Selective effects of a 4-oxystilbene derivative on wild and mutant neuronal chick $\alpha 7$ nicotinic receptor

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1 We assessed the pharmacological activity of triethyl-(β -4-stilbenoxy-ethyl) ammonium (MG624), a drug that is active on neuronal nicotinic receptors (nicotinic AChR). Experiments on the major nicotinic AChR subtypes present in chick brain, showed that it inhibits the binding of [¹²⁵I]- α Bungarotoxin (α Bgtx) to the $\alpha 7$ subtype, and that of [³H]-epibatidine (Epi) to the $\alpha 4\beta 2$ subtype, with K_i values of respectively 106 nM and 84 μ M.

2 MG624 also inhibited ACh elicited currents (I_{ACh}) in the oocyte-expressed $\alpha 7$ and $\alpha 4\beta 2$ chick subtypes with half-inhibitory concentrations (IC_{50}) of respectively 109 nM and 3.2 μ M.

3 When tested on muscle-type AChR, it inhibited [¹²⁵I]- α Bgtx binding with a K_i of 32 μ M and ACh elicited currents (I_{ACh}) in the oocyte-expressed $\alpha 1\beta 1\gamma\delta$ chick subtype with an IC_{50} of 2.9 μ M.

4 The interaction of MG624 with the $\alpha 7$ subtype was investigated using an $\alpha 7$ homomeric mutant receptor with a threonine-for-leucine 247 substitution (L247T $\alpha 7$). MG624 did not induce any current in oocytes expressing the wild type $\alpha 7$ receptor, but did induce large currents in the oocyte-expressed L247T $\alpha 7$ receptor. The MG624 elicited current (I_{MG624}) has an EC_{50} of 0.2 nM and a Hill coefficient nH of 1.9, and is blocked by the nicotinic receptor antagonist methyllycaconitine (MLA).

5 These binding and electrophysiological studies show that MG624 is a potent antagonist of neuronal chick $\alpha 7$ nicotinic AChR, and becomes a competitive agonist following the mutation of the highly conserved leucine residue 247 located in the M2 channel domain.

Keywords: MG624; neuronal nicotinic receptors; muscle nicotinic receptors; subtypes; α Bungarotoxin; epibatidine; methyllycaconitine; oocytes; chick

Abbreviations: ACh, Acetylcholine; Abs, Antibodies; α Bgtx, α Bungarotoxin; Epi, epibatidine; Carb, carbamylcholine; KD, kilodalton; K_d , dissociation constant; MLA, methyllycaconitine; mAbs, monoclonal antibodies; d-Tub, d-Tubocurarine

Introduction

Molecular biology studies have revealed the presence of several nicotinic AChR subtypes in vertebrate brain and ganglia, each of which has a distinct pattern of expression (Sargent, 1993; Role & Berg, 1996).

These subtypes are believed to be pentameric ion channels consisting of distinct homologous subunits that are coded by 11 genes ($\alpha 2$ – $\alpha 9$ and $\beta 2$ – $\beta 4$). Expression studies have demonstrated that these subtypes are both pharmacologically and functionally distinguishable, and can be formed homomERICALLY from the $\alpha 7$, $\alpha 8$ and $\alpha 9$ subunits, or as heteromeric complexes containing one or more α subunits (different from those forming the homomeric receptors) and one or more β subunits. The diversity and complexity of the nAChR present in vertebrate brain and ganglia has also been confirmed using biochemical and immunological techniques. An α Bgtx receptor containing both the $\alpha 7$ and $\alpha 8$ subunits is present (Gotti *et al.*, 1994) together with nAChR subtypes containing three or four different nAChR subunits, such as $\alpha 3\alpha 4\beta 2$, $\alpha 4\alpha 5\beta 2$ and $\alpha 3\alpha 5\beta 2\beta 4$, (Conroy *et al.*, 1992; Vernallis *et al.*, 1993; Conroy & Berg, 1995). An $\alpha 4\beta 2\beta 3\beta 4$ subtype has been found in rat cerebellum (Forsayeth & Kobrin, 1997).

Pharmacological and functional studies have shown that the major nicotinic AChR subtypes present in vertebrate brain are the $\alpha 4\beta 2$ and $\alpha 7$ subtypes (Albuquerque *et al.*, 1997; Gotti *et al.*, 1997a; McGehee & Role, 1995). The $\alpha 4\beta 2$ subtype

represents more than 80% of the high affinity agonist binding sites, and is known to be located at least presynaptically where it can modulate neurotransmitter release (Wonnacott, 1997). The $\alpha 7$ subtype, which is as abundant as the $\alpha 4\beta 2$ subtype, binds α Bgtx with high affinity and contains the $\alpha 7$ subunit. When expressed in oocytes, the $\alpha 7$ subunit forms functional homomeric receptor-channels with a low affinity for ACh, desensitizes very rapidly, and has a non linear current-voltage relationship (Couturier *et al.*, 1990; McGehee & Role, 1995). This homomeric $\alpha 7$ subtype is not only highly permeable to Ca^{2+} , but Ca^{2+} binding to sites located on the extracellular part of the $\alpha 7$ receptor can potentiate its responses to the natural ACh neurotransmitter (Galzi *et al.*, 1996).

A mutated form of the $\alpha 7$ subunit has been engineered (L247T $\alpha 7$), in which the substitution of the conserved leucine 247 for threonine in the M2 channel domain greatly changes the functional and pharmacological properties of $\alpha 7$ receptors leading to a higher affinity for ACh, a reduced rate of desensitization, and no current rectification. Furthermore a number of competitive antagonists of the $\alpha 7$ wild type become agonists of the L247T $\alpha 7$ receptor. These alterations in the functional and pharmacological properties of the mutated receptor make it a good model for studying the structure and function of the $\alpha 7$ subtype and its drug interactions (Revah *et al.*, 1991; Bertrand *et al.*, 1993; 1997).

As the pharmacological properties and physiological functions of nicotinic AChR subtypes are still largely unknown, considerable efforts have been made to investigate their

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structure in order to provide information that can be used to design more selective compounds. Much of the recent increase in nicotinic ligand research is due to the growing evidence that nicotinic AChRs are involved in brain function and play a central role in the physiopathology of a number of disorders (Lèna & Changeux, 1997; Dani & Heinemann, 1996; Lindstrom, 1997; Kuryatov *et al.*, 1997; Gotti *et al.*, 1997a).

It has long been known that 4-oxystilbene derivatives have ganglioplegic activity and are able to antagonise nicotine-induced tremors in rabbits (Mantegazza & Tommasini, 1955). We therefore decided to test the nicotinic AChR selectivity of these compounds by means of binding and electrophysiological studies on the two subtypes most expressed in vertebrate brain: the $\alpha 7$ and $\alpha 4\beta 2$ subtypes. We chose triethyl-(β -4-stilbenoxy-ethyl) ammonium (MG624) (Figure 1) because it retains the pharmacological activity of the 4-oxystilbene compounds (Mantegazza & Tommasini, 1955) and it is the most potent 4-oxystilbene derivative in inhibiting the binding of [125 I]- α Bgtx to a heterogeneous receptor population of $\alpha 7$ -containing receptors and the least potent in inhibiting the binding of [3 H]-Epi to the $\beta 4$ and $\beta 2$ -containing receptors (Gotti *et al.*, 1998).

The aim of this study was to gain more information on the nicotinic specificity of this compound, by investigating its binding to the individual neuronal subtypes, and comparing the results with those obtained in the same subtypes expressed in oocytes. In order to clarify its mechanism of action, we also investigated its effects on the mutated L247T $\alpha 7$ receptor.

Methods

Antibody production and characterization

The peptides and antibodies (Abs) were produced as previously described (Gotti *et al.*, 1994). Anti- $\alpha 7$ and anti- $\beta 2$ chick-specific antibodies (Abs) were produced in rabbits by means of immunization with peptides chosen from the most divergent region of the $\alpha 7$ and $\beta 2$ subunits (the cytoplasmic loop between M3 and M4), and also against a peptide located at the COOH terminal of the $\beta 2$ subunit.

The anti-muscle type Ab was mAb73, a mAb that is specific for the $\beta 1$ subunit (Tzartos *et al.*, 1986), which was prepared as previously described (Gotti *et al.*, 1997b).

The specificity of anti- $\alpha 7$ Abs has been previously reported by Gotti *et al.* (1994; 1997c). The specificity of the anti- $\beta 2$ Abs, those directed against the cytoplasmic peptide, as well as those directed against the COOH peptide, was measured in immunoprecipitation experiments using receptors labelled with the nicotinic agonist [3 H]-Epi (Gerzanich *et al.*, 1995). Both of our Abs were capable of immunoprecipitating $95 \pm 2\%$ of the [3 H]-Epi high affinity labelled receptors, an amount that was almost identical to that immunoprecipitated by mAb 270, which specifically recognizes the $\beta 2$ subunit (Whiting *et al.*,

1987) and, in our hands, immunoprecipitated 90% of the [3 H]-Epi high affinity sites.

mAb 270, which was raised against chicken brain nicotinic AChR and directed against the $\beta 2$ subunit (Whiting *et al.*, 1987), and mAb35, which was raised against muscle-type AChR, recognizes the $\alpha 1$ subunit and cross-reacts with the $\alpha 5$ subunit (Conroy *et al.*, 1992), was purified from a hybridoma cell line obtained from the American Type Culture Collection. mAb 299, which was raised against rat brain nicotinic AChR and directed against the $\alpha 4$ subunit (Whiting & Lindstrom, 1988), and mAb 313, which was raised against the fusion protein containing the putative cytoplasmic of the $\alpha 3$ subunit (Whiting *et al.*, 1991b), were both purchased from RBI.

Anti-muscle type receptor subunits mAbs, 155 (anti $\alpha 1$), 148 (anti $\beta 1$), 162 (anti γ) and 137 (anti δ) (Tzartos *et al.*, 1986) were a generous gift of Dr Tzartos.

Receptor immobilization by subunit-specific antibodies

The affinity-purified Abs were bound to the wells at a concentration of 10 μ g/ml by means of overnight incubation at 4°C in phosphate buffer pH 7.5. On the following day, the wells were washed in order to remove the excess of unbound Abs, and then incubated overnight at 4°C with 200 μ l of 2% Triton X-100 extract obtained from COL membranes and prepared as described below (in the case of the $\alpha 7$ and $\beta 2$ -containing receptors), or with purified AChR foetal calf muscle in the case of mAb 73. After overnight incubation with the extract, the wells were washed and the presence of immobilized receptor was revealed by means of [125 I]- α Bgtx (in the case of the $\alpha 7$ subtype or muscle AChR) or [3 H]-Epi binding (in the case of the $\alpha 4\beta 2$ subtype).

AChR subtype preparation

The extracts containing the neuronal $\alpha 7$ and $\alpha 4\beta 2$ subtypes were obtained from chick optic lobes (COLs) as previously described (Gotti *et al.*, 1994). For every experiment, 18 g of COL, were homogenized in an excess of Na phosphate pH 7.4 50 mM, NaCl 1 M, EDTA 2 mM, EGTA 2 mM and PMFS 2 mM for 2 min in an ultra Turrax homogenizer; the homogenate was then diluted and centrifuged for 1.5 h at 60,000 $\times$ g.

This homogenization, dilution and centrifugation procedure was performed three times, after which the pellets were collected, rapidly rinsed with Na phosphate 50 mM, NaCl 50 mM, EDTA 2 mM, EGTA 2 mM, PMFS 2 mM, and then resuspended in the same buffer containing a mixture of 5 μ g ml $^{-1}$ of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A and aprotinin (Sigma). Triton X-100, at a final concentration of 2%, was added to the washed membrane, and the membrane extracted for 2 h at 4°C. The extract (100 ml) was then centrifuged for 1.5 h at 60,000 $\times$ g and recovered.

$\alpha 7$ subtype Previous experiments (Gotti *et al.*, 1994) have shown that 60–70% of the α Bgtx receptors present in COL extract contain the $\alpha 7$ subunit ($\alpha 7$ subtype), and 20–25% contain both the $\alpha 7$ and $\alpha 8$ subunits ($\alpha 7$ – $\alpha 8$ subtype). In order to obtain an extract containing only the $\alpha 7$ subtype, we immunodepleted the original extract by means of incubation with anti- $\alpha 8$ antibodies bound to Sepharose 4B.

Immunoprecipitation experiments with anti- $\alpha 7$ and anti- $\alpha 8$ subunit-specific Abs were performed in order to verify the depletion of the subtypes containing the $\alpha 8$ subunit, and the depleted extract was plated on wells coated with anti- $\alpha 7$ Abs.

Figure 1 Chemical structure of the MG624 compound.

For the immunopurification of the $\alpha 7$ subtype, the $\alpha 8$ -depleted extract was then incubated overnight with anti- $\alpha 7$ Abs bound to Sepharose and after the resin had been extensively washed, the bound receptors were eluted with 0.2 M glycine pH 2.2, dialyzed, concentrated and lyophilized.

$\alpha 4\beta 2$ subtype Using [^3H]-Epi labelled receptors and Abs directed against the different nicotinic AChR subunits, we found that, although COL extract contains a number of nicotinic AChR subtypes ($\alpha 3\beta 2$, $\alpha 3\beta 4$, and $\alpha 4\alpha 5\beta 2$, $\alpha 4\beta 2$), the $\alpha 4\beta 2$ subtype represents 65–70% of the high affinity agonist binding sites.

In order to remove the $\alpha 3$ and $\beta 4$ -containing receptors, the extract (20 ml for each round) was first incubated with 5 ml of Sepharose-4B and bound anti- $\alpha 3$ Abs, and then with 5 ml of Sepharose-4B and bound anti- $\beta 4$ Abs (1 mg ml $^{-1}$ of purified Abs). The flowthrough was then incubated twice with Sepharose 4B with bound anti- $\alpha 5$ Abs in order to remove the $\alpha 4\alpha 5\beta 2$ subtype. Immunoprecipitation experiments with anti-subunit specific Abs and mAbs were performed in order to verify the depletion of the subtypes containing the $\alpha 3$, $\beta 4$ and $\alpha 5$ subunits, and the extract was then plated on wells coated with anti $\beta 2$ Abs.

For $\alpha 4\beta 2$ subtype immunopurification, the $\alpha 3$, $\beta 4$, and $\alpha 5$ depleted extract was incubated overnight with anti- $\beta 2$ Abs bound to Sepharose, and the receptors recovered as described for the $\alpha 7$ subtype.

Muscle subtype The $\alpha 1\beta 1\gamma\delta$ subtype was purified from foetal calf muscles, as previously described with minor modifications; the purified receptor was then plated on wells coated with mAb 73, a mAb directed against the $\beta 1$ subunit present in the muscle-type receptor (Gotti *et al.*, 1997b).

For $\alpha 1\beta 1\gamma\delta$ subtype immunopurification, the receptors purified on the α Bgtx column were incubated overnight with anti- $\beta 1$ mAbs bound to Sepharose, and the receptors processed as described for the $\alpha 7$ subtype.

Binding of nicotinic ligands to immobilized subtypes

The subtypes immobilized by the corresponding subunit-specific Abs were incubated overnight at 20°C with 250 μl of [^3H]-Epi or with 250 μl [^{125}I]- α Bgtx. All of the incubations were performed using a buffer containing (mM) Tris-HCl pH 7.5; NaCl 150; KCl 5; MgCl $_2$ 1; CaCl $_2$ 2.5; 2 mg ml $^{-1}$ BSA and 0.05% Tween 20 (buffer C). The [^3H]-Epi saturation binding experiments to the $\alpha 4\beta 2$ subtype were carried out in triplicate at concentrations ranging from 0.005–5 nM; for each [^3H]-Epi concentration, unspecific binding in the presence of 100 nM of cold Epi was evaluated. At the concentration of 2.5 nM [^3H]-Epi, a parallel inhibition experiment using 5–6 different concentration of cold Epi (from 1 nM–1000 nM) was performed.

The [^{125}I]- α Bgtx saturation binding experiments were performed in triplicate using concentrations of 0.01–10 nM with non-specific binding being determined in parallel by means of incubation in the presence of 1 μM α Bgtx. At the concentration of 3 nM [^{125}I]- α Bgtx, a parallel inhibition experiment using 5–6 different concentrations of cold α Bgtx (from 1 nM–1000 nM) was performed.

Testing direct saturation binding (the first 7–8 concentrations of respectively 0.005–5 nM for [^3H]-Epi and 0.01–10 nM for [^{125}I]- α Bgtx) and displacement (the last 5–6 concentrations of 1–1000 nM for both ligands) (Rovati *et al.*, 1991) in the same experiment make it possible to use the LIGAND programme to fit both the saturation and competition data

in order to search for the possible presence of sites with different affinity for Epi. This is because high Epi concentrations can be reached without using excessive amounts of labelled ligand (the competition part of the curve) and at the same time, adequate radioactivity can be retained in the lower concentration range (the saturation part of the curve).

In order to assess the pharmacological profiles of the immuno-immobilized subtypes we performed competition experiments using MG624, d-Tubocurarine (d-Tub) and MLA.

The drugs were dissolved in buffer C just before use and serial dilutions were preincubated for 30 min at RT. Subsequently a final concentration of 0.1 nM [^3H]-Epi was added to the $\alpha 4\beta 2$ subtype, and a final concentration of 0.5 nM or 0.3 nM of [^{125}I]- α Bgtx was added to the $\alpha 7$ or $\alpha 1\beta 1\gamma\delta$ subtypes. The $\alpha 4\beta 2$ and $\alpha 7$ subtypes were incubated overnight at 20°C, the $\alpha 1\beta 1\gamma\delta$ subtype was incubated for 48 h at 20°C.

After incubation, the wells were washed seven times with ice-cold PBS containing 0.05% Tween 20, and the bound radioactivity was recovered by means of incubation with 200 μl of 2N NaOH for 2 h. The bound radioactivity was then determined by means of liquid scintillation counting in a β counter in the case of [^3H]-Epi, or direct counting in a γ counter in the case of [^{125}I]- α Bgtx.

Data analysis The experimental data obtained from the saturation binding experiments were analysed by means of a non-linear least square procedure using the LIGAND program as described by Munson & Rodbard (1980). The parameters of the [^3H]-Epi binding to the $\alpha 4\beta 2$ subtype and [^{125}I]- α Bgtx binding to the $\alpha 7$ and $\alpha 1\beta 1\gamma\delta$ receptors obtained from at least three separate experiments were analysed using the LIGAND program. An 'extra sum of squares' F-test was performed by the program LIGAND to evaluate the different binding models statistically.

The K_i values of all of the tested drugs were determined by means of LIGAND, using the data obtained from three independent experiments. The experimental data from all of the subtypes were fitted to models with one and two binding site classes in order to test whether the fits were significantly better for the one- or two-binding site model (Munson & Rodbard, 1980).

cDNAs and oocyte injection

The cDNAs encoding the chick neuronal nicotinic AChR subunits and the chick muscle-type subunits were kindly provided by Dr Marc Ballivet. Full length cDNAs encoding wild type $\alpha 7$, L247T $\alpha 7$, the neuronal $\alpha 4\beta 2$ subtype or the muscle-type $\alpha 1\beta 1\gamma\delta$ receptor were expressed as previously described (Palma *et al.*, 1996a). The preparation of oocytes and nuclear injection procedures are detailed elsewhere (Bertrand *et al.*, 1991). Stage VI oocytes were injected intranuclearly (10 nl) with cDNA clones (0.2 mg ml $^{-1}$) using a pressure micro injector (Eppendorf, Germany) and a Singer micro manipulator (U.K.).

Electrophysiological recordings

Membrane currents were recorded 2–4 days after injection using a voltage-clamp technique with two microelectrodes filled with 3 M KCl. The oocytes were placed in a recording chamber (volume, 0.1 ml) and continuously perfused with Ringer's oocyte medium (mM): NaCl 82.5; KCl 2.5; CaCl $_2$ 2.5; MgCl $_2$ 1; HEPES 5/adjusted to pH 7.4 with NaOH) at

controlled room temperature (20–22°C). The recordings were usually made in the presence of atropine (0.5 μM) in order to prevent the stimulation of the muscarinic AChRs sometimes present in native oocytes (Miledi *et al.*, 1989). Since atropine can affect nicotinic responses, the results obtained in five oocytes (two donors) pre-treated or not with MG624 (100 nM) for 30 s before applying ACh (100 μM) in the presence of atropine (0.5 μM) were compared with those elicited in oocytes not treated with atropine. Atropine did not alter I_{ACh} nor reduce the I_{ACh} induced by MG624 in any of the examined oocytes. To construct dose/response relationships, the oocyte membrane potential was held at -60 mV, and the drugs applied at 3 min intervals. After each determination, control currents were elicited, twice in order to check for possible current rundown. The drugs were dissolved in Ringer's medium and applied by superfusing the oocytes at a rate of 12 ml min^{-1} . In the experiments designed to block nicotinic AChRs, the nicotinic blocker MLA (Palma *et al.*, 1996a) was coapplied with the neurotransmitter after MLA pre-treatment for 40 s. The solutions were exchanged by using electromagnetic valves (Type III; General Valve, U.S.A.). MG624 was dissolved just before the experiments.

Experimental analysis

The current records were digitized at 50–150 Hz using an analogue-to-digital converter (Digidata 1200 Interface, Axon Instruments, U.S.A.), and computer stored for subsequent analysis using pClamp 6.0.2 routines (Axon Instruments, U.S.A.). To estimate the half-inhibitory concentration (IC_{50}) of MG624, as well as the half dissociation constant (EC_{50}) of ACh, the data were fitted, by means of least-square routines (included in Sigma Plot, Jandel, Germany), to the following Hill equations:

$$I/I_{\text{max}} = \text{IC}_{50}n_{\text{H}} / ([\text{MG624}]n_{\text{H}} + \text{IC}_{50}n_{\text{H}}) \quad (1)$$

$$I/I_{\text{max}} = [\text{ACh}]n_{\text{H}} / ([\text{ACh}]n_{\text{H}} + \text{EC}_{50}n_{\text{H}}) \quad (2)$$

where $[\text{MG624}]$ and $[\text{ACh}]$ are the drug doses, n_{H} the Hill coefficient, and I_{max} the maximum response. For more details, see Miledi *et al.*, 1989 and Palma *et al.*, 1996a.

Materials

The lyophilized αBgtx , anti-protease inhibitors, cholinergic ligands, Triton X-100, anti-rabbit and anti-rat antisera were purchased from Sigma, U.S.A.; non-radioactive Epi, MLA, mAb 313, mAb 299 from RBI; CnBr-activated Sepharose 4BCL from Pharmacia, Sweden; [^{125}I]- αBgtx , [^{125}I]-Prot.A and [^3H]-Epi from Amersham, U.K.; and the reagents for gel electrophoresis from Bio-Rad Laboratories, U.S.A.

Results

Subunit composition of immunoimmobilized subtypes

In order to check the subunit composition of the receptors immuno-immobilized by our anti- $\alpha 7$, anti- $\beta 2$ and anti- $\beta 1$ Abs, we used the same Abs to immunopurify the receptors and determine their subunit composition by Western blotting. Figure 2A shows the results of the $\alpha 7$ subtype probed with anti- $\alpha 7$ (lane 4), anti- $\alpha 8$ Abs (lane 5), anti- $\alpha 3$ (mAb 313, lane 1), anti- $\alpha 4$ (mAb 299, lane 2), anti- $\alpha 5$ (mAb 35, lane 3) and anti- $\beta 2$ (lane 6) and anti- $\beta 4$ Abs (lane 7). Only the anti- $\alpha 7$ Abs were specifically able to recognize a peptide of the expected M_r of 57 KD.

Figure 2B shows the results of the $\alpha 4\beta 2$ immunopurified receptors probed with the same Abs and mAbs. Only the $\alpha 4$ -specific mAb 299 (lane 9) and our anti- $\beta 2$ Abs (lane 12) were able to recognize single polypeptides of respectively 66 and 54 KD. As expected, the anti- $\alpha 7$ Abs (lane 11) did not recognize any peptide (in agreement with previously reported data from Vernallis *et al.*, 1993; Gotti *et al.*, 1994), thus showing that the $\alpha 7$ -containing receptors are not promiscuous with the heteromeric receptors in terms of subunit(s).

Figure 2 Western blot analysis of the subunit composition of the immuno-immobilized nicotinic AChR subtypes: $\alpha 7$ (A), $\alpha 4\beta 2$ (B) and $\alpha 1\beta 1\gamma\delta$ (C). The receptors, which were immunopurified as described in Methods, were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose and probed with the indicated subunit-specific Abs or mAbs used at concentrations of 5–10 $\mu\text{g}/\text{ml}$. The anti- $\alpha 7$, $\alpha 8$, $\beta 2$ and $\beta 4$ Abs were those produced by us and described in Methods, whereas anti- $\alpha 3$ (mAb 313), anti- $\alpha 4$ (mAb 299) and anti- $\alpha 5$ (mAb 35) were commercially available mAbs. When mAbs were used, the blots were incubated for a further 2 h with anti-rat IgG diluted 1:1000. The bound Abs were revealed by means of [^{125}I]-Protein A. The molecular weight markers (top to bottom) are 97 KD, 66 KD, 45 KD, 31 KD and 21 KD.

We have also probed the $\alpha 7$ and $\alpha 4\beta 2$ subtypes for the presence of the $\alpha 2$ subunit (data not shown) using mAb 323 (a generous gift of Dr Lindstrom and used for the localization of the $\alpha 2$ subunit by Ullian & Sargent (1995) but we did not have any labelling. This mAb certainly recognizes the $\alpha 2$ subunit in immunolocalization but there are no data as to whether it works on Western blotting, and so we do not know if the lack of signal was due to the absence of the subunit or a lack of recognition.

We did not check for the presence of the $\alpha 6$ subunit in these subtypes because no specific Abs are available, but on the basis of the work of Fucile *et al.* (1998), showing undetectable levels of mRNA for the $\alpha 6$ subunit in the chick optic tectum, we believe that, if the $\alpha 6$ -containing subtype is present in COL it is certainly a minor subtype.

We also probed the purified $\alpha 1\beta 1\gamma \delta$ muscle-type subtype with mAbs specific for the $\alpha 1$ (mAb 155), $\beta 1$ (mAb 148), γ (mAb 162) and δ (mAb 137) subunits (Tzartos *et al.*, 1986); the results are shown in Figure 2C. Apart from mAb 148 (lane 15), which recognized the $\alpha 1$ subunit of M_r 42 KD in addition to the $\beta 1$ subunit of M_r 49 KD, the other mAbs only recognized the peptides of M_r 42, 55 and 58 KD corresponding to the $\alpha 1$ (lane 14), γ (lane 16) and δ subunits (lane 17).

Since it has been reported that the $\alpha 7$ subunit is present in embryonic muscle (Corriveau *et al.*, 1995), we probed the $\alpha 1\beta 1\gamma \delta$ receptors with our anti- $\alpha 7$ Abs produced against the chick $\alpha 7$ subunit, as well as with our anti- $\beta 2$ and anti- $\beta 4$ Abs, but we could not determine the presence of any of the three subunits (lanes 18, 19, 20) in this subtype.

Ligand binding to $\alpha 7$ and $\alpha 4\beta 2$ chick neuronal subtypes

The binding of [125 I]- α Bgtx to the immuno-immobilized $\alpha 7$ subtype has a K_d of 0.58 nM [Coefficient of variation (CV) = 17%], and MG624 competitively displaced this [125 I]- α Bgtx binding in a concentration-dependent manner with a K_i of 106 nM (CV 20%) (see Table 1). In the same experiments, we also tested two nicotinic antagonists as controls, (the $\alpha 7$ selective MLA and the non-selective d-Tub) for their ability to inhibit [125 I]- α Bgtx binding to the $\alpha 7$ subtype, and found K_i

values of 1.15 nM (CV 21%) for MLA and 1.57 μ M (CV 26%) for d-Tub (see Table 1). Figure 3A shows a representative binding experiment and the inhibition of the binding induced by MG624, MLA and d-Tub.

Several nicotinic AChR subtypes containing the $\beta 2$ subunit ($\alpha 3\beta 2$, $\alpha 3\beta 2\beta 4$, $\alpha 4\alpha 5\beta 2$, $\alpha 4\beta 2$) are present in COL membrane extract. The K_i value on the $\beta 2$ -containing subtypes that we have previously reported (K_i 69.5 μ M; Gotti *et al.*, 1998) is an average of the K_i values of the different subtypes. In order to determine the K_i value of only the $\alpha 4\beta 2$ subtype, we performed selective immunodepletion experiments to obtain an extract that only contained $\alpha 4\beta 2$ receptors. The $\alpha 4\beta 2$ subtype bound [3 H]-Epi with a K_d of 73 pM (CV 18%); the K_i values of MG624, MLA and d-Tub were respectively 84 μ M > 50 μ M and 19.6 μ M (see Table 1).

Figure 3B shows the results of a representative experiment of [3 H]-Epi binding to the $\alpha 4\beta 2$ subtype, and the inhibition of this binding by MG624, MLA and d-Tub.

Ligand binding to the muscle $\alpha 1\beta 1\gamma \delta$ subtype

Binding experiments showed that MG624 has a selective action toward the neuronal $\alpha 7$ subtype which, like the $\alpha 8$ and $\alpha 9$ subtypes, binds α Bgtx. Given that the superfamily of nicotinic AChRs includes the muscle $\alpha 1\beta 1\gamma \delta$ subtype (which, in addition to the $\alpha 7$, $\alpha 8$ and $\alpha 9$ subtypes, also binds α Bgtx), we tested the effects of MG624 on it by means of binding and inhibition experiments. The K_d of [125 I]- α Bgtx binding to the $\alpha 1\beta 1\gamma \delta$ subtype obtained from foetal calf muscle was 0.33 nM (CV 29%); the K_i of MG624 in competing with the [125 I]- α Bgtx binding was 32 μ M (CV 28%). As a control, we also tested the MLA and d-Tub antagonists, and found that their K_i were > 50 μ M and 98 nM (see Table 1).

Figure 3C shows a representative experiment of [125 I]- α Bgtx binding to the immuno-immobilized $\alpha 1\beta 1\gamma \delta$ subtype and the inhibition of the binding by MG624, MLA and d-Tub.

All of the competition data relating to the binding of the three drugs to the $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma \delta$ subtypes were fitted for one or two classes of binding site: and there was always a statistically better fit for the presence of only a single class of binding sites.

Effects of MG624 on wild type neuronal $\alpha 7$ nicotinic AChRs

Oocytes injected with wild type $\alpha 7$ subunit cDNA responded to ACh with an inward current (I_{ACh}) whose peak amplitude depended on the concentration of the transmitter. With 100 μ M ACh (EC_{50} ACh), applied to oocytes held at -60 mV, the mean current amplitude averaged 1.1 μ A (23 oocytes; three donors, 23/3; range: 99 nA to 3.9 μ A); and decayed with a time to 10% decay ($T_{0.1}$) of 0.13 ± 0.01 s (mean $\pm$ s.d.). Neither the non-injected oocytes nor the oocytes expressing $\alpha 7$ nicotinic AChRs, responded to MG624 (10 nM–1 μ M) with a detectable response above a noise level of 4 ± 1 nA. When MG624 was co-applied with ACh, the I_{ACh} amplitude was no different from the control. On the contrary, when the oocytes were pretreated with MG624 for 20–30 s before co-applying ACh, the I_{ACh} amplitude was considerably reduced. As no further reduction was observed when MG624 pretreatment was extended as much as 30–60 min, all of the other experiments were carried out using an exposure time of 30 s before the co-application of ACh. With this procedure MG624 (100 nM) reduced I_{ACh} peak amplitude by 59.7% at 100 μ M ACh without affecting current decay ($T_{0.1} = 0.13 \pm 0.03$ s). The inhibition of I_{ACh} by MG624 was dose-dependent; the IC_{50} was 109 nM

Table 1 Relative affinities (K_i) of MG624, MLA and d-Tub for immunoimmobilized subtypes, and MG624 relative inhibitory potencies (IC_{50}) on ACh-evoked responses in oocyte-expressed chick subtypes

Subtype	MG624 K_i (nM)	MLA K_i (nM)	d-Tub K_i (nM)	MG624 IC_{50} (nM)
$\alpha 7$	106 (20)	1.15 (21)	1570 (26)	109
$\alpha 4\beta 2$	84 000 (20)	> 50 000 (21)	19 600 (38)	3200
$\alpha 1\beta 1\gamma \delta$	32 200 (26)	> 50 000 (18)	98 (25)	2900

The K_d s of [125 I]- α Bgtx for the $\alpha 7$ and $\alpha 1\beta 1\gamma \delta$ subtypes were respectively 0.58 nM (CV = 17%) and 0.33 nM (CV = 29%), and the K_d of [3 H]-Epi for the $\alpha 4\beta 2$ was 73 pM (CV = 18%). The K_i values were derived from [125 I]- α Bgtx saturation and competition binding curves to the $\alpha 7$ and $\alpha 1\beta 1\gamma \delta$ subtypes, and [3 H]-Epi saturation and competition binding curves to the $\alpha 4\beta 2$ subtype. The curves obtained from three separate experiments were fitted using a non-linear least squares analysis program and the F test (Munson & Rodboard (1980)). The numbers in brackets represent the per cent CV. The MG624 IC_{50} values were obtained from inhibition experiments performed in oocyte-expressed subtypes using increasing concentrations of MG624 for 30 s before the application of ACh at their EC_{50} values (100 μ M for the $\alpha 7$ subtype, 2 μ M for the $\alpha 4\beta 2$ and 10 μ M for the $\alpha 1\beta 1\gamma \delta$ subtype).

$\alpha 7$ subtype $\alpha 4\beta 2$ subtype $\alpha 1\beta 1\gamma\delta$ subtype

Figure 3 Binding of nicotinic ligands to immuno-immobilized subtypes. (A) Saturation curves of total and specific [125 I]- α Bgtx binding to the $\alpha 7$ subtype (left), and the inhibition curves of specific [125 I]- α Bgtx binding to the immuno-immobilized subtype by nicotinic antagonists (right). For total binding (●), the immuno-immobilized $\alpha 7$ subtype was incubated overnight at 20°C with the indicated concentrations of [125 I]- α Bgtx and, for aspecific binding (○), also in the presence of 1 μ M cold α Bgtx. The total, aspecific binding and inhibition curves shown are those obtained from a representative experiment. (B) Saturation curves of total and aspecific [3 H]-Epi binding to $\alpha 4\beta 2$ (left) and the inhibition curves of specific [3 H]-Epi binding to the $\alpha 4\beta 2$ immuno-immobilized subtype by nicotinic antagonist (right). For total binding (●), the immuno-immobilized subtype was incubated overnight at 20°C with the indicated concentrations on [3 H]-Epi and, for aspecific binding (○), also in the presence of 100 nM cold α Bgtx. The total, aspecific binding and inhibition curves shown are those obtained from a representative experiment. (C) Saturation curves of total (●) and aspecific (○) [125 I]- α Bgtx binding to the $\alpha 1\beta 1\gamma\delta$ subtype (left), and the inhibition curves of specific [125 I]- α Bgtx binding to the immuno-immobilized subtype by nicotinic antagonists (right). The binding experiments were performed as described in A, except for the fact that the incubation time was 48 h at 20°C.

(Figure 4A, lower part) and remained similar when the Ca^{2+} was substituted by an equimolar concentration of Ba^{2+} (three oocytes, data not shown).

In order to exclude a possible channel block by MG624, we tested MG624 inhibition at different potentials because one of the characteristics of a channel blocker is that the block is voltage dependent. Our findings showed that the block of MG624 was not voltage dependent (Figure 5A) and the degree of block at different voltages was constant (as shown in Figure 5B). In addition, as previously reported (Palma *et al.*, 1996a, b), the I_{ACh} -voltage relationship showed a high degree of rectification at positive potentials, and this pattern was not modified by MG624 (100 nM) even though the current amplitudes were markedly reduced (Figure 5A). All of these findings together indicate that the effect of MG624 on $\alpha 7$ nicotinic AChR function is consistent with voltage-independent inhibition, and thus not compatible with a channel block.

Inhibitory effects of MG624 on the $\alpha 4\beta 2$ and on the muscle $\alpha 1\beta 1\gamma\delta$ subtypes

In order to compare the potency of MG624 in inhibiting the function of wild type $\alpha 7$ vs $\alpha 4\beta 2$ or $\alpha 1\beta 1\gamma\delta$ nicotinic AChR subtypes, experiments were performed in oocytes injected with

either $\alpha 4\beta 2$ or $\alpha 1\beta 1\gamma\delta$ subunit cDNAs. The upper part of Figure 4B shows a typical recording obtained from oocytes injected with $\alpha 4\beta 2$ subunits exposed to 2 μM ACh (left), 2 μM ACh + 1 μM MG624 (middle) and the recovery after 3 min washes (right). Upper part of Figure 4C shows a typical recording obtained from oocytes injected with $\alpha 1\beta 1\gamma\delta$ subunits exposed to 10 μM ACh (left), 10 μM ACh + 1 μM MG624 (middle) and the recovery after 3 min washes (right). The lower part of the same figures shows the dose-dependent inhibition of I_{ACh} by MG624 in the two subtypes. The inhibition was obtained by incubation with increasing concentrations of MG624 for 30 s before the application of ACh at about its EC_{50} value. The IC_{50} and n_{H} values were 3.2 μM and 1.2 ($n = 7$) for the $\alpha 4\beta 2$ subtype (ACh, 2 μM ; Fucile *et al.*, 1997, and 2.9 μM and 0.9 (ACh, 10 μM ; Mileo *et al.*, 1995) for the $\alpha 1\beta 1\gamma\delta$ subtype, thus indicating that the half inhibitory concentrations of MG624 for $\alpha 4\beta 2$ or $\alpha 1\beta 1\gamma\delta$ nicotinic AChRs were much higher than those of $\alpha 7$ nicotinic AChRs.

Effects of MG624 on L247T $\alpha 7$ nicotinic AChR

In order to obtain more information concerning the nature of the inhibitory activity of MG624, we tested it on the mutated L247T in which it is known that some nicotinic antagonists

Figure 4 MG624 dose- I_{ACh} response curves for neuronal and muscle nicotinic AChRs expressed in *Xenopus* oocytes. (A) $\alpha 7$ subunit cDNA-injected *Xenopus* oocyte; Top: current traces in an oocyte voltage-clamped at -60mV (ACh 100 μM , horizontal bar) before (left), during (middle), and after (right) treatment with MG624 (100 nM). Dashed or continuous line, MG624 and ACh applications, respectively. Note similar I_{ACh} decay in control vs treated oocyte. Bottom: peak current (mean $\pm$ s.e.mean) evoked by ACh coapplied with MG624 at the concentrations indicated in the abscissa, were normalized (in percentages) to the response to 100 μM ACh. Ordinate: normalized percentage I_{ACh} . The oocytes ($n = 12$, three donors) were pretreated for 30 s with MG624 at the same concentrations as those used for the MG624 coapplications with ACh. $\text{IC}_{50} = 109$ nM and $n_{\text{H}} = 2.5$ (estimated using equation 1, see Methods. (B) $\alpha 4\beta 2$ subunit cDNAs-injected oocytes. Top: ACh 2 μM , and MG624 1 μM . Bottom: peak current (mean $\pm$ s.e.mean) ($n = 7$, two donors) evoked by ACh coapplied with MG624 at the concentrations indicated in the abscissa, were normalized (in percentages) to the response to 2 μM ACh. The best fit gave an IC_{50} value for MG624 of 3.2 μM and an $n_{\text{H}} = 1.2$. Abscissa, ordinate, drug pretreatment, holding potentials and bars as in A. (C) $\alpha 1\beta 1\gamma\delta$ subunits cDNA-injected oocytes. Top: ACh, 10 μM and MG624 1 μM . Bottom: peak current (mean $\pm$ s.e.mean) ($n = 6$, two donors) evoked by ACh coapplied with MG624 at the concentrations indicated in the abscissa, were normalized (in percentages) to the response to 10 μM ACh. The best fit gave an IC_{50} value for MG624 of 2.9 μM and $n_{\text{H}} =$ value of 0.9. Abscissa, ordinate, drug pretreatment, holding potentials and bars as in A and B.

behave as agonists. In contrast to its failure to generate currents in oocytes expressing wild type $\alpha 7$ nicotinic AChR, MG624 applied alone to oocytes expressing L247T $\alpha 7$ nicotinic AChRs gave rise to an inward current (I_{MG624}) whose amplitude depended on the concentration of the drug. The MG624 dose- I_{MG624} relationship (Figure 6A) gave EC_{50} and n_{H} values for MG624 of 0.2 nM and 1.9, thus indicating that the level of cooperativity was similar, but the apparent affinity of MG624 for the L247T $\alpha 7$ mutant was 1000 times greater than that of ACh (MG624 EC_{50} = 0.2 nM and ACh EC_{50} = 0.2 μM ; Palma *et al.*, 1996a,b). The mean amplitude of I_{MG624} was 2.5 μA (range: 434 nA to 6 μA ; holding potential, -60 mV; 15/3) when MG624 was bath-applied at a concentration of 0.2 nM (EC_{50} MG624), and that of I_{ACh} was 2.1 μA (range: 80 nA to 5.8 μA , same oocytes) with an ACh concentration of 0.2 μM (EC_{50} ACh) (Figure 6B left and middle).

It is well known that the potent competitive nAChR antagonist MLA reduces the holding current required to clamp the cell expressing L247T $\alpha 7$ nicotinic AChRs vs wild type $\alpha 7$ (Betrand *et al.*, 1997). As expected the L247T $\alpha 7$ mutant-injected oocytes acutely exposed to MLA (500 pM, 6 oocytes/two donors) gave rise to an MLA-induced outward current and became unresponsive to MG624 (Figure 6C).

In order to investigate whether ACh alters the apparent affinity and cooperativity of the L247T $\alpha 7$ mutant for MG624,

Figure 5 Voltage independent inhibition of I_{ACh} by MG624 in oocytes expressing homomeric wild type $\alpha 7$ nAChRs. (A) Current-voltage relationships in an oocyte held at -60 mV. Peak currents evoked at various test potentials by ACh (100 μM , ■) or by ACh plus MG624 (100 nM, □) (representative of ten experiments, two donors). The solid lines represent two-order polynomial data fits. Note rectification at positive potentials and similar curve profiles. (B) Inhibition of I_{ACh} (ACh 100 μM) by MG624 (100 nM) at various holding potentials. The relative block was calculated as $[1 - (I_{\text{ACh}} + \text{MG624})/I_{\text{ACh}}] \times 100$. Each point is the mean $\pm$ s.e.mean of ten oocytes. ACh was applied at 3 min intervals, and the oocytes were pretreated with MG624 as in Figure 4.

the MG624 dose- I_{MG624} relationship was examined in the presence of ACh. The dose-response relationship fitted to a Hill equation shifted to the right in the presence of 0.2 μM ACh (Figure 6A), giving EC_{50} and n_{H} values of 0.67 nM and 1.5 respectively (10 oocytes/two donors) a finding that is consistent with a competitive action of MG624 on the L247T $\alpha 7$ mutant receptor.

Analysis at different transmembrane voltages showed that, as in the case of I_{ACh} , the amplitude of I_{MG624} increased almost linearly with potential (Figure 7). This linearity was maintained even at high MG624 concentrations, a behaviour that is incompatible with an open channel blockade. Furthermore, the reversal potential (V_{r}) of I_{MG624} was similar to that previously reported for I_{ACh} (Palma *et al.*, 1996b), thus indicating that the ionic basis of the responses to both substances was the same.

Figure 6 Current responses in oocytes expressing L247T $\alpha 7$ nicotinic AChRs. (A) Dose-response relationships for MG624 (○) and MG624 plus 0.2 μM ACh (●). The peak currents were normalized to the current evoked by MG624 at 10 nM. Each point represents the mean value ($n=10$, two donors); for the sake of simplicity, s.e.mean values $<0.3\%$ are not reported. The oocytes were held at -60 mV. (○): EC_{50} = 0.23 nM and n_{H} = 1.9 (estimated using equation 2, see Methods). (●): EC_{50} = 0.67 nM and n_{H} = 1.5. The average I_{max} was -3708 ± 551 nA for the MG624 currents, and -3273 ± 746 nA for ACh + MG624. (B) Current responses in the same oocyte held at -60 mV to 0.2 μM ACh (left), 0.2 nM MG624 (right); (C) I_{MG624} (MG624, 10 nM) blocked by MLA (500 pM). Note the outward current evoked by MLA and the absence of response to MG624. (Representative of six experiments, two donors). Dashed lines: MG624, continuous line: MLA application; holding potential as in A.

Figure 7 I_{MG624} and I_{ACh} membrane voltage relationships in an oocyte injected with L247T $\alpha 7$ subunit cDNA. The curves were obtained using voltage-ramps ranging from -90 mV to $+30$ mV. The MG624 and ACh concentrations were 0.2 nM and 0.2 μ M, respectively. Note the same curve profiles for both drugs and the same equilibrium potential (-13 mV).

Discussion

In a previous study, we demonstrated that two oxystilbene derivatives (MG624 and F3: Gotti *et al.*, 1998) are selective ligands for neuronal α Bgtx receptors and provided a preliminary characterization of the antagonistic effects of this class of compounds. In the present study, the interaction of MG624 with the $\alpha 7$ subtype has been better characterized by means of binding and electrophysiological studies, and its antagonistic selectivity towards the $\alpha 7$ homomeric chick subtype further demonstrated by comparing its effects with those obtained using the neuronal $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ muscle chick AChR subtypes. Furthermore, in order to obtain more information concerning the compound's mechanism of action, we tested its effect on the mutated L247T $\alpha 7$ subtype.

The binding studies showed that MG624 has a K_i of 106 nM, which is lower than that reported in our previous study (27 nM). This difference is probably due to the fact that the binding experiments in this study were performed using a purified $\alpha 7$ subtype (see Figure 2), whereas the previous data were obtained in a mixed population of $\alpha 7$ and $\alpha 7$ - $\alpha 8$ subtypes. In order to have a more complete pharmacological profile of the $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ subtypes used in the binding studies, we also tested two other nicotinic antagonists: the $\alpha 7$ -selecting antagonist MLA and the non selective d-Tub. We found that the K_i of MLA for the $\alpha 7$ subtype was in close agreement with published data (Ward *et al.*, 1990), whereas the K_i values for the $\alpha 4\beta 2$ subtype and the $\alpha 1\beta 1\gamma\delta$ muscle were higher than previously reported. The K_i values determined for d-Tub were very similar to those reported by other authors using the same nicotinic subtypes (Anand *et al.*, 1993; Whiting *et al.*, 1991a; Colquhoun & Rang 1976).

The specificity and selectivity of MG624 revealed by the binding studies was confirmed using the oocyte-expressed chick $\alpha 7$ subtype, MG624 had a dose-dependent inhibitory action on $\alpha 7$ nicotinic AChR, and this block did not increase at

hyperpolarizing potentials; the constant degree of the block at different voltages strongly suggests that the effect of this drug is not due to an open channel blocking mechanism. Moreover, when inhibited by MG624, the $\alpha 7$ nicotinic AChR showed positive cooperativity and the difference between the binding affinity and the IC_{50} to block $\alpha 7$ homomers was minimal. These results suggest that MG624 is a competitive inhibitor of the $\alpha 7$ receptor subtype.

Further information on the MG624 $\alpha 7$ subunit binding site comes from the experiments made using the mutated L247T $\alpha 7$ subtype (Revah *et al.*, 1991; Bertrand *et al.*, 1992). In particular, when tested on this mutated receptor, $\alpha 7$ antagonists can be classified into at least two main groups: the first includes the classical nicotinic antagonists (such as hexamethonium, d-Tub and dihydro- β -erythroidine), which elicit current responses; the second includes the selective $\alpha 7$ antagonists, (α Bgtx and MLA) which do not induce any currents but are capable of blocking the currents induced by hexamethonium, d-Tub and dihydro- β -erythroidine. Our results show that, when applied alone to L247T $\alpha 7$ mutant receptors at concentrations of even 500 times less than the IC_{50} of the $\alpha 7$ wild type, MG624 is still capable of giving rise to large currents that are blocked by low concentrations of MLA.

The current elicited by MG624 is linearly related to voltage and, as has been previously suggested by Bertrand *et al.*, 1992, incompatible with an open channel block mechanism. This effect distinguishes MG624 from both d-Tub and hexamethonium, both of which elicit currents that are reduced at hyperpolarized potentials. Furthermore, unlike that of 5-hydroxytryptamine (Palma *et al.*, 1996b), the action of MG624 on the L247T $\alpha 7$ receptor appears to be competitive in nature, since the receptor affinity for MG624 is influenced by ACh. The current elicited by MG624 in L247T $\alpha 7$ mutant receptors is blocked by the competitive antagonist MLA; moreover, these receptors show similar positive cooperativity despite the fact that they have a higher affinity for MG624 than for ACh. These findings indicate that MG624 activates L247T $\alpha 7$ receptors by acting on the same ACh site in the extracellular domain.

In conclusion, the MG624-induced competitive inhibition of the [125 I]- α Bgtx binding on $\alpha 7$ containing receptor (Gotti *et al.*, 1998), the linearity of the I-V relationship on the $\alpha 7$ receptors also in the presence of MG624, and the effects of ACh on the concentration-response relationship for MG624 in the mutated L247T $\alpha 7$, strongly suggest that MG624 is a selective competitive antagonist of the $\alpha 7$ receptor. The binding site of this drug only partially overlaps that of MLA (which blocks the effect of MG624 on the mutated receptor) because, unlike MLA, MG624 is capable of activating the mutated $\alpha 7$ receptor.

The binding studies showed that MG624 also binds to the chick neuronal $\alpha 4\beta 2$ and muscle $\alpha 1\beta 1\gamma\delta$ subtypes, and this was confirmed by its antagonist effect on oocyte-expressed subtypes. However, MG624 was respectively 30 and 27 times less potent on the $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ subtypes than on the $\alpha 7$ subtype.

Whereas the K_i and IC_{50} for the $\alpha 7$ subtype were almost identical, we found a difference between the K_i values and the IC_{50} for the $\alpha 4\beta 2$ and $\alpha 1\beta 1\gamma\delta$ subtypes (see Table 1) and can only speculate that this may be due to the presence of some essential and still uncloned subunit(s) in native receptors that can influence the affinity of drug binding (although we do not have any proof of such a presence) and/or the variability in the results obtained when different expression systems are used for electrophysiological experiments (Covernton *et al.*, 1994; Lewis *et al.*, 1997; Fucile *et al.*, 1997).

There is increasing evidence that the brain contains a large number of pharmacologically distinct nicotinic AChR subtypes that may be involved in mediating specific functions and behaviours. Experimental findings show that the $\alpha 7$ receptor is certainly involved in the modulation of synaptic transmission, CNS development and sensory gating (reviewed in Albuquerque *et al.*, 1997; Role & Berg 1996; Gotti *et al.*, 1997a), but further *in vivo* studies are necessary in order to clarify the complete physiological role of this subtype.

In vivo studies of $\alpha 7$ -mediated functions are hampered by the limited availability of $\alpha 7$ -specific ligands. Although a number of nicotinic agonists have recently been discovered (GTS-21, ABT418, Epibatidine) (Decker *et al.*, 1995; Gotti *et al.*, 1997a), all of them are more potent towards the $\alpha 4\beta 2$ subtype than towards the $\alpha 7$ subtype. The characterization of anabaseine as a potent agonist of α Bgtx-sensitive receptors has recently been reported (Kem *et al.*, 1997), but it has low K_i and EC_{50} values not only for $\alpha 7$ -containing receptors, but also for the $\alpha 4\beta 2$ and muscle type receptors.

The currently available $\alpha 7$ -selective antagonists are: α Bgtx, MLA and α conotoxin IMI; α Bgtx and MLA bind to and block the $\alpha 7$ subtype with very high affinity, specificity and potency, and both are more potent than MG624 in blocking $\alpha 7$ -

containing receptors, with an IC_{50} of respectively 0.52 nM and 25 pM (Johnson *et al.*, 1995; Palma *et al.*, 1996a). α Conotoxin IMI also specifically blocks the $\alpha 7$ homomeric receptor with an IC_{50} of 220 nM (Johnson *et al.*, 1995) a value that is higher than that of α Bgtx and MLA but very close to that of MG624. Although they are very specific, due to their toxic effects, these toxins are not very easy to use in *in vivo* studies aimed at delineating the roles of different nicotinic AChR subtypes in normal physiology.

In conclusion, MG624 is a powerful and selective competitive antagonist for the $\alpha 7$ subtype, and could serve as a lead structure for the development of new drugs with even greater $\alpha 7$ selectivity.

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