins.

Key words: Hair cells; Ion channels; Nicotinic receptors; Serotonin receptors

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are complexes of protein subunits which co-assemble to form an ion channel gated through the binding of the neurotransmitter acetylcholine (ACh) to its ligand-binding site [1]. A diversity of gene subunits have been cloned in recent years. The α9 and α10 subunits are distant members of the nAChR family. When expressed in *Xenopus laevis* oocytes they form either α9 homomeric or α9α10 heteromeric receptor channel complexes that display a very distinct pharmacological profile falling into neither the nicotinic nor the muscarinic subdivision of the pharmacological classification scheme of cholinergic receptors [2–5]. The α9 and α10 genes exhibit a unique and restricted expression pattern which includes the hair cells of the mammalian cochlea [2,5]. A receptor composed of both α9 and α10 subunits has been proposed to mediate synaptic transmission between efferent cholinergic olivocochlear fibers and sensory auditory hair cells [5].

The α9 and α10 genes are not always coordinately expressed. A comparison of expression patterns for the α9 and α10 subunit genes has revealed that cells in several adult rat tissues transcribe α9, but not the α10 subunit: these include adult cochlear inner hair cells, nasal epithelium, sternohyoid muscle, and base of the tongue [2,5]. In the absence of uncharacterized nAChR subunits, nicotinic cholinergic function in these cells may be subserved, at least in part, by homomeric α9 nAChRs. Therefore, the properties of homomeric α9 receptors is still a matter of interest.

In spite of the fact that when expressed in *X. laevis* oocytes α9 homomeric and α9α10 heteromeric receptors exhibit a quite similar pharmacological profile, macroscopic currents recorded from α9α10 injected oocytes are 10–100 times bigger than those recorded from oocytes injected with α9 cRNA [5]. This might result from differences in efficiency in subunit folding and assembly of membrane receptors and/or coupling between agonist binding and channel opening. Moreover, ACh fails to evoke currents in α9 or α9α10-transfected mammalian cell lines, suggesting lack of expression of these receptors in a variety of recombinant systems (Katz, Elgoyhen and Bouzat, unpublished observations). It has been demonstrated that the inefficient folding and cell-surface expression of several nAChR subunits can be enhanced by replacing the C-terminal region of nAChR subunits with the corresponding region of the subtype 3A serotonin receptor (5HT3$_A$) subunit [6,7]. Although responses to ACh of the α7/5HT3$_A$ chimera are not bigger than those reported for the α7 receptor when expressed in *Xenopus* oocytes [6], this heterologous system can be used to rapidly screen for functional chimeras, which can be subsequently expressed in mammalian cell lines. Chimeric nicotinic/5HT3$_A$ receptors have been extensively used in order to identify molecular determinants underlying the pharmacological diversity of nAChRs both in cell lines and *X. laevis* expression systems [7–12].

In the present study we describe the functional properties of a nicotinic α9/5HT3$_A$ chimeric receptor expressed in *X. laevis* oocytes. It preserves ligand-binding properties of α9 and channel properties of 5HT3$_A$.

MATERIALS AND METHODS

A full-length α9 rat cDNA constructed in the vector pGEMHE was used as described previously [5]. A rat 5HT3$_A$ cDNA was kindly provided by Dr D. Johnson and S. Heinemann (The Salk Institute for Biological Studies, La Jolla, CA). The chimeric α9/5HT3 receptor was obtained by three consecutive PCRs in order to obtain a construct comprising the N-terminus of the α9 receptor and the C-terminus of the 5HT3$_A$ receptor. Leu209 (α9 numbering, equivalent to Val201 of the α7-5HT3), at the beginning of the putative MI transmembrane domain, was selected as the junction point. The construct was subcloned into the pGEMHE vector and sequenced. cRNA was synthesized using the mMessage mMachine T7 transcription kit (Ambion, Austin, TX).

Experimental procedures for oocyte maintenance and electrophysiological recordings under two-electrode voltage-clamp were as described previously [2,3]. During electrophysiological recordings, oocytes were continuously superfused (~10 ml/min) with normal frog saline comprising (mM): 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES buffer, pH 7.2. In order to prevent the activation of the native oocyte's Ca^{2+}-sensitive chloride current, I_{ClCa}, by Ca^{2+} entering through the α9 receptor [2], oocytes were incubated with the membrane permeant Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoximethyl ester (BAPTA-AM, 100 μM) for 3 h prior to electrophysiological recordings when indicated. Under our experimental conditions, the effectiveness of this treatment to buffer rises in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was tested by eliciting Ca^{2+} entrance through voltage-gated Ca^{2+} channels (depolarizing steps from −100 to +20 mV). Transient outward currents disappeared after treatment with BAPTA-AM even in frog saline solutions containing 10 mM Ca^{2+}, indicating that the rise in $[Ca^{2+}]_i$ after Ca^{2+} entry through voltage-gated Ca^{2+} channels was buffered and, therefore, was unable to activate the transient I_{ClCa} (data not shown). Current–voltage (I/V) curves were derived from peak current values at different holding potentials. All other experiments were performed at a holding potential of −70 mV. Concentration–response curves were normalized to the maximal agonist peak response in each oocyte. For the inhibition curves, antagonists were co-applied with 100 μM ACh and responses were referred to as a percentage of this value. The mean ± s.e.m. of peak current responses of ≥ 3–5 oocytes/experiment are represented. Agonist concentration–response and inhibition curves were iteratively fitted as described previously [3].

Multiple comparisons of IC_{50} values were performed with a one-way ANOVA followed by Tukey's test. $p < 0.05$ was considered significant. Drugs purchased from either Sigma Chemical Co. (St. Louis, MO) or RBI (Natik, MA), were dissolved in distilled water as 10 mM stocks and stored aliquoted at −20°C.

RESULTS

Experimental data obtained with a chimeric α7/5HT3$_A$ receptor have been intepreted as suggesting that members of the family of Cys-loop receptors share a highly conserved architectural framework designed to mediate signal transduction [6]. Following this model, a chimeric receptor comprising the N-terminal region of the nicotinic α9 subunit and the C-terminal part (comprising all transmembrane domains) of the 5HT3$_A$ receptor subunit, should preserve the ligand binding properties of the α9 nAChR. As predicted, when expressed in *X. laevis* oocytes, the α9/5HT3$_A$ receptor responded to ACh (Fig. 1). Figure 1b shows representative traces of α9 and α9/5HT3$_A$ receptors to ACh (10 and 100 μM, respectively) and of 5HT3$_A$ receptors

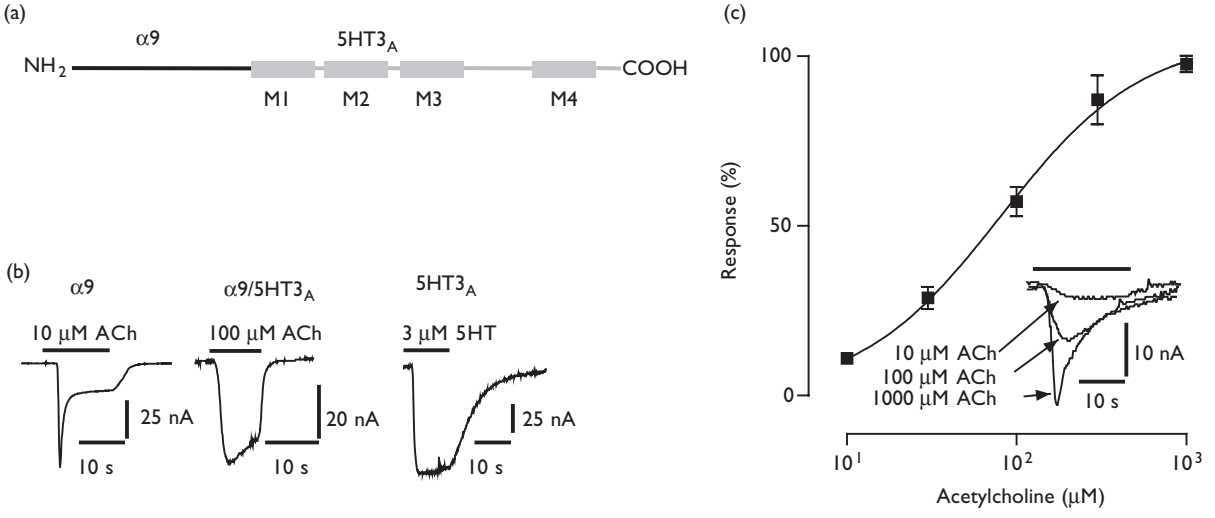

Fig. 1. (**a**) Schematic representation of the α9/5HT3$_A$ chimera. (**b**) Representative traces of α9 and α9/5HT3$_A$ to ACh, and of 5HT3$_A$ receptors to serotonin. (**c**) Concentration–response curves to ACh. Peak current values were normalized and referred to the maximal peak response to ACh. The mean and s.e.m. of three experiments per group are shown. The inset shows representative traces to 10, 100 μM and 1 mM ACh.

to 3 μM serotonin. The concentrations used are near the EC_{50} values for each receptor under our experimental conditions, (present results and [2,13]). As described previously [2], ACh-evoked responses through α9 receptors displayed a fast peak that rapidly decayed to a plateau level. This fast peak represents the activation of a Cl^- current in response to elevation of intracellular Ca^{2+} due to Ca^{2+} entry through the highly Ca^{2+} permeable α9 receptor [14]. ACh-evoked responses in α9/5HT3$_A$ injected oocytes lacked this fast peak component, as did responses evoked by serotonin in 5HT3$_A$ receptors (Fig. 1b). Responses of α9/5HT3$_A$ receptors were not modified after the incubation of the oocytes for 3 h in the presence of the fast Ca^{2+} chelator BAPTA-AM (100 μM, data not shown), indicating a very low or negligible Ca^{2+} permeability. As shown in Fig. 1c, responses to ACh of the α9/5HT3$_A$ receptor were concentration-dependent with an EC_{50} of 70 ± 6.2 μM and Hill coefficient of 1.2 ± 0.1 ($n = 5$). Current amplitudes to 100 μM ACh were usually smaller than those obtained with the wild type α9 receptor and ranged from 11 to 49 nA ($n = 21$). On the other hand, as expected for a receptor whose binding site corresponds to that of a nAChR, the chimeric receptor did not respond to serotonin ($n = 3$).

Methyllycaconitine, a drug selective for brain α-bungarotoxin binding sites [15,16], is a potent competitive blocker of the α9 receptor with an IC_{50} of 1 nM [3]. As shown in Fig. 2, MLA also potently blocked the α9/5HT3$_A$ chimera with an IC_{50} of 9 ± 1 nM and a Hill coefficient of 1.7 ± 0.6 ($n = 3$).

A characteristic feature of the recombinant nicotinic α9 receptor is that when expressed in *X. laevis* oocytes it is not activated by nicotine. Moreover, nicotine is a competitive antagonist of this receptor [2,3]. In addition, α9 is also blocked by the muscarinic cholinergic antagonist atropine, and by the glycine receptor antagonist strychnine [2–4]. As shown in Fig. 3 the α9/5HT3$_A$ chimera preserved this unusual pharmacological profile. Thus, it was blocked by these three compounds with a similar rank order of potency,

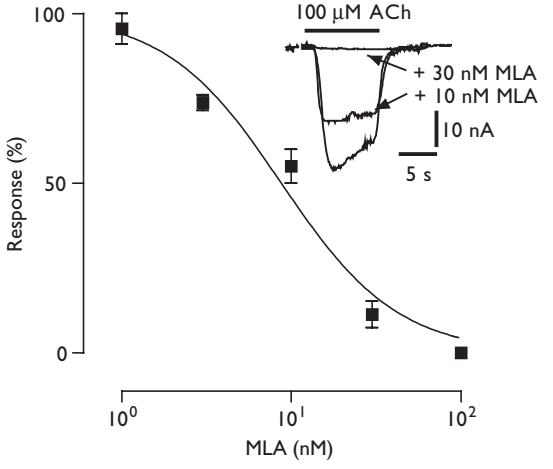

Fig. 2. Inhibition curves performed in the presence of increasing concentrations of MLA. MLA was co-applied with 100 μM ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and s.e.m. of three experiments per point are shown. The inset shows representative traces to 100 μM ACh, either alone or in the presence of 10 and 30 nM MLA.

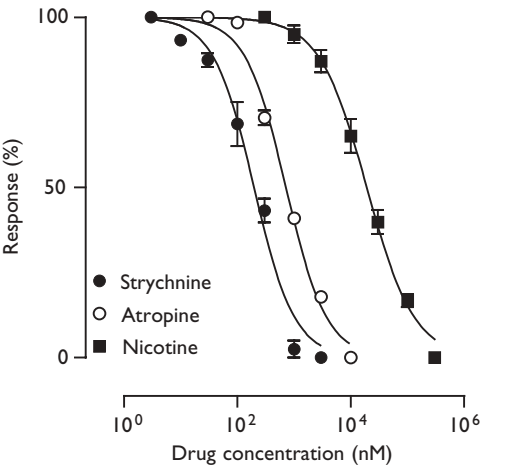

Fig. 3. Block of the α9/5HT3$_A$ chimera by strychnine, atropine and nicotine. Inhibition curves were performed in the presence of increasing concentrations of the antagonists co-applied with 100 μM ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and s.e.m. of five experiments per point are shown.

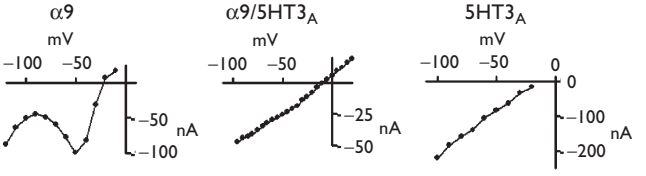

Fig. 4. Current–voltage relationships derived from peak currents at different holding potentials. Reversal potentials for all three receptors were approximately −15 mV. Representative traces of three experiments per receptor are shown.

strychnine > atropine > nicotine, as described previously for the α9 receptor. The IC_{50} values and Hill coefficients were, respectively, 0.22 ± 0.04 μM and 1.2 ± 0.1; 0.63 ± 0.03 μM and 1.2 ± 0.1; 19 ± 3 μM and 1.0 ± 0.1.

As expected for a molecule that contains the C-terminus of the 5HT3$_A$ receptor, the I–V curve for the chimeric receptor was almost linear (Fig. 4). This differs from the characteristic I–V curve described for the α9 nAChR [2] and resembles that described for the 5-HT3$_A$ and the α7-5HT3$_A$ receptors [6].

DISCUSSION

In the present study we report the construction of a functional chimeric receptor composed of the N-terminus of the nicotinic α9 subunit and the C-terminus of the serotonin type A receptor subunit. When expressed in *X. laevis* oocytes this chimeric receptor is functional and exhibits the unusual pharmacological properties which are a signature of the recombinant α9 receptor, but channel properties of the 5-HT3$_A$. Thus, the α9/5HT3$_A$ chimeric receptor was blocked by MLA, nicotine, atropine and strychnine, whereas it exhibited the current-voltage relationship of the 5-HT3$_A$ receptor. Moreover, currents through the chimeric receptor lacked the fast peak component subserved

by a Ca^{2+}-activated Cl^- conductance, typical of the ACh-evoked responses through α9 receptors [2]. This probably results from the fact that the chimera contains the channel domains of the $5HT3_A$ receptor and that the relative Ca^{2+} to monovalent permeability of these receptors is at least nine times lower than that of the nicotinic α9 receptor [14,17]. This is further supported by the observation that the ACh-evoked responses through the α9/$5HT3_A$ chimera were not modified by the fast Ca^{2+} chelator BAPTA-AM, thus precluding a major contribution of a Ca^{2+}-activated Cl^- conductance. Taken together, the present results demonstrate that, as reported for the α7/$5HT3_A$ chimera [6], functional properties that are expected to be determined by the extracellular domain on the one hand, or by the channel domain on the other, are found to be associated with the corresponding protein segment in the chimera. In addition, the successful coupling of the neurotransmitter binding site of one receptor to the ion channel of the other, further supports the notion of the presence of two independent protein domains with functional specificity, which behave as elementary units within the receptor-channel complex [6].

The fact that the introduction of the C-terminus of the $5HT3_A$ to the nicotinic α9 receptor did not enhance the magnitude of the ACh-evoked responses in *X. laevis* oocytes was not totally unexpected. Responses to ACh of the α7/$5HT3_A$ chimera are not bigger than those reported for the α7 receptor when expressed in *Xenopus* oocytes [6]. Differences in current amplitude between α9 homomeric receptors and α9α10 heteromeric receptors in *Xenopus* oocytes probably do not derive from an inefficient surface expression of α9 receptors in this heterologous system, since binding studies with α-bungarotoxin show similar expression levels [18]. Thus, α9-subunits are probably properly synthesized by the oocyte machinery and correctly inserted in the plasma membrane where they form high-affinity α-bungarotoxin binding sites.

Inefficient folding and/or membrane surface expression of several neuronal nAChRs has been reported for a variety of host cells [10,11,19]. For example, although when expressed in *X. laevis* oocytes the α7 receptors respond to ACh with robust inward currents [20], most attempts to reconstitute α7 nAChRs in cell lines have failed [11]. In the case of the α7 and α8 nAChRs, this has been overcome by the construction of the α7 and α8/$5HT3_A$ chimeras, suggesting that structural determinants within the transmembrane domains and/or the intracellular loop of the α7 and α8 proteins are in part responsible for the observed phenomena. Acetylcholine fails to evoke currents in α9 or α9α10-transfected mammalian cell lines, suggesting lack of expression of these receptors in a variety of recombinant systems (Katz, Elgoyhen and Bouzat, unpublished observations). The finding that α9/$5HT3_A$ responds to ACh in *X. laevis* oocytes, indicates functional coupling between the ligand-binding and the channel domains of the chimeric receptor. This observation, together with the fact that the chimera now bears the structural determinants from the $5HT3_A$ receptor required for efficient folding and/or enhanced membrane surface expression of neuronal nicotinic α-bungarotoxin binding receptors (α7 and α8) in cell lines, opens the future possibility of expressing a functional α9/$5HT3_A$ chimera in a variety of heterologous systems including mammalian cell lines.

CONCLUSIONS

We present experimental evidence of functional coupling between the ligand-binding and the channel domains of a α9/$5HT3_A$ chimeric receptor when expressed in *X. laevis* oocytes. This indicates the presence of two independent protein domains with functional specificity, which behave as elementary units within the receptor-channel complex. The eventual possibility of expressing the α9/$5HT3_A$ chimera in a variety of cell lines would facilitate further approaches in the pharmacological characterization of α9-containing nAChRs.

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