0026-895X/04/6502-453–460$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:453–460, 2004

Vol. 65, No. 2
2811/1127570
Printed in U.S.A.

Pharmacological Properties of $\alpha9\alpha10$ Nicotinic Acetylcholine Receptors Revealed by Heterologous Expression of Subunit Chimeras

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Received July 10, 2003; accepted November 12, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The nicotinic acetylcholine receptor (nAChR) $\alpha9$ and $\alpha10$ subunits are expressed primarily within hair cells of the inner ear and have been implicated in auditory processing. Although functional recombinant nAChRs generated by the coexpression of $\alpha9$ and $\alpha10$ in *Xenopus laevis* oocytes have been described previously, there have been no reports of the successful heterologous expression of $\alpha9\alpha10$ nAChRs in cultured cell lines. In this study, subunit chimeras ($\alpha9\chi$ and $\alpha10\chi$) have been constructed that contain the extracellular, ligand binding domain of the $\alpha9$ or $\alpha10$ subunits fused to the C-terminal domain of the 5-hydroxytryptamine type 3A ($5HT_{3A}$) subunit. Specific high-affinity binding of the nicotinic radioligand [^{3}H]methyllycaconitine was detected in membrane preparations of mammalian cells transfected with $\alpha9\chi$ or $\alpha10\chi$ alone, but significantly higher levels of binding were detected when $\alpha9\chi$ and $\alpha10\chi$ were cotransfected, providing evidence of a requirement for coassembly of $\alpha9$ and $\alpha10$ for the efficient formation of a nicotinic binding site. The pharmacological profile of $\alpha9\chi\alpha10\chi$ receptors, determined by equilibrium radioligand binding studies, is broadly similar to that determined previously by electrophysiological studies conducted with native and recombinant $\alpha9\alpha10$ nAChRs. In agreement with evidence that $\alpha9\alpha10$ nAChRs exhibit an atypical pharmacological profile, we have identified specific high-affinity binding of several non-nicotinic ligands including strychnine (a glycine receptor antagonist), bicuculline (a $GABA_A$ receptor antagonist), and atropine (a muscarinic acetylcholine receptor antagonist). Results have also been compared with radioligand binding data conducted with a previously described $\alpha7/5HT_{3A}$ ($\alpha7\chi$) subunit chimera.

Nicotinic acetylcholine receptors (nAChRs) are a heterogeneous family of pentameric neurotransmitter-gated ion channels assembled from an extensive group of transmembrane subunits. It is common for vertebrate nAChRs to be classified as either "muscle-type" or "neuronal" depending on whether they are expressed at the neuromuscular junction or within the central or peripheral nervous system (Lukas et al., 1999). Five muscle-type subunits ($\alpha1$, $\beta1$, γ, δ, and ϵ) and 10 neuronal subunits ($\alpha2$–$\alpha8$ and $\beta2$–$\beta4$) have been identified. More recently, two additional vertebrate nAChR subunits ($\alpha9$ and $\alpha10$) have been identified by molecular cloning (Elgoyhen et al., 1994, 2001; Lustig et al., 2001). Expression of the $\alpha9$ and $\alpha10$ subunits is largely restricted to the hair cells of the inner ear (Elgoyhen et al., 1994, 2001; Hiel et al., 1996; Lustig et al., 2001); consequently, nAChRs containing $\alpha9$ and $\alpha10$ do not fall conveniently into the conventional classification of either muscle-type or neuronal nAChRs.

Nicotinic receptors expressed in outer hair cells of the cochlea have been demonstrated to be responsible for modulating auditory nerve responses to acoustic stimulation and protection from acoustic overstimulation (Sridhar et al., 1997). A role for $\alpha9$-containing nAChRs in auditory processing has been suggested by in vivo studies conducted with transgenic (Vetter et al., 1999) and wild-type animals (Luebke and Foster, 2002).

When expressed alone in *Xenopus laevis* oocytes, the $\alpha9$ subunit, but not the $\alpha10$ subunit, is capable of generating functional homomeric nAChRs (Elgoyhen et al., 1994, 2001; Sgard et al., 2002). Significantly, when $\alpha9$ and $\alpha10$ are coexpressed in oocytes, considerably larger whole-cell currents are observed, suggesting that these subunits may normally coassemble to form heteromeric complexes (Elgoyhen et al., 2001; Sgard et al., 2002). Despite successful studies in oocytes, there have been no reports of the heterologous expression of $\alpha9$- or $\alpha10$-containing nAChRs, as either homomeric or heteromeric complexes, in other expression systems. Although much can be achieved by characterization of oocyte-expressed nAChRs, some approaches to the pharmacological characterization of $\alpha9\alpha10$ nAChRs (e.g., equilibrium binding

This work was supported by grants from the Wellcome Trust.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; MLA, methyllycaconitine; ACh, acetylcholine; αBTX, α-bungarotoxin.

studies) have been hindered by the lack of a suitable cultured cell-based expression system. Difficulties have also been reported in the heterologous expression of other nAChR subunits (in particular the $\alpha7$ and $\alpha8$ subunits) in cultured mammalian cell lines (Puchacz et al., 1994; Quik et al., 1996; Cooper and Millar, 1997, 1998; Kassner and Berg, 1997; Rangwala et al., 1997; Chen et al., 1998; Sweileh et al., 2000), which has led to the conclusion that appropriate subunit folding and assembly events are influenced strongly by the nature of the host cell type (Millar, 1999). It has also been demonstrated that the relatively 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 5-hydroxytryptamine receptor ($5HT_{3A}$) subunit (Eiselé et al., 1993; Blumenthal et al., 1997; Rangwala et al., 1997; Cooper and Millar, 1998; Quiram and Sine, 1998; Cooper et al., 1999; Harkness and Millar, 2001).

In this study, we constructed chimeric subunit cDNAs (which, for brevity, are referred to as $\alpha9\chi$ and $\alpha10\chi$) containing the extracellular N-terminal domain of the rat $\alpha9$ or $\alpha10$ subunits fused to the transmembrane and intracellular domain of the mouse $5HT_{3A}$ subunit. Coexpression of these subunit chimeras in mammalian cells generates a high-affinity binding site for nicotinic ligands. The pharmacological properties of this chimeric $\alpha9\chi\alpha10\chi$ receptor have been examined by equilibrium radioligand binding and compared with data derived previously from electrophysiological studies conducted on recombinant and native $\alpha9\alpha10$ nAChRs (Housley and Ashmore, 1991; Fuchs and Murrow, 1992; Rothlin et al., 1999; Verbitsky et al., 2000; Elgoyhen et al., 2001).

Materials and Methods

Molecular Biology. The rat nAChR $\alpha9$ and $\alpha10$ subunit cDNAs (Elgoyhen et al., 1994; Elgoyhen et al., 2001) were provided by Drs. Jim Boulter (University of California, Los Angeles, CA) and Belén Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). The mouse $5HT_{3A}$ subunit cDNA (Maricq et al., 1991) was provided by Dr. David Julius (University of California, San Francisco, CA). Chimeric subunit cDNAs similar to $\alpha7^{(V201)}/5HT_{3A}$ described previously (Eiselé et al., 1993; Cooper and Millar, 1998) were constructed containing the extracellular N-terminal domain of the $\alpha9$ or $\alpha10$ subunit fused to the transmembrane and intracellular domain of the mouse $5HT_{3A}$ subunit. A BclI site was introduced into the $\alpha9$ and $\alpha10$ cDNAs by polymerase chain reaction at positions analogous to Val201 in the $\alpha7$ sequence, just before the first putative transmembrane region, M1. The N-terminal $\alpha9$ and $\alpha10$ polymerase chain reaction fragments were subcloned into plasmid expression construct pRK5-$\alpha7^{(V201)}/5HT_{3A}$, described previously (Cooper et al., 1999), after removal of the $\alpha7$ sequence by restriction digestion, to create pRK5-$\alpha9^{(L209)}/5HT_{3A}$ and pRK5-$\alpha10^{(L206)}/5HT_{3A}$.

Cell Culture and Transfection. The mammalian cell line tsA201, derived from the human embryonic kidney 293 cell line, was obtained from Dr. William Green (University of Chicago, Chicago, IL). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) containing 2 mM L-GlutaMAX (Invitrogen) 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 transiently transfected using Effectene transfection reagent (QIAGEN, Crawley, UK) according to the manufacturer's instructions. In all cases, cells were transfected overnight and assayed for expression approximately 40 to 48 h after transfection. To ensure that levels of radioligand binding were not influenced by differences in the amount of subunit cDNA, when subunits were expressed singly and in combination, the amount of each subunit plasmid DNA and also the total amount of plasmid DNA was kept constant. This was achieved by inclusion of empty plasmid expression vector when single subunits were transfected.

Radioligand Binding to Cell Membrane Preparations. Binding studies with [^{3}H]methyllycaconitine ([^{3}H]MLA; specific activity, 26 Ci/mmol; Tocris Cookson Ltd, Avonmouth, UK) to cell membrane preparations were performed essentially as described previously (Lansdell and Millar, 2000b; Harkness and Millar, 2002). Membranes (typically 10–100 μg of protein) were incubated with radioligand for 150 min at 4°C in a total volume of 300 μl in the presence of protease inhibitors leupeptin (2 μg/ml) and pepstatin (1 μg/ml). For saturation binding experiments, nonspecific binding was determined by the addition of 1 mM carbachol, 1 mM nicotine, and 10 μM MLA to triplicate samples. For competition binding assays with [^{3}H]MLA, nonspecific binding for cells transfected with the $\alpha9\chi$ and $\alpha10\chi$ chimeric cDNA was determined by addition of 10 mM nicotine, 10 mM carbachol, and 100 μM *d*-tubocurarine. For cells transfected with $\alpha7\chi$ cDNA, nonspecific binding was determined in the presence of 3 μM MLA. Radioligand binding was assayed by filtration onto 0.5% polyethylenimine-presoaked Whatman GF/B filters followed by rapid washing with ice-cold 10 mM phosphate buffer using a Brandel cell harvester and radioactivity determined by scintillation counting. Care was taken to ensure that the number of receptor binding sites used for binding studies was low enough to avoid significant (>10%) ligand depletion at low concentrations of radioligand. Preliminary experiments were conducted to ensure that incubation times were long enough to enable radioligand binding to reach equilibrium. Curves for equilibrium binding were fitted with the Hill equation by equally weighted least-squares (CVFIT program; David Colquhoun, University College, London). In all cases, the calculated Hill coefficients (n_H) did not differ significantly from 1. Protein concentrations were determined by a detergent-compatible protein assay using bovine serum albumin standards (Bio-Rad, Hercules, CA).

Radioligand Binding to Intact Cells. Levels of cell-surface radioligand binding were determined with intact transfected cells. Because of concerns that [^{3}H]MLA might be able to cross the cell membrane (Davies et al., 1999), a protocol similar to that described previously (Whiteaker et al., 1998) was used to determine levels of cell-surface radioligand binding. Transfected cells, grown in 10-cm plates, were gently resuspended in HBSS. Intact cells (typically 5×10^5 cells) were incubated in HBSS in a total volume of 300 μl with 15 nM [^{3}H]MLA for 2 h on ice. Specific binding to cell surface receptors was determined by competition with 1 mM ACh, a membrane-impermeant ligand. This concentration of ACh was found to block >95% of total [^{3}H]MLA binding to cell membrane preparations. Despite concerns about membrane permeation of MLA, levels of nonspecific [^{3}H]MLA binding to intact cells, determined by competition with ACh, were very low (typically <3% total binding), suggesting little significant entry of [^{3}H]MLA into cells during the time course of the experiment. In addition, levels of nonspecific binding were not significantly different when binding was performed on intact cells in competition with a combination of membrane-permeant and -impermeant ligands (1 mM nicotine, 1 mM carbachol, and 10 μM MLA), which would be expected to block binding of [^{3}H]MLA to surface and intracellular sites. To determine the proportion of specific binding on the cell surface, binding assays with intact cells were performed in parallel with assays on cell membrane preparations (as described above). Samples were harvested onto GF/B glass fiber filters in ice-cold phosphate-buffered saline, as described above, and assayed by scintillation counting.

***X. laevis* Oocyte Expression and Electrophysiology.** Adult female *X. laevis* frogs were obtained from Blades Biological (Edenbridge, UK). Acetylcholine (ACh) chloride and α-bungarotoxin (αBTX) were purchased from Sigma. *X. laevis* oocytes (stage V–VI) were removed from schedule 1 sacrificed frogs and defolliculated

manually after treatment with collagenase type I (4 mg/ml calcium-free Barth's solution) for 90 min at room temperature. Plasmids containing the $\alpha9\chi$ and $\alpha10\chi$ subunit chimera coding sequences were suspended in distilled water and injected into the nuclei of the oocytes within 4 h after harvesting, using a variable volume microinjector (Drummond, Broomall, PA). Approximately 2 ng of $\alpha9\chi$ or $\alpha10\chi$ cDNA was injected in a total volume of 18.4 nl/oocyte. After injection, oocytes were incubated at 18°C in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 50 mg/l neomycin; pH adjusted to 7.6 with NaOH; osmolarity, 235 mOsM). Experiments were performed on oocytes after 3 to 5 days of incubation. Oocytes were placed in a recording chamber (internal diameter, 3 mm), which was continuously perfused with a saline solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, 10 mM HEPES; pH adjusted to 7.3 with NaOH; osmolarity, 235 mOsM) at a rate of ~10 ml/min. Dilutions of drugs in external saline were prepared immediately before the experiments and applied by switching between control and drug-containing saline using a BPS-8 solution exchange system (ALA Scientific Inc., Westbury, NY). Unless otherwise indicated, agonist applications were alternated with 2 min of superfusion with agonist-free saline to allow the receptors to recover from desensitization. Oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–2.5 MΩ) and voltage clamped using a GeneClamp 500B amplifier (Axon Instruments, Union City, CA). The external saline was clamped at ground potential by means of a virtual ground circuit using a silver/silver chloride reference electrode and a platinum/iridium current-passing electrode. The membrane potential was held at −60 mV, unless otherwise indicated. The current needed to keep the oocyte membrane at the holding potential was measured. Membrane currents were low-pass filtered (four-pole low pass Bessel filter, −3dB at 100 Hz), digitized (300 Hz), and stored on disk for off-line computer analysis. The experiments were performed at room temperature.

Results

Rat nAChR α9 and α10 subunit cDNAs were introduced by transient transfection into cultured human embryonic kidney tsA201 cells. Membranes were isolated from cells co-transfected with α9 and α10 cDNAs and from cells transfected with α9 alone or α10 alone. Binding studies were performed with [^{3}H]MLA, a nicotinic receptor antagonist that has been reported to bind with high apparent affinity to recombinant α9α10 nAChRs expressed in *X. laevis* oocytes (Verbitsky et al., 2000), but no specific radioligand binding was detected. Further binding studies were performed with a range of nicotinic radioligands including [^{125}I]αBTX, [^{3}H]epibatidine, and [^{3}H]methylcarbamylcholine, none of which revealed specific high-affinity binding. This observation is consistent with previous studies which have failed to detect functional nAChRs in mammalian (human embryonic kidney 293) cells transfected with α9 and α10 cDNAs (Elgoyhen et al., 1994, 2001; Lustig et al., 2001).

Several nAChR subunits (including α1, α4, α7, α8, and β2) have been shown to form a high-affinity nicotinic ligand binding site efficiently when expressed as chimeric subunits in which their extracellular N-terminal domains are fused to the C-terminal region of the $5HT_{3A}$ receptor subunit (Eiselé et al., 1993; Blumenthal et al., 1997; Rangwala et al., 1997; Cooper and Millar, 1998; Quiram and Sine, 1998; Cooper et al., 1999). For this reason, subunit chimeras were constructed that contained the extracellular domains of the α9 and α10 subunits, respectively, fused to the C-terminal domain of $5HT_{3A}$ (for brevity, these are referred to herein as $\alpha9\chi$ and $\alpha10\chi$).

To examine whether these chimeras were able to generate functional receptors, as has been demonstrated for the α7/$5HT_{3A}$ chimera (Eiselé et al., 1993; Blumenthal et al., 1997; Rangwala et al., 1997; Cooper and Millar, 1998; Quiram and Sine, 1998; Cooper et al., 1999), the $\alpha9\chi$ and $\alpha10\chi$ cDNAs in pRK5 were injected into *X. laevis* oocytes. Two-electrode voltage-clamp recordings were used to obtain clear evidence for the expression of functional receptors, although responses were significantly smaller than those observed with the α7/$5HT_{3A}$ chimera (Eiselé et al., 1993; Blumenthal et al., 1997; Rangwala et al., 1997; Cooper and Millar, 1998; Quiram and Sine, 1998; Cooper et al., 1999). Small whole-cell responses to ACh were detected in oocytes injected with either $\alpha9\chi$ alone (13.6 ± 7.7 nA; $n = 10$) or with $\alpha10\chi$ alone (40.4 ± 39.8 nA; $n = 24$), demonstrating that both of these chimeras were able

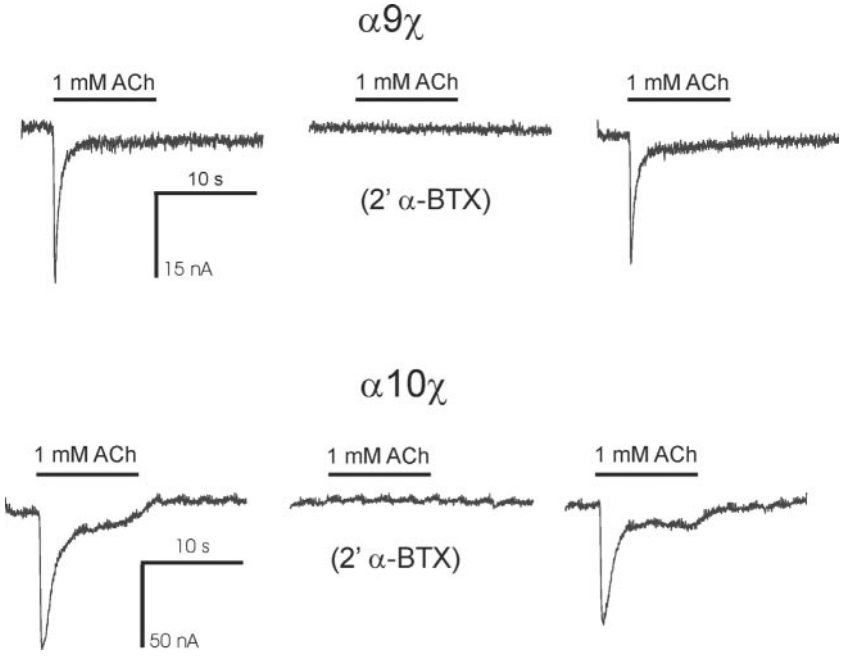

Fig. 1. Functional responses in *X. laevis* oocytes injected with $\alpha9\chi$ and $\alpha10\chi$ subunit cDNAs. Application of 1 mM ACh to oocytes expressing $\alpha9\chi$ or $\alpha10\chi$ nAChRs induced small, transient inward currents that were completely abolished after 2-min preapplication of 1 μM αBTX. The inhibitory effect of αBTX on both chimeric nAChRs was almost completely reversed after 4- to 6-min of washout of the toxin.

to form functional homomeric receptors (Fig. 1). Responses detected in oocytes coinjected with the $\alpha9\chi$ and $\alpha10\chi$ cDNAs (12.5 ± 3.9 nA; $n = 19$) were not significantly larger than those recorded with either subunit alone. In all cases, responses to ACh were blocked completely and reversibly by a 2-min application of 1 μM αBTX (Fig. 1).

To examine whether problems that have been encountered in the heterologous expression of $\alpha9$ and $\alpha10$ nAChRs in mammalian cell lines could be alleviated by expression of nAChR/5HT$_3$R chimeras, the $\alpha9\chi$ and $\alpha10\chi$ subunit cDNAs were introduced by transient transfection into tsA201 cells. Radioligand binding performed on membrane preparations derived from cells transfected with either the $\alpha9\chi$ chimera alone or the $\alpha10\chi$ chimera alone revealed specific binding with the nicotinic antagonist [^{3}H]MLA at levels significantly above background (Fig. 2). However, when $\alpha9\chi$ and $\alpha10\chi$ were coexpressed, substantially higher levels of specific [^{3}H]MLA binding were observed (Fig. 2). Levels of [^{3}H]MLA binding detected in cells cotransfected with $\alpha9\chi$ and $\alpha10\chi$ were considerably higher than with $\alpha9\chi$ alone (4.3 ± 0.3 fold, $n = 4$) or with $\alpha10\chi$ alone (21.2 ± 7.5 fold, $n = 6$) (Fig. 3).

Saturation radioligand binding performed on cells cotransfected with $\alpha9\chi$ and $\alpha10\chi$ revealed that [^{3}H]MLA bound with high affinity (K_d = 7.5 ± 1.2 nM, $n = 5$) (Fig. 3). The low levels of radioligand binding to $\alpha9\chi$ and $\alpha10\chi$, when expressed alone, prevented an accurate determination of the affinity of [^{3}H]MLA binding to homomeric $\alpha9\chi$ and $\alpha10\chi$ receptors.

Equilibrium competition binding studies were performed with a range of ligands to determine their affinity for $\alpha9\chi\alpha10\chi$ receptors (Fig. 4; Table 1). The nicotinic antagonist αBTX bound with high affinity (66 ± 22 nM). Other nicotinic ligands bound with lower affinities, including *d*-tubocurarine (0.3 ± 0.1 μM), 1,1-dimethyl-4-phenylpiperazinium (2.0 ± 0.4 μM), ACh (2.7 ± 2.5 μM), carbachol (10.4 ± 2.2 μM), methylcarbachol (38.7 ± 19.2 μM), and nicotine (42.9 ± 6.2 μM). Specific competition binding was also observed with the glycine receptor convulsant strychnine, the GABA receptor antagonist bicuculline, and the muscarinic antagonist atropine (66 ± 7 nM, 0.6 ± 0.3 μM, and 9.9 ± 1.3 μM, respectively). The rank order of K_i values is in good agreement with the relative potencies of these ligands identified from electrophysiological assays on both recombinant $\alpha9$ and $\alpha9\alpha10$ nAChRs expressed in *X. laevis* oocytes and with native nAChRs expressed in hair cells (Housley and Ashmore, 1991; Fuchs and Murrow, 1992; Rothlin et al., 1999; Verbitsky et al., 2000) (Table 1).

A further series of radioligand binding studies were performed on tsA201 cells transfected with a $\alpha7^{(V201)}$/5HT$_{3A}$ subunit chimera, $\alpha7\chi$ (Eiselé et al., 1993; Cooper and Millar, 1998). The rationale for these studies was to enable a comparison of $\alpha7\chi$ receptors with $\alpha9\chi\alpha10\chi$ receptors and with radioligand binding data that have been reported previously for native $\alpha7$ nAChRs (Anand et al., 1993; Davies et al., 1999). Thus, whereas radioligand binding data determined with $\alpha9\chi\alpha10\chi$ have been compared with EC$_{50}$/IC$_{50}$ values for $\alpha9\alpha10$ receptors (Table 1), a direct comparison is possible

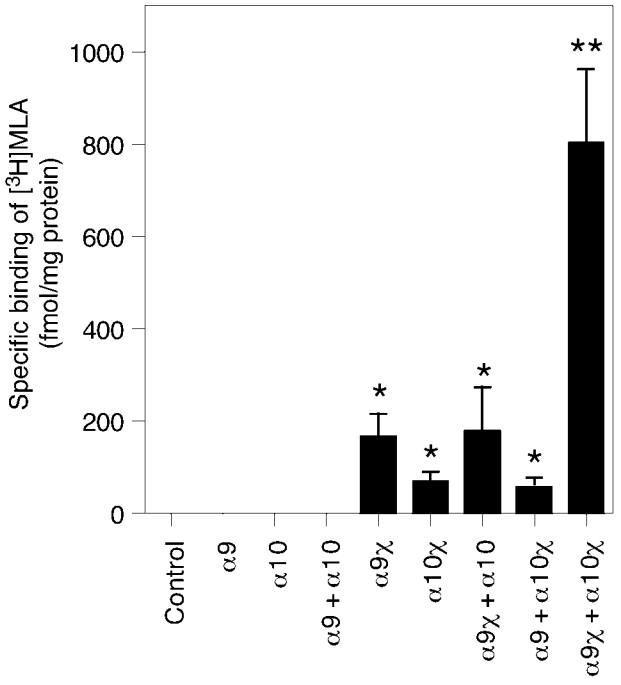

Fig. 2. Specific binding of [^{3}H]MLA to cell membranes prepared from tsA201 cells transiently transfected with wild-type ($\alpha9$ and $\alpha10$) and chimeric ($\alpha9\chi$ and $\alpha10\chi$) subunits. Data are presented as means of four to seven independent experiments performed in triplicate with [^{3}H]MLA (15 nM) and show significant levels of radioligand binding in cells transfected with $\alpha9\chi$ (166 ± 50 fmol/mg protein; $n = 5$), $\alpha10\chi$ (68 ± 22 fmol/mg protein; $n = 5$), and $\alpha9\chi\alpha10\chi$ (802 ± 160 fmol/mg protein; $n = 6$). Significance was determined by two-tailed Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

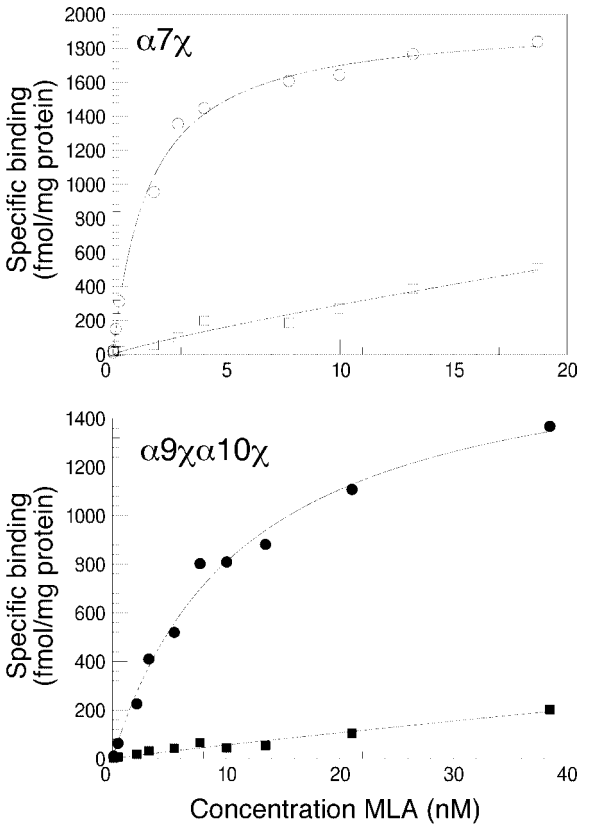

Fig. 3. Specific binding of [^{3}H]MLA to $\alpha9\chi\alpha10\chi$ and $\alpha7\chi$ receptors expressed in tsA201 cells. Equilibrium saturation radioligand binding was performed on membranes prepared from tsA201 cells transfected with $\alpha9\chi$+$\alpha10\chi$ (closed symbols) or $\alpha7\chi$ (open symbols) subunit chimeras. Data presented for specific binding (circles) and nonspecific binding (squares) are from a single experiment performed in triplicate but are typical of four to five independent determinations, giving mean K_d values of 7.5 ± 1.2 nM ($\alpha9\chi\alpha10\chi$) and 1.2 ± 0.2 nM ($\alpha7\chi$).

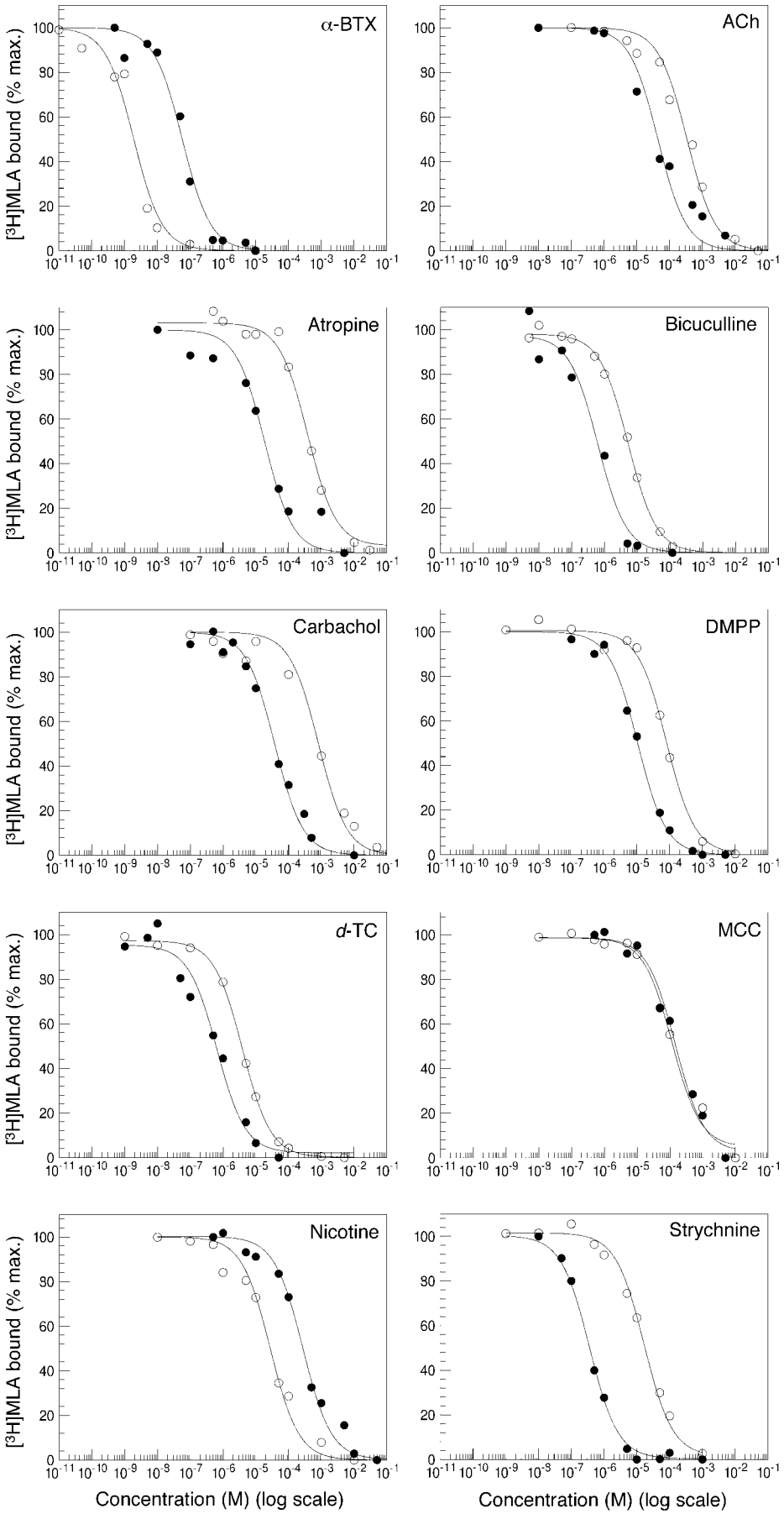

Fig. 4. Pharmacological characterization of $\alpha9\chi\alpha10\chi$ and $\alpha7\chi$ receptors expressed in tsA201 cells. Equilibrium radioligand binding of [^{3}H]MLA was performed with a variety of competing ligands to cells transfected with $\alpha9\chi\alpha10\chi$ (●) and $\alpha7\chi$ (○). Data points are means of triplicate samples. Each graph is from a single experiment but is typical of three to four individual experiments. Mean K_i values are presented in Tables 1 and 2.

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TABLE 1

Pharmacological properties of $\alpha9\chi$ $\alpha10\chi$ nAChRs and comparison with native and recombinant receptors. Ligands are listed in order of their decreasing affinity as determined by equilibrium radioligand binding experiments with $\alpha9\chi$ $\alpha10\chi$ receptors expressed in tsA201 cells. The rank order for these ligands is in good general agreement with estimates of agonist/antagonist potency derived from functional assays performed with either recombinant $\alpha9$ or $\alpha9\alpha10$ nAChRs expressed in *X. laevis* oocytes or with native nAChRs expressed in hair cells. K_d values (for MLA) were determined by equilibrium saturation binding, whereas K_i values (for all other ligands) were determined by competition binding performed with [^{3}H]MLA.

Ligand	$\alpha9\chi\alpha10\chi$ tsA201 Cells(K_d/K_i)[a]	$\alpha9$ Oocytes (EC_{50}/IC_{50})[b]	$\alpha9\alpha10$ Oocytes (EC_{50}/IC_{50})[c]	$\alpha9\alpha10$ Hair Cells (EC_{50}/IC_{50})[d]
MLA	7.5 ± 1.2 nM	1 nM	N.D.	N.D.
α-Bungarotoxin	66 ± 22 nM	4 nM	nM range	nM range
Strychnine	66 ± 7 nM	18 nM	20 nM	nM range
d-Tubocurarine	290 ± 90 nM	300 nM	110 nM	nM range
Bicuculline	0.6 ± 0.3 μM	0.8 μM	1 μM	~1 μM
DMPP	2.0 ± 0.4 μM	N.D.	N.D.	N.D.
Acetylcholine	2.7 ± 2.5 μM	11 μM	14 μM	7 μM
Atropine	9.9 ± 1.3 μM	1 μM	1 μM	mM range
Carbachol	10.4 ± 2.2 μM	64 μM	N.D.	87 μM
Methylcarbachol	38.7 ± 19.2μM	30 μM	N.D.	N.D.
Nicotine	42.9 ± 6.2 μM	32 μM	4 μM	mM range

N.D., not determined; DMPP, 1,1-dimethyl-4-phenlypiperazinium.
[a] Data (this study) are means of four to five experiments each performed in triplicate.
[b] Data from Verbitsky et al. (2000).
[c] Data from Elgoyhen et al. (2001).
[d] The subunit composition of native nAChRs expressed in hair cells is assumed to be $\alpha9\alpha10$. Data from ooctyes were from cells injected with $\alpha9$ alone or $\alpha9+\alpha10$, as indicated. Data from several studies, as reviewed by Verbitsky et al. (2000) and Elgoyhen et al. (2001).

between equilibrium radioligand binding data from $\alpha7\chi$ and $\alpha7$ nAChRs (Table 2). A similar pharmacological profile of recombinant $\alpha7\chi$ and native $\alpha7$ nAChRs would argue that nAChR/5HT$_{3A}$ subunit chimeras reflect the ligand binding properties of corresponding wild-type nAChRs.

Saturation radioligand binding experiments performed with tsA201 cells transfected with $\alpha7\chi$ revealed high-affinity binding of [^{3}H]MLA (1.2 ± 0.2 nM). This is in close agreement with estimates of the affinity of [^{3}H]MLA binding to rat brain $\alpha7$ nAChRs (1.9 ± 0.3 nM; Davies et al., 1999). As illustrated in Table 2, competition binding studies performed on tsA201 cells transfected with $\alpha7\chi$ reveal a pharmacological profile similar to that of the native $\alpha7$ nAChR (Anand et al., 1993; Davies et al., 1999).

TABLE 2

Pharmacological properties of $\alpha7\chi$ nAChRs and comparison with native $\alpha7$ receptors. Ligands are listed in order of their decreasing affinity as determined by equilibrium radioligand binding experiments with $\alpha7\chi$ receptors expressed in tsA201 cells. The rank order for these ligands is in good general agreement with estimates of binding affinity determined with native $\alpha7$ nAChRs expressed in rat or chick brain. K_d values (for MLA) were determined by equilibrium saturation binding, whereas K_i values (for all other ligands) were determined by competition binding performed with [^{3}H]MLA. The subunit composition of native $\alpha7$-containing nAChRs is uncertain, but data from rat brain are expected to correspond predominantly to homomeric $\alpha7$ (Chen and Patrick, 1997; Drisdel and Green, 2000). Data from chick brain are expected to represent binding to a mixed population $\alpha7$, $\alpha8$, and $\alpha7\alpha8$ nAChRs (Keyser et al., 1993).

Ligand	$\alpha7\chi$ tsA201 Cells (K_d/K_i)[a]	$\alpha7$ Native (Brain)(K_d/K_i)
MLA	1.2 ± 0.2 nM	1.9 ± 0.3 nM[b]
α-Bungarotoxin	0.3 ± 0.1 nM	1.8 ± 0.5 nM[b]
Bicuculline	1.4 ± 0.2 μM	N.D.
d-Tubocurarine	1.6 ± 0.2 μM	N.D.
Strychnine	6.7 ± 0.9 μM	5.4 ± 0.5 μM[c]
Nicotine	28.8 ± 8.7 μM	6.1 ± 1.1 μM[b]
DMPP	28.9 ± 5.3 μM	N.D.
Methylcarbachol	90 ± 34 μM	10.6 ± 0.6 μM[b]
Atropine	140 ± 11 μM	198 ± 10 μM[c]
Acetylcholine	129 ± 39 μM	103 ± 8.7 μM[c]
Carbachol	698 ± 248μM	580 ± 205 μM[c]

N.D., not determined; DMPP, 1,1-dimethyl-4-phenlypiperazinium.
[a] Data (this study) are means of three experiments each performed in triplicate.
[b] Data from Davies et al. (1999), from rat brain $\alpha7$ nAChR.
[c] Data from Anand et al. (1993), from chick brain $\alpha7$ nAChR.

It has been shown previously that nAChR/5HT$_3$R subunit chimeras, such as $\alpha4\chi$, $\alpha7\chi$, $\alpha8\chi$, and $\beta2\chi$ are expressed at high levels on the cell surface of transfected mammalian cells (Cooper and Millar, 1998; Cooper et al., 1999; Harkness and Millar, 2002). To examine whether $\alpha9\chi\alpha10\chi$ receptors were expressed on the surface of tsA201 cells, we examined the ability of [^{3}H]MLA to bind to intact transfected cells. A potential concern with using [^{3}H]MLA to assay levels of cell-surface binding is the possibility that this ligand might be able to cross the cell membrane (Davies et al., 1999). To circumvent this potential problem, we adopted a strategy that has been employed previously (Whiteaker et al., 1998), in which specific radioligand binding to cell-surface sites is determined by competition with acetylcholine, a membrane-impermeant ligand. Binding studies performed with intact tsA201 cells indicated high levels of specific cell-surface [^{3}H]MLA binding (9.5 ± 2.2 fmol/10^6 cells; n = 3). This approach has been used successfully even with membrane-permeant radioligands such as [^{3}H]epibatidine (Whiteaker et al., 1998). Levels of nonspecific [^{3}H]MLA binding (as determined by competition with ACh), however, were very low (< 3% of total binding), suggesting little significant entry of [^{3}H]MLA into intact cells within the time course of the experiment. Radioligand binding studies performed in parallel with cell membrane preparations indicated that specific cell-surface [^{3}H]MLA binding to $\alpha9\chi\alpha10\chi$ represented 83 ± 16% of total specific binding. This is similar to levels of cell-surface expression that have been reported with other nAChR/5HT$_3$R subunit chimeras expressed in tsA201 cells (Harkness and Millar, 2002).

Discussion

Several previous studies have reported inefficient folding and/or cell-surface expression of nAChR subunits when expressed heterologously in a variety of host cell types (Puchacz et al., 1994; Quik et al., 1996; Cooper and Millar, 1997, 1998; Kassner and Berg, 1997; Rangwala et al., 1997; Chen et al., 1998; Sweileh et al., 2000). A common feature of these studies is that inefficient folding and cell-surface expression of

nAChRs can be attributed to sequences present within the C-terminal (transmembrane and intracellular) subunit domains (Eiselé et al., 1993; Blumenthal et al., 1997; Rangwala et al., 1997; Cooper and Millar, 1998; Quiram and Sine, 1998; Cooper et al., 1999; Harkness and Millar, 2001). Heteromeric complexes formed by the coexpression of $\alpha4\chi$ and $\beta2\chi$ chimeras, for example, are expressed on the cell surface of transfected mammalian kidney cells at levels up to 25-fold higher than $\alpha4\beta2$ nAChRs (Cooper et al., 1999). High levels of functional cell-surface receptors have been detected in several cell lines after expression of $\alpha7\chi$ or $\alpha8\chi$ subunit chimeras, including host cell types in which few, if any, correctly folded nAChRs can be detected after expression of wild-type $\alpha7$ or $\alpha8$ (Eiselé et al., 1993; Cooper and Millar, 1998).

When expressed in *X. laevis* oocytes, the $\alpha9$ subunit forms functional homomeric nAChRs (Elgoyhen et al., 1994). In contrast, the $\alpha10$ subunit has been reported to be unable to generate functional homomeric nAChRs, but coexpression of $\alpha9$ and $\alpha10$ facilitates expression of functional (heteromeric) receptors (Elgoyhen et al., 2001). In the present study, no evidence for specific binding of nicotinic radioligands was detected in mammalian cells transfected with $\alpha9$ and $\alpha10$. It has been possible, however, to detect specific high-affinity binding of [^{3}H]MLA in a mammalian cell line transfected with chimeric $\alpha9\chi$ and $\alpha10\chi$ subunits. This has permitted, for the first time, a detailed pharmacological characterization of recombinant $\alpha9\alpha10$-type nAChRs by equilibrium radioligand binding. Successful heterologous expression of $\alpha9\chi$ and $\alpha10\chi$ subunit chimeras is consistent with previous studies with chimeras such as $\alpha7^{(V201)}/5HT_{3A}$ (Eiselé et al., 1993; Cooper and Millar, 1998) and provides further evidence that inefficient folding of neuronal nAChR subunits into a conformation recognized by nicotinic radioligands can be attributed to their C-terminal region.

Whereas low levels of [^{3}H]MLA binding were observed in cells transfected with either $\alpha9\chi$ or $\alpha10\chi$ alone, significantly higher levels of binding were detected in cells cotransfected with $\alpha9\chi$ and $\alpha10\chi$, providing evidence that heteromeric coassembly allows more efficient formation of a nicotinic binding site. This is consistent with oocyte coexpression studies of wild-type $\alpha9$ and $\alpha10$, which reveal that responses in oocytes coinjected with $\alpha9$ and $\alpha10$ are ~100-fold larger than in oocytes injected only with $\alpha9$ (Elgoyhen et al., 2001). The importance of appropriate heteromeric subunit-subunit interactions between N-terminal domains has also been illustrated by several previous studies (performed with mutated and chimeric nAChR subunits) (Gu et al., 1991; Yu and Hall, 1991; Sumikawa, 1992; Lansdell and Millar, 2000a).

The presence of the entire N-terminal extracellular ligand-binding domain of the $\alpha9$ and $\alpha10$ subunits within the $\alpha9\chi$ and $\alpha10\chi$ chimeras would suggest that such chimeras might exhibit pharmacological properties that mimic those of native $\alpha9\alpha10$ receptors. Electrophysiological studies on recombinant $\alpha9$-containing receptors expressed in *X. laevis* oocytes have revealed somewhat atypical pharmacological properties with modulation by a diverse collection of ligands, including those known to bind with high affinity to muscarinic acetylcholine receptors, glycine receptors, and γ-aminobutyric acid receptors (Rothlin et al., 1999; Verbitsky et al., 2000; Elgoyhen et al., 2001). The pharmacological profile for the chimeric $\alpha9\chi\alpha10\chi$ receptor constructed in this study correlates well with data obtained from electrophysiological assays on both recombinant $\alpha9$ and $\alpha9\alpha10$ nAChRs expressed in *X. laevis* oocytes and native nAChRs expressed in hair cells (Housley and Ashmore, 1991; Fuchs and Murrow, 1992; Rothlin et al., 1999; Verbitsky et al., 2000). The assumption that the $\alpha9\chi\alpha10\chi$ receptor can serve as a viable model for investigation of the $\alpha9\alpha10$ receptor is supported by our comparison of equilibrium binding data obtained with the $\alpha7^{(V201)}/5HT_{3A}$ chimera with data obtained with native $\alpha7$ nAChRs (Anand et al., 1993; Davies et al., 1999). Comparison of competition binding data conducted with $\alpha7\chi$ and $\alpha9\chi\alpha10\chi$ reveals that MLA, αBTX, and nicotine bind with higher affinity to $\alpha7\chi$ than to $\alpha9\chi\alpha10\chi$, whereas all other ligands tested bind with higher affinity to $\alpha9\chi\alpha10\chi$ than to $\alpha7\chi$ (Tables 1 and 2). This is in agreement with previous studies reporting that $\alpha9\alpha10$ nAChRs exhibit an atypical pharmacological profile (Rothlin et al., 1999; Verbitsky et al., 2000; Elgoyhen et al., 2001) and $\alpha7$ receptors demonstrate a more conventional nAChR profile (Anand et al., 1993; Davies et al., 1999). Differences in the affinity of ligands for $\alpha9\chi\alpha10\chi$ and $\alpha7\chi$ nAChRs can be seen from a comparison of Tables 1 and 2, in which ligands are listed in order of decreasing affinities for the two subunit combinations.

The finding that $\alpha9\chi$ is able to generate a functional homomeric ion channel is consistent with the ability of both the $\alpha9$ subunit and the $5HT_{3A}$ subunit to form homomeric ligand-gated ion channels (Maricq et al., 1991; Elgoyhen et al., 1994). Despite the inability of wild-type $\alpha10$ to generate functional homomeric nAChRs when expressed in oocytes (Elgoyhen et al., 2001), we have found that the $\alpha10\chi$ chimera is able to do so. This suggests that the wild-type $\alpha10$ subunit contains an extracellular domain capable of forming a nicotinic agonist/antagonist binding site (when folded into an appropriate conformation), despite the inability of the wild-type $\alpha10$ subunit to form a functional homomeric ion channel. Interestingly, it has also been reported that a chimera containing the N-terminal region of $\alpha9$ fused to the C-terminal region of $\alpha10$ produces functional ion channels in oocytes (Sgard et al., 2002). Thus, despite the inability of $\alpha10$ to form functional homomeric nAChRs in heterologous expression systems, subunit chimeras containing either the N terminus of $\alpha10$ (with the C terminus of $5HT_{3A}$) or the C terminus of $\alpha10$ (with the N terminus of $\alpha9$) are able to do so.

Our observation that functional responses detected in oocytes coinjected with the $\alpha9\chi$ and $\alpha10\chi$ cDNAs were not significantly larger than those obtained with either chimeric subunit alone is in contrast to coexpression studies of wild-type $\alpha9$ and $\alpha10$ in oocytes. Whole-cell responses in oocytes coinjected with wild-type $\alpha9$ and $\alpha10$ subunits are, typically, ~100-fold larger than observed with the $\alpha9$ subunit alone (Elgoyhen et al., 2001). This discrepancy between chimeric and wild-type subunits is surprising given the synergistic effect upon [^{3}H]MLA binding that is observed when the two chimeric subunits are coexpressed in mammalian cells. The lack of a clear synergistic effect upon whole-cell current responses when $\alpha9\chi$ and $\alpha10\chi$ are coexpressed in oocytes, together with clear evidence for such an effect with the wild-type $\alpha9$ and $\alpha10$ subunits, may be a consequence of differences in their transmembrane pore-forming domains. In the homomeric $\alpha9\chi$ channels, homomeric $\alpha10\chi$ channels, and heteromeric $\alpha9\chi\alpha10\chi$ channels, the presumed transmembrane domain is provided by the $5HT_{3A}$ subunit. If the coupling between agonist binding and channel opening domains in these chimeric subunits is relatively inefficient in

both homomeric and heteromeric complexes, this may explain why significantly larger whole-cell responses are not observed when $\alpha9\chi$ and $\alpha10\chi$ are coexpressed.

In summary, by constructing and heterologously expressing $\alpha9\chi$ and $\alpha10\chi$ subunit chimeras, we have been able for the first time to examine by radioligand binding the pharmacological properties of recombinant $\alpha9\alpha10$-type nAChRs in a mammalian cell line. The equilibrium binding studies reported here, conducted with cells cotransfected with $\alpha9\chi$ and $\alpha10\chi$, are in good general agreement with previous studies of wild-type $\alpha9\alpha10$ nAChRs conducted by electrophysiological techniques (Rothlin et al., 1999; Verbitsky et al., 2000). In particular, we have demonstrated specific high-affinity binding of the nicotinic antagonist MLA together with evidence for an atypical pharmacological profile for $\alpha9\alpha10$ nAChRs.

Acknowledgments

We thank Drs Jim Boulter, Belén Elgoyhen, and David Julius for generously providing the $\alpha9$, $\alpha10$, and $5HT_{3A}$ subunit cDNAs.

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