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4-oxystilbene compounds are selective ligands for neuronal nicotinic αBungarotoxin receptors

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1 Starting from the structure of an old 4-oxystilbene derivate with ganglioplegic activity (MG624), we synthesized two further derivates (F2 and F3) and two stereoisomers of F3 (F3A and F3B), and studied their selective effect on neuronal nicotinic acetylcholine receptor (AChR) subtypes.

2 MG 624, F3, F3A and F3B inhibited of ^{125}I-αBungarotoxin (αBgtx) binding to neuronal chick optic lobe (COL) membranes, with nM affinity, but inhibited ^{125}I-αBgtx binding to TE671 cell-expressed muscle-type AChR only at much higher concentrations.

3 We immobilized the α7, β2 and β4 containing chick neuronal nicotinic AChR subtypes using anti-subunit specific antibodies. MG 624, F3, F3A and F3B inhibited ^{125}I-αBgtx binding to the α7-containing receptors with nM affinity, but inhibited ^{3}H-Epi binding to β2-containing receptors only at very high concentrations (more than 35 μM); their affinity for the β4-containing receptors was ten times more than for the β2-containing subtype.

4 Both MG624 and F3 compounds inhibited the ACh evoked currents in homomeric oocyte-expressed chick α7 receptors with an IC_{50} of respectively 94 and 119 nM.

5 High doses of both MG 624 and F3 depressed the contractile response to vagus nerve stimulation in guinea pig nerve-stomach preparations although at different IC_{50}s (49.4 *vs* 166.2 μM) The effect of MG624 on rat nerve-hemidiaphragm preparations was 33 times less potent than that of F3 (IC_{50} 486 *vs* 14.5 μM).

6 In conclusion, MG624 and F3 have a high degree of antagonist selectivity for neuronal nicotinic αBgtx receptors containing the α7 subunit.

Keywords: 4-oxystilbene; neuronal nicotinic receptors; subtypes; αBungarotoxin receptors; αBungarotoxin; Epibatidine; chick optic lobe

Introduction

Neuronal nicotinic acetylcholine receptors (AChRs) are widely distributed in the peripheral and central nervous systems. They are known to be involved in the control of ganglionic transmission and complex brain processes such as attention, memory, locomotor activity, nociception, temperature control and excitability; furthermore, they are also involved in a number of brain pathologies and in smoking addiction (reviewed in Dani & Heinemann, 1996; Role & Berg, 1996; Gotti *et al*., 1997a). They are localised in both postsynaptic structures, where they are responsible for nicotinic transmission, as well as in presynaptic boutons, where they modulate the release of a number of neurotransmitters (reviewed in Wonnaccott, 1997).

Neuronal nicotinic AChRs are a family of ACh-gated cation channels, which have a pentameric structure that is similar to that of muscle AChRs. Eight alpha (α2–α9) and three beta (β2–β4) subunits coding for neuronal nicotinic AChR subunits have been cloned in vertebrates. Expression studies in heterologous systems have demonstrated that at least two classes of nicotinic receptors can be generated: one by only expressing a single type of α subunit, as in the case of the α7, α8 and α9 subtypes (homomeric receptors), and the other by means of the pairwise combination of one kind α subunit other than that forming the homomeric receptors and one kind of β subunit (heteromeric receptors). Binding studies have also confirmed the presence of the two classes: the first includes the receptors containing either α7 or α8 subunits, or both, and bind αBungarotoxin (αBgtx) with high affinity ; the second includes the receptors that bind nicotinic agonists with very high affinity (pM-nM) but do not bind αBgtx. The various subtypes have different pharmacological and electrophysiological properties (reviewed in Sargent, 1993; Papke, 1993; McGehee & Role, 1995; Gotti *et al*., 1997a).

The role and function of most of these neuronal nicotinic AChR subtypes are difficult to establish because individual neurons can coexpress various subunit combinations. Recent immunoprecipitation and immunolocalization experiments have demonstrated that the subunit composition of native receptors is more complex than was first thought, with certain subtypes being made up of as many as three or four different subunits (Conroy & Berg, 1995; Forsayeth & Kobrin, 1997).

The most abundant subtype found in vertebrate brain is the α4β2 subtype, whereas little or no α4 subunit is present in autonomic neurons, whose major subtype contains the α3 and β4 subunits. The α7-containing subtype accounts for most of the high affinity αBgtx binding sites present in both the central

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and peripheral nervous systems (reviewed in McGehee & Role, 1995).

One of the goals of recent research in the nicotinic field has therefore been to find substances that specifically recognise different receptor subtypes in order to help in the characterisation of neuronal nicotinic AChRs and, possibly, in the development of drugs that may be useful in the treatment of some of the pathologies in which neuronal nicotinic AChRs are involved. A number of old and new compounds have been investigated in both binding and functional studies, but few of them show selective specificity for neuronal nicotinic AChR subtypes (recently reviewed by Gotti *et al*., 1997a).

Recent advances in the search for novel neuronal nicotinic AChRs ligands include the discovery of 2-(3-pyridyloxymethyl) azacyclic compounds **I** that have subnanomolar affinity for rat brain [^{3}H]-(−)-cytisine binding sites (Abreo *et al*., 1996), as well as their analogues **II**, in which the pyridine ring has been bioisosterically replaced by a substituted aryl moiety (Elliott *et al*., 1996) (Figure 1). The K_i values of the latter compounds for rat brain [^{3}H]-(−)-cytisine binding sites ranges from 3 to >10,000 nM depending on the aromatic substitution. Electron-withdrawing groups at the *meta* position improve K_i values, whereas substitution at the *orto* and *para* positions is generally unfavourable.

On the basis of these results and the previously known effects of 4-oxystilbene derivatives on ganglionic neuronal nicotinic AChRs (Mantegazza & Tommasini, 1955), we decided to explore compounds **1-3**, (*S*)-**3** and (*R*)-**3** (Figure 2) as potentially subtype-selective neuronal nicotinic AChR ligands.

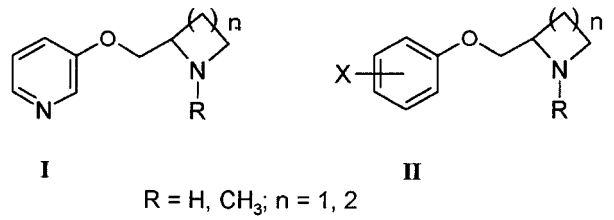

Figure 1 Chemical structure of 2-(3-pyridyloxymethyl) azacyclic compounds (**I**) (taken from Abreo *et al*., 1996) and their analogues (**II**) (taken from Elliott *et al*., 1996).

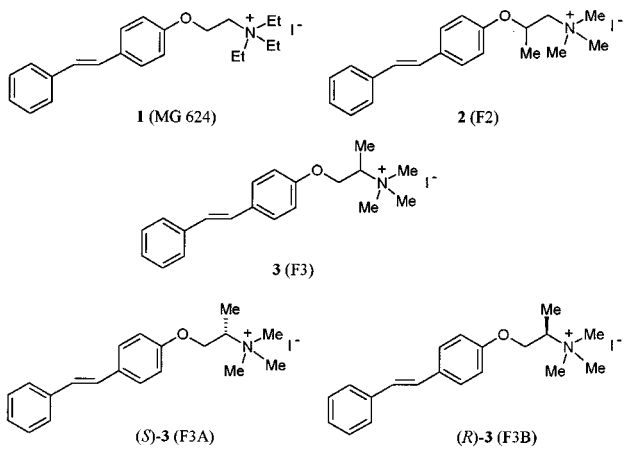

Figure 2 Chemical structure of 4-oxystilbene compounds.

Methods

Chemical synthesis

The synthesis of N,N,N-triethyl-2-(4-*trans*-stilbenoxy)ethylammonium iodide **1** (MG 624) was described by Cavallini *et al*. (1953). N,N,N-trimethyl-2-(4-*trans*-stilbenoxy)propylammonium iodide **2** (F2) and N,N,N-trimethyl-1-(4-*trans*-stilbenoxy)-2-propylammonium iodide **3** (F3) were prepared by treating the corresponding N,N-dimethyl derivatives with iodomethane. A mixture of F2 and F3 was previously obtained from *trans*-4-hydroxylstilbene and 2-dimethylaminoisopropyl chloride and then resolved by means of chromatography before conversion to the quaternary ammonium salts **2** and **3**.

Racemic N,N-dimethyl-1-(4-*trans*-stilbenoxy)-2-propylamine, the immediate precursor of **3**, was subjected to optical resolution with dibenzoyltartaric acid to yield the two enantiomeric tertiary amines, that were subsequently transformed into the quaternary ammonium salts (*S*)- and (*R*)- **3** (F3A and F3B) (see Figure 2).

Preparation of chick optic lobe and retina membranes and extracts

Chick optic lobes (COLs) and retinas were dissected from 1-day old chickens, immediately frozen in liquid nitrogen and stored at −80°C for later use. No differences in the binding properties of fresh and frozen tissues were observed (Gotti *et al*., 1994, 1997b). For every experiment, the two types of membranes were separately homogenized in an excess of 50 mM Na phosphate pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA and 2 mM PMFS for 2 min in an ultraTurrax homogenizer. The homogenate was then diluted and centrifuged for 1 h at 60,000 g. This procedure of homogenization, dilution and centrifugation was done three times, after which the pellets were collected, rapidly rinsed with 50 mM Na phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM PMFS and then resuspended in the same buffer containing a mixture of 5 μg/ml 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 membranes and the membranes were extracted for 2 h at 4°C.The extract was then centrifuged for 1.5 h at 60,000 g, and the clear supernatant recovered.

TE671 cell membranes

TE671 cells obtained from the American Type Culture Collection were grown at confluency for ten days in petri dishes in RPMI medium containing 10% fetal calf serum, 2 mM glutamine, and 100 mg/ml penicillin and streptomycin The cells were detached using phosphate buffered saline (PBS) containing 1 mM PMFS, washed twice by centrifugation, resuspended in 50 mM Tris HCl pH 7.4 containing 50 mM Na Cl 2 mM EDTA, 2 mM EGTA mM and then homogenized. A 5 μg/ml mixture of the protease inhibitors leupeptin, bestatin, pepstatin A, aprotinin and 1 mM PMFS was added to the membrane homogenate.

Peptide synthesis and polyclonal antibody production

The peptides and antibodies (Abs) were produced as previously described (Gotti *et al*., 1994). Anti-α7 and anti-β2 chick-specific antibodies (Abs) were produced in rabbits by

means of immunization with peptides chosen from the most divergent region of the α7 and β2 subunits (the cytoplasmic loop between M3 and M4) and against peptides located at the COOH terminal for the β2 and β4 subunits.

The specificity of the polyclonal Abs was tested in ELISA for possible crossreactivity with other peptides obtained from AChR subunits, as well as in immunoprecipitation experiments and on Western blots of purified receptors, and the Abs were found to be specific for the subtypes containing the corresponding subunit. The Abs raised against the α7, β2 and β4 peptides were purified on an affinity column made by coupling the corresponding peptide to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Receptor immobilization by subunit-specific antibodies

The affinity-purified Abs were bound to the wells by overnight incubation at 4°C at a concentration of 10 μg/ml 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 μl of 2% Triton X-100 extract obtained from COL membranes in the case of the α7 and β2-containing receptors, and from chick retina extract in the case of the β4-containing receptors. After overnight incubation with the extract, the wells were washed and the presence of immobilized receptor was revealed by means of ^{125}I-αBgtx or ^{3}H-Epi binding.

Receptor subtype immunopurification

The chick optic lobe (COL) and retina extracts were prepared as described above. The α7-containing receptors were immunopurified as previously described (Gotti *et al*., 1994). The β2-containing receptors were purified from the COL extract and, in order to remove the β4-containing receptors, the extract (20 ml for each round) was first incubated with 5 ml of Sepharose-4B with bound anti-β4 Abs (1 mg/ml of purified Abs) and then the flowthrough of this column was incubated twice with Sepharose 4B with bound anti-β2 Abs. The β4-containing receptors were purified from chick retina extract, which was first incubated with 5 ml of Sepharose-4B and bound anti-β4 Abs (1 mg/ml of purified Abs). The bound receptors were eluted with 0.2 M glycine (pH 2.2) or with the peptides used for the immunization.

After elution of receptors with either glycine or peptides, the receptors were desalted and concentrated on a Centricon 10 microconcentrator (Amicon).

The recovery at each immunoprecipitation step was determined by means of ^{125}I-αBgtx or ^{3}H-Epi binding and by quantitative immunoprecipitation of the receptors present in the solution before and after each immunopurification as previously described (Gotti *et al*., 1994).

^{3}H-Epibatidine and ^{125}I-αBungarotoxin binding

Membranes All of the membranes used for the binding assays were previously washed by centrifugation with a buffer containing 50 mM Tris-HCl pH 7, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ (buffer A). Saturation experiments were performed by incubating aliquots of COL, and TE671 membranes with ^{125}I-αBgtx at 4°C for 24 and 48 h respectively in the same buffer A plus 2 mg/ml BSA. To the incubation mixture a final concentration of 5 μg/ml of the protease inhibitors leupeptin, bestatin, pepstatin A, aprotinin and 2 mM PMFS was added in order to block possible proteolysis during the long incubation time of the assays.

Non-specific binding (averaging 25–30% of total binding) was determined in parallel by means of incubation in the presence of 2 μM unlabeled αBgtx. At the end of the incubation, the samples were filtered on GFC filters presoaked in PBS + 1% BSA through a Brandell-apparatus and the filters counted in a γ counter.

Saturation experiments with ^{3}H-Epi were performed overnight by incubating aliquots of COL membranes with ^{3}H-Epi concentrations ranging from 0.005 to 5 nM at 20°C.

Non-specific binding (averaging 10–15% of total binding) was determined in parallel by means of incubation in the presence of 100 nM–1 μM unlabeled Epi.

Immobilized subtypes The subtypes immobilized by the corresponding subunit-specific Abs were incubated overnight at 20°C with 300 μl of ^{3}H-Epi ranging from 0.005–5 nM, or with 300 μl ^{125}I-αBgtx ranging from 0.01–20 nM. All the incubations were performed in a buffer A plus 2 mg/ml BSA and 0.05% Tween 20. Specific labeled ligand binding was defined as total binding minus the binding in the presence of 100 nM cold Epi or 2 μM αBgtx. The inhibition of ^{3}H-Epi and ^{125}I-αBgtx binding to the immobilized subtypes by the tested compounds, was measured by preincubating the indicated concentrations of compounds for 30 min at RT, followed by overnight incubation with 0.05 nM ^{3}H-Epi or 0.25 nM ^{125}I-αBgtx at 20°C.

The subtypes immobilized by the corresponding subunit-specific Abs were incubated overnight at 20°C with 300 μl of ^{3}H-Epi or ^{125}I-αBgtx. The binding experiments of ^{3}H-Epi and ^{125}I-αBgtx were performed according to a mixed protocol combining both saturation (the first 7–8 concentrations of respectively 0.005–5 nM and 0.01–20 nM) and displacement curves (the last 3–4 concentrations of 50–1000 nM for both ligands) (Rovati *et al*., 1991).

By effectively combining both saturation and competition protocols in a single curve, high ligand concentrations can be reached without using excessive amounts of labeled ligand (the competition part of the curve), while retaining adequate radioactivity in the lower concentration range (the saturation part of the curve). To study the interaction of the heterologous ligands, 0.05 nM of ^{3}H-Epi or 0.25 nM ^{125}I-αBgtx were used in displacement studies: the non-specific binding was respectively 10–15% and 25–30% of the total binding for ^{3}H-Epi and ^{125}I-αBgtx, (calculated by LIGAND as one of the unknown parameters of the model).

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

Data analysis The experimental data obtained from the saturation binding experiments to membranes or immunoimmobilized subtypes were analyzed by means of a non-linear least square procedure using the LIGAND program as described by Munson and Rodbard (1980). The calculated binding parameters were obtained by simultaneously fitting two independent experiments for the membranes and three independent experiments. An 'extra sum of squares' F-test was performed by the LIGAND program to evaluate the different binding models statistically (i.e., one site *vs* two site models, comparison of the binding parameters, etc.) (Munson & Rodbard, 1980).

The K_i values of all of the tested drugs were determined by means of LIGAND, using the data obtained from 3–4 independent experiments.

The equilibrium ligand binding data were analysed by means of the LIGAND computer program, (Munson & Rodbard, 1980). Models of increasing complexity were considered. The selection of the best fitting model and evaluation of the statistical significance of the parameter difference was based on the F-test for the extra sum of square principle (Draper & Smith, 1966).

A statistical level of significance of $P<0.05$ was accepted. The calculated binding parameters were obtained by simultaneously fitting three independent experiments (six curves, three homologous and three heterologous curves). Binding is expressed as the ratio of the bound concentration over total ligand concentration (B/T) *vs* the logarithm of total concentration. The total concentration is the sum of 'hot' and 'cold' ligand. All of the curves shown were computer generated.

Oocyte injection and electrophysiological recordings

The cDNA encoding the chick neuronal nicotinic α7 subunit was kindly provided by Dr Marc Ballivet. Full-length cDNA encoding wild type α7 was expressed as previously described (Palma *et al*., 1996). Stage VI oocytes were injected intranuclearly (10 nl) with cDNA clones (0.2 mg/ml) using a pressure micro injector (Eppendorf, Germany)

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) continuously perfused with oocyte Ringer's medium (82.5 mM NaCl/2.5 mM KCl/2.5 mM $CaCl_2$/1 mM $MgCl_2$/5 mM HEPES/adjusted to pH 7.4 with NaOH) at controlled room temperature (20–22°C), usually in the presence of atropine (0.5 μM) (Miledi *et al*., 1989).

In order to construct dose/response relationships, the oocyte membrane potential was held at −60 mV and the drugs were applied at 3 min intervals.

To estimate the half-inhibitory concentration (IC_{50}) of MG 624 and F3, the data were fitted to Hill equations using least-square routines (included in *Sigma Plot*, Jandel, Germany): $I/I_{max} = IC_{50}\ nH/([MG\ 624\ or\ F3]nH + IC_{50}\ nH)$ (*1*) where [MG 624] is the drug dose, nH is the Hill coefficient, and I_{max} the maximum response. For more details, see Miledi *et al*. (1989) and Palma *et al*. (1996).

In vitro *preparations*

To reveal the selectivity and functional activity of the compounds on ganglionic and muscle type AChRs, we studied their effects on guinea pig vagus nerve-stomach and rat phrenic nerve hemidiaphragm preparations. The rats and guinea pigs were killed and exsanguinated under pentobarbital anaesthesia. The guinea pig stomachs and attached vagus nerves were removed and mounted in a 100 ml organ bath according to the method described by Paton and Vane (1963). Using insulated platinum ring electrodes, the nerves were stimulated with rectangular 0.2 msec pulses at a frequency of 20 Hz and a voltage of 0.2 V for a period of 10 s every 2 min. The contractions of the stomach were recorded by means of a pressure transducer (HP267BC).

The isolated left phrenic nerve-hemidiaphragm was prepared as described by Bulbring (1946). Indirect stimulation of the hemidiaphragm was elicited via a bipolar silver wire electrode on the phrenic nerve using 1 ms pulses at a frequency of 0.1 Hz, and a voltage three times greater than that necessary to produce maximal twitches. Direct stimulation was elicited using stimuli of the same type and frequency delivered via a field electrode. The contraction of the muscle was recorded using an isometric transducer (HP-FTA 100-1).

The data for both preparations were obtained in seven separate experiments. For both tissues, the IC_{50} and 95% confidence limits were calculated from the dose-response curves of MG 624 and F3.

Results

Antibody specificity

Since the validity of our pharmacological experiments on the immobilized subtypes is completely dependent on the Abs used to immunoimmobilize the subtypes, special care was taken to characterize the specificity of the Abs used by means of immunoprecipitation and Western blotting experiments.

Anti-α7 Abs When tested by immunoprecipitation, the anti α7-Abs (mean ± sem; $n=3$) were capable of immunoprecipitating 95% of the ^{125}IαBgtx receptors present in the COL extract: 70 ± 2% of receptors contain the α7 subunit and 25 ± 0.8% contain both the α7 and α8 subunit, as already reported by Gotti *et al*. (1994).

Since COL extract contains a large number of receptors that bind ^{3}H-Epi with high affinity, we tested our anti-α7 Abs for their capacity to immunoprecipitate the high affinity ^{3}H-Epi labeled receptors, and found that they could not immunoprecipitate any of the ^{3}H-Epi labeled receptors. The anti-α7 Abs were used to immunopurify the α7-containing receptors from COL extract and to study the subunit composition of these receptors. Figure 3 (left) shows the immunopurified α7 receptors run on SDS–PAGE probed with the anti-α7, anti-α8, anti-β2 and anti-β4 Abs. In this immunopurified α7 containing receptor, both anti-α7 and anti-α8 Abs were capable of recognizing a single band of M_r 57 KD (albeit at different intensities) whereas the anti-β2 and anti-β4 Abs could not recognize any peptide.

The recognition by the anti-α8 Ab is not due to cross-reactivity, but to the presence of the α7-α8 subtype that contains both the α7 and α8 subunits (Gotti *et al*., 1994, 1997b).

Anti-β2 Abs The anti-β2 Abs, directed against the cytoplasmic peptide, as well as those directed against the COOH peptide, were capable of immunoprecipitating 95 ± 2% of the ^{3}H Epi high affinity labeled receptors. The amounts of receptor immunoprecipitated by these polyclonal Abs were almost identical to those immunoprecipitated by mAb 270, which specifically recognizes the β2 subunit (Whiting *et al*., 1987) and, in our hands, immunoprecipitated 90% of the ^{3}H-Epi high affinity site.

In order to identify the other subunits present in the COL extract together with the β2 subunit, we performed immunoprecipitation experiments using subunit specific mAbs. To this end, we used anti-α3 (mAb 313), anti-α4 (mAb 299), anti-α5 (mAb 35), and anti-β4 Abs, and found that they respectively immunoprecipitated 15 ± 2, 70 ± 4, 28 ± 2 and 10 ± 1% of the ^{3}H-Epi high affinity binding sites. The results of these immunprecipitation experiments together with those of immunodepletion experiments not reported here, suggest that the β2-containing receptors represent an heterogeneous population of receptor subtypes containing the α3β2β4, α3β2,

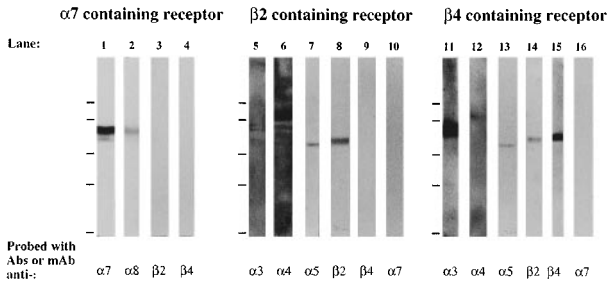

Figure 3 Western blot analysis of the immunoisolated receptor subtypes. (*Left*) α7 containing receptors purified from chick optic lobe extract using anti-α7 Abs, (*Center*) β2 containing receptors purified from chick optic lobe extract using anti-β2 Abs and (*Right*) β4 containing receptors purified from retina extract using anti-β4 Abs. The immunopurified receptors 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 μg/ml. The anti α7, α8, β2 and β4 Abs were those produced by us and described in Methods section, whereas the anti α3(mAb 313), anti α4(mAb 299) and anti-α5 (mAb35) were the Abs commercially available. The bound Abs were revealed by means of ^{125}I-Protein A. When mAbs were used, the blots were incubated for a further 2 h with anti-rat IgG diluted 1 : 1000. The molecular weight markers (top to bottom) are 97 kD, 66 kD, 45 kD, 31 kD and 21 kD.

α4α5β2 or α4β2 subunits; in this population, the pure α4β2 subtype certainly represents 65% of the ^{3}H-Epi-binding, the α4 α5β2 represents 20±4% and the other subtypes together represent no more than 15%.

Our immunoprecipitation experiments using anti-subunit specific Abs detected the presence of a small amount of β4 containing receptors (10±3%) in COL extract. In order to check the real specificity of our anti-β2 Abs, we first immunodepleted the COL extract of the β4 containing receptors and then used the anti-β2 Abs to immunopurify the β2-containing receptor. The immunopurified receptor was then probed with the anti-α3 (mAb 313), anti-α4 (mAb 299), anti-α5 (mAb35), anti-α7, anti-β2 and anti-β4 Abs. The results are shown in Figure 3 (middle). mAb 313, mAb 299, mAb35 and anti-β2 Abs recognized only a single peptide of respectively M_r 57±1, M_r 66, M_r 52±1 and 54±1 kD; the anti-α7 and anti-β4 Abs did not recognize any peptide.

Anti-β4 Abs The anti-β4 Abs in chick retina extract were tested in 3 separate experiments and found to immunoprecipitate 95±2% of the ^{3}H-Epi high affinity binding, whereas mAb 313, mAb 299, mAb 35 and anti-β2 Abs respectively immunoprecipitated 66±2, 55±3, 17±1,5 and 40±0.8%, thus suggesting the presence of several neuronal nicotinic AChR subtypes also in this tissue, although those containing both the α3 and β4 subunits represent the large majority (≈70%).

Using anti-β4 Abs, we immunopurified the β4-containing chick retina receptors, ran them on SDS–PAGE and probed them with subunit-specific mAbs and Abs. The results are shown in Figure 3 (right). mAb 313, mAb 299, mAb35, anti-β2 and anti-β4 recognized a single peptide of respectively 57, 66, 52, 54 and 54 kD, whereas the anti-α7 Abs did not recognize any peptide.

The molecular masses of the α3, α5, α7, α8, β2 and β4 subunits, determined by Western blot corresponded to the expected sizes deduced from their cDNA sequences, whereas the molecular mass of the α4 subunit was slightly lower.

In conclusion, on the basis of the results of our previous (Gotti *et al*., 1994, 1997b) and present experiments, the polyclonal Abs raised against the peptides of the α7, β2 and β4 subunits specifically recognize the native receptors containing those subunits, both in immunoprecipitation and after SDS–PAGE.

Table 1 Comparison of MG 624, F2, F3, F3A, and F3B binding affinities at the COL and TE671 membrane bound receptors

Tissue: Ki	*Col* μM	*TE671* μM
MG 624	0.055 (14)	70 (20)
F2	26.5 (33)	196 (16)
F3	0.109 (29)	77 (19)
F3A	0.092 (46)	59 (22)
F3B	0.094 (39)	38.2 (21)
^{125}I-αBgtx affinity (Kd)	2 nM (27)	0.5 nM (20)

The Kd and K_i values were derived from ^{125}I-αBgtx saturation and competition binding curves to the COL and TE671 membranes. The curves obtained from two separate experiments were fitted using a non-linear least squares analysis program and the F test (Munson and Rodboard). The numbers in brackets represent the % of CV.

Ligand binding to COL and TE671 membranes

The binding of ^{125}I-αBgtx to COL membranes has a Kd value of 2 nM [coefficient of variation (CV) = 27%] and a B_{max} of 362±35 fmol/mg of protein. MG 624, F2 and F3, and the two stereoisomers of F3 (F3A and F3B) competitively displaced ^{125}I-αBgtx binding in a concentration-dependent manner although with different potencies; the K_i values reported in Table 1, which were obtained from the competition experiments, were respectively: 0.055, 26.5, 0.109, 0.092 and 0.094 μM.

The least potent of the five compounds was F2 (481 and 243 times less potent than MG 624 and F3 respectively); the two F3 enantiomers had very similar potencies, thus indicating their lack of stereoselectivity.

In addition to the receptor class that binds αBgtx with high affinity, chick brain membranes also express another class of nicotinic receptors that do not bind αBgtx but bind nicotinic agonists with high affinity. This is composed of several subtypes (α4β2, α4β2α5, α3β2) and is labeled with high affinity by the newly discovered nicotinic agonist Epi (Gerzanich *et al*., 1995).

We used ^{3}H-Epi as a ligand of this class of receptors and found that it binds to chick optic lobe membranes with a Kd of 64 pM (CV = 25%) and a B_{max} of 86±12 fmol/mg of protein. When tested in competition experiments using compound concentrations, ranging from 1 nM to 50 μM, none of the five compounds could inhibit ^{3}H-Epi binding to COL membrane.

In order to obtain a complete pharmacological profile of these compounds, we also tested them on membranes obtained from TE671 cells, which are known to express a muscle type nicotinic AChR. We found that ^{125}I-αBgtx binds to this receptor with a Kd of 0.52 nM (CV = 20%) and a B_{max} of 133±10 fmol/mg of protein; all five compounds inhibited binding only at very high concentrations, with K_i values respectively of 70, 196, 77, 59 and 38.2 μM, and with a very similar potencies (see Table 1).

Ligands binding to immobilized subtypes

Previous experiments (Gotti *et al*., 1994) as well as those reported above for the anti-α7 and anti-β2 Abs, showed that

anti-α7 Abs immunoprecipitate 90% of the ^{125}I-αBgtx binding sites and that anti-β2 Abs immunoprecipitate more then 90% of the high affinity ^{3}H-Epi sites present in COL extract. This indicates that the binding of ^{125}I-αBgtx is predominantly to an α7-containing subtype, whereas that of the high affinity ^{3}H-Epi is to a β2-containing subtype. In the competition experiments performed on COL membranes, we found that MG 624 and F3 were selective in inhibiting ^{125}I-αBgtx binding, whereas they did not have any effect on the inhibition of ^{3}H-Epi. In order to identify the subtype target of these compounds, we decided to test them on subtypes specifically immuno-immobilized by the corresponding Abs. Furthermore, since these compounds are reported to have ganglioplegic effects, we also decided to test them on the α3β4 containing subtype, a ganglionic type of nicotinic receptor which is present in high concentrations in the chick retina.

Using subunit-specific antibodies, we immobilized the α7- and β2-containing receptors present in the Triton X-100 extract obtained from COL, and the β4-containing receptors present in the Triton X-100 extract obtained from chick retina. The receptors immobilized on the anti-α7 specific Abs have a Kd for ^{125}I-αBgtx of 0.5 nM (CV = 27%). The K_i of the receptors for the compounds are shown in Table 2. Figure 4 shows the inhibition curves of MG 624, F2 and F3 on the binding of ^{125}I-αBgtx. The most potent compound in inhibiting ^{125}I-αBgtx binding to the α7 subtype was MG 624: the order of potency of MG 624 > F3 > F2.

The immobilized β2-containing receptors gave a Kd for ^{3}H-Epi of 36 pM (CV = 37%). The K_i values of the compounds are reported in Table 2. Figure 5 shows the inhibition curves of MG 624, F2 and F3 on the binding of ^{3}H-Epi to the β2-containing subtypes. Confirming the results obtained on membranes, for MG 624 and F3, K_i values of respectively 69.5 and 39 μM were more than three (for MG624) and almost three (for F3) orders of magnitude less potent in displacing ^{3}H-Epi to the β2-containing subtype than in displacing ^{125}I-αBgtx to the α7 subtype. F2 also had a very low potency on this subtype, with a K_i = 374 μM.

In order to obtain a complete pharmacological profile of the effects of the compounds on the different neuronal nicotinic AChR subtypes, we tested them on the β4-containing subtypes immunoimmobilized on the anti-β4 Abs. ^{3}H-Epi binds to these receptors with a Kd of 20 pM (CV = 27); Figure 6 shows the inhibition curves of MG 624, F2 and F3 on the binding of ^{3}H-Epi to the β4 containing subtypes (K_i values in Table 2).

Table 2 Comparison of MG 624, F2, F3, F3A and F3B binding affinities to the immunoimmobilized subtypes

Subtype: Ki	α7 μM		β4 μM		β2 μM	
MG 624	0.027	(31)	8.3	(20)	69.5	(25)
F2	27.4	(25)	14.3	(21)	374	(38)
F3	0.050	(29)	4.01	(26)	39	(25)
F3A	0.070	(40)	3.84	(33)	75	(33)
F3B	0.081	(35)	4	(38)	45	(31)
Ligand affinity (Kd):	0.5 nM (27) ^{125}I-αBgtx		20 pM (27) ^{3}H-Epi		36 pM (37) ^{3}H-Epi	

The Kd and K_i values were derived from ^{125}I-αBgtx saturation and competition binding curves to the α7 subtype and ^{3}H-Epi saturation and competition binding curves to β2 and β4 containing subtypes. The curves obtained from three separate experiments were fitted using a non-linear least squares analysis program and the F test (Munson and Rodboard). The numbers in brackets represent the % of CV.

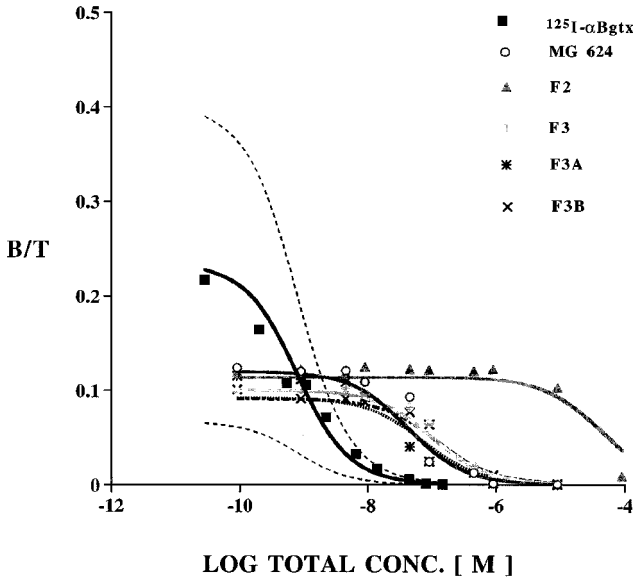

Figure 4 Binding of ^{125}I-αBgtx to immobilized α7-containing receptors and its inhibition by MG 624, F2, F3, F3A and F3B. The binding of ^{125}I-αBgtx to immobilized α7-containing receptors and its inhibition by MG 624, F2 F3, F3A and F3B were performed according to a mixed protocol combining both saturation (the first 7–8 concentrations of respectively 0.01–20 nM) and displacement curves (the last 3–4 concentrations of 50–2000 nM). The α7-containing receptors (immunoimmobilized as described in Methods section) were incubated for 30 min at 20°C with the indicated compound concentrations. ^{125}I-αBgtx (at a final concentration of 0.25 nM) was then added and left overnight. The curves were obtained by fitting the data from three separate experiments each performed in triplicate using the LIGAND programme. The dotted lines represent the 95% c.l.: for the sake of clarity, these are only shown for the ^{125}I-αBgtx curve.

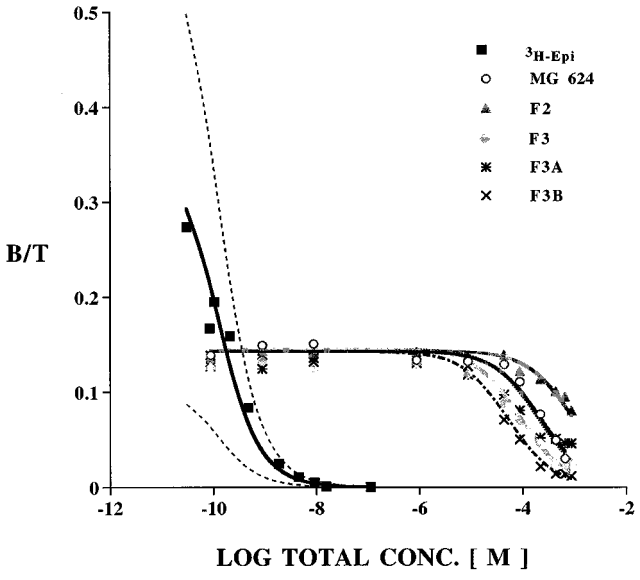

Figure 5 Binding of ^{3}H-Epi to immobilized β2 containing receptors and its inhibition by MG 624, F2, F3, F3A and F3B. The binding of ^{3}H-Epi to immobilized β2 containing receptors and the inhibition of the binding by MG 624, F2, F3, F3A and F3B were performed according to a mixed protocol combining both saturation (the first 7–8 concentrations of respectively 0.005–5 nM) and displacement curves (the last 3–4 concentrations of 50–1000 nM). The immunoimmobilized β2-containing receptors were incubated for 30 min at 20°C with the indicated concentration of compounds. ^{3}H-Epi (at a final concentration of 50 pM) was then added and left overnight. The curves were obtained and processed as described in Figure 4. The dotted lines represent the 95% c.l.: for the sake of clarity, only the ^{3}H-Epi curve is shown.

MG 624, F2 and F3 were approximately 10 times more potent in inhibiting the binding of ^{3}H-Epi to the β4-containing than to the β2-containing subtypes. None of the compounds showed any selectivity toward the β2 or β4-containing receptor subtypes.

Neither of the stereoisomers of F3, which was the most active compound on β2 and β4-containing receptors, showed any increase in specificity for either of the receptor subtypes.

The nature of the inhibition of MG624 and F3 on the binding of ^{125}I-αBgtx to the immobilized α7-containing receptors was investigated by performing two saturation binding experiments in the presence and absence of 0.3 μM MG624 or F3. Scatchard analysis of the ^{125}I-αBgtx binding in the absence and presence of MG624 or F3 indicated that competitive inhibition occurred, as the slope decreased in the presence of each of the compounds whereas the B_{max} was not changed.

Effects of MG624 and F3 on oocyte-expressed chick α7 subtype

As the binding experiments showed that MG 624 and F3 were the most interesting compounds, we tested their biological effects on the functional activity of the chick homomeric α7 subtype expressed in oocyte.

Oocytes injected with α7 subunit cDNA responded to ACh with an inward current (IACh) whose peak amplitude depended on the ACh concentration; this current was blocked by the α7-selective antagonist αBgtx. With the EC_{50} concentration of 100 μM ACh, applied to oocytes held at −60 mV, the mean current amplitude was 594±127 nA (mean±sem) (9 oocytes, 2 donors).

Neither the non-injected oocytes nor those expressing α7 neuronal nicotinic AChRs, responded to MG 624 or F3 (10 nM–1 μM) when applied alone. To compare the potency of MG624 and F3 in inhibiting α7 subtype ACh elicited currents, experiments were performed in α7-injected oocytes exposed to increasing concentrations of MG624 or F3, 30 s before applying ACh at about its EC_{50} value. Both compounds completely blocked the ACh-evoked currents at a concentration of 1 μM; the IC_{50} and n_H values of MG624 for the α7 receptor were 94 nM and 1.2 ($n=6$), those of F3 were 119 nM and 1.3 ($n=3$).

In vitro *experiments*

The biological activity of MG624 and F3 compounds was further characterized by investigating their effects on ganglionic and muscle-type synaptic transmission. It was already known that MG 624 had very strong ganglioplegic activity (Cavallini *et al*., 1953; Mantegazza & Tommasini, 1955) and very weak antimuscarinic property *in vivo*; furthermore, it has been reported that it has no activity on the neuromuscular junction. In order to confirm these results and to compare them with those of F3, we tested the *in vitro* effects of MG 624 and F3 on two different tissue preparations: the guinea pig vagus nerve-stomach and the rat phrenic nerve-hemidiaphragm preparations.

Vagus nerve-stomach preparation The guinea-pig vagus nerve contains axons that synapse with the post-postganglionic cholinergic neurons which mediate the contractile phase of the smooth muscle of the stomach. The typical responses elicited by nerve stimulation, and those elicited by the addition of ACh, before and after the addition of the compounds are shown in Figure 7 and Table 3. Both compounds depressed the contractile response obtained by electrical field stimulation of the vagus, although with different IC_{50} values in μM, of 49.4 (40.4–60.3) for MG 624 and 166.2 for F3 (129.6–213.1), but were totally ineffective on the stomach contractions obtained by direct stimulation of the muscarinic receptors by exogenously added ACh.

Phrenic nerve-hemidiaphragm To verify whether MG 624 and F3 can have an effect on muscle-type AChRs, we tested them on the phrenic nerve-hemidiaphragm preparation. (Figure 8 and Table 3). We found that, MG 624 had very little activity

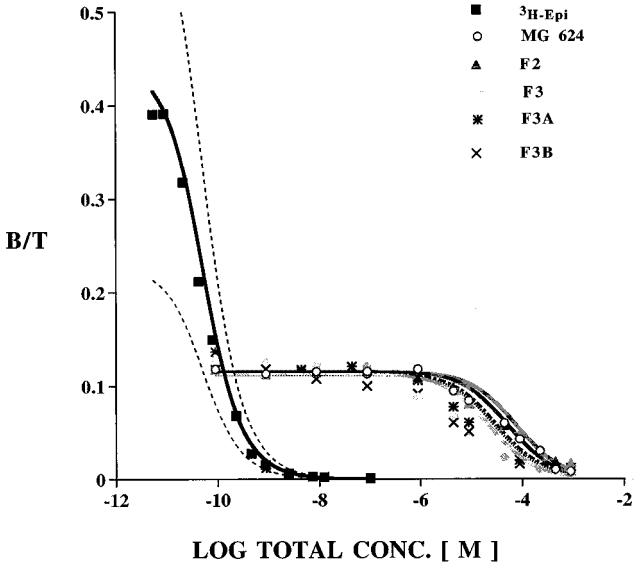

Figure 6 Binding of ^{3}H-Epi to immobilized β4 containing receptors and its inhibition by MG 624, F2, F3, F3A and F3B. The binding of ^{3}H-Epi to the immobilized β4 containing subtype and the inhibition of the binding by MG 624, F2, F3, F3A and F3B were performed as described in Figure 5. The curves were obtained as described in Figure 4. The dotted lines represent the 95% c.l.: for the sake of clarity, only the ^{3}H-Epi curve is shown.

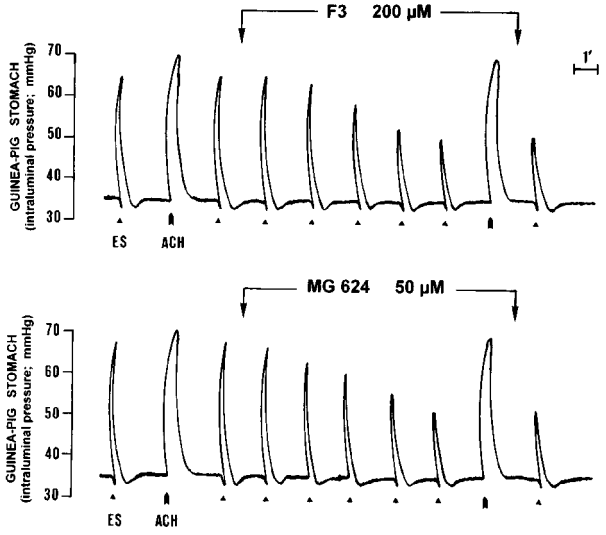

Figure 7 Typical tracings in two different experiments showing the effect of MG624 and F3 on guinea-pig stomach contractions induced by electrical stimulation (ES) of the vagus nerve and acetylcholine (ACh = 5 μM). ES: 0.2 ms; 20 Hz, 0.2 V for 10 s every 2 min.

on the evoked contractions ($IC_{50}=485.9$ μM) whereas F3 ($IC_{50}=14.4$ μM) was more than 33 times more potent; neither compound influenced the muscle contraction obtained by means of direct muscle stimulation.

These *in vitro* data show that MG 624 has inhibitory activity on guinea pig stomach parasympathetic ganglia but very weak activity on the rat hemidiaphragm neuromuscular junction. F3 had the opposite effects: a good neuromuscular blocking activity and a ganglioplegic effect only at 10 times higher concentrations.

Discussion

The data reported here indicate that the two 4-oxystilbene derivatives, the old drug MG624 and the newly synthesized F3, are potent nicotinic antagonists that are apparently selective towards neuronal αBgtx receptors.

Binding studies

To investigate whether MG 624, F2, F3, F3A and F3B have a selective activity towards the different neuronal nicotinic AChR subtypes present in ganglia and in the CNS, we studied their inhibition of the binding of ^{125}I-αBgtx (a toxin that binds the α7 and α8 neuronal nicotinic AChR subtypes in vertebrates) and ^{3}H-Epi (which binds to the β2 and β4-containing neuronal nicotinic AChR subtypes with pM affinity). We first investigated the binding of these compounds to crude neuronal membranes and then performed binding experiments on defined immunoimmobilized neuronal nicotinic AChR subtypes.

Table 3 Estimation of MG 624 and F3 IC_{50} values obtaining from dose-response curves of the vagus nerve-stomach and phrenic nerve hemidiaphragm

	Vagus nerve-stomach Ic_{50} (μM)	*Phrenic nerve-hemidiaphragm* IC_{50} (μM)
MG 624	49.9 (40.5–60.3)	485.9 (405.4–582.3)
F3	166.2 (129.6–213.1)	14.4 (12.0–17.4)

The data for both preparations were obtained in seven separate experiments. For both tissues, the IC_{50} and 95% confidence limits (in brackets) were calculated from the dose-response curves of MG 624 and F3.

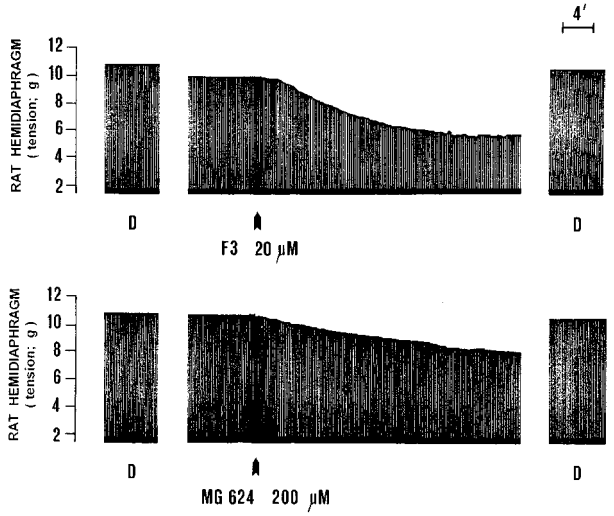

Figure 8 Typical mechanograms of two different experiments showing the effect of MG624 and F3 on rat hemidiaphragm twitches indirectly evoked by electrically stimulating the phrenic nerve. Indirect electrical stimulation: 0.1 Hz, 1 ms, 0.5 V. Direct electrical stimulation (D): 0.1 Hz, 1 ms, 2 V.

Both MG624 and F3 compounds inhibited the binding of ^{125}I-αBgtx to the α7-containing receptors with nanomolar affinity (27 and 50 nM respectively), but inhibited the binding of ^{3}H-Epi to the β4-containing and β2-containing subtypes at concentrations that were two to three orders of magnitude higher. The same compounds inhibited the binding of ^{125}I-αBgtx to the muscle-type AChR with a potency similar to that found at the β2-containing subtype. These compounds affected the subtypes with the following order of potency: α7-containing $>>$ β4-containing $>$ β2-containing = muscle-type receptors.

The results showing the selectivity towards the α7 subtype were obtained in chick optic lobe, a neuronal tissue consisting of 60–70% of receptors that only contain the α7 subunit and the other 20–25% of receptors containing both the α7 and α8 subunits (both subtypes may also contain other unidentified subunits) (Gotti *et al*., 1994). Our binding studies clearly show that these drugs are active on α7 and α7-α8 containing receptors, but we cannot exclude the possibility that they are also active on α8-containing receptors.

Only very high concentrations of our compounds affect β2 containing receptors, which we have shown here to be a heterogeneous class of receptors. Although with approximately one-hundredth of the potency shown towards the α7 subtype, both MG624 and F3 showed an affinity (in the low μM range) for the receptors containing the β4 subunit (see Table 2), which also contain the α3 subunit, as revealed by Western blot and immunoprecipitation analyses using subunit specific antibodies.

Although F3 is twice as active as MG624 on α3β4-containing receptors it is not selective, since it blocks both muscle and α7 receptors (see Table 3). However, its relatively low K_i value may make it useful in the study of the biophysical and functional characteristics of α3β4 containing receptors.

Functional studies

The data in the literature, and our own *in vitro* results and preliminary electrophysiological data from oocyte-expressed subtypes, showed that MG 624 and F3 are nicotinic antagonists that bind to the α7 subtype with nM affinity. The MG 624 and F3 concentrations required to block the response of the ACh-activated homomeric chick α7 subtype expressed in oocytes (94 and 119 nM respectively) are in close agreement with those required to inhibit the binding of ^{125}I-αBgtx to the α7-containing subtype (27 and 50 nM respectively).

Although MG624 and F3 show similar potency in binding studies towards the α7-containing subtype, and also towards the muscle-type AChR, our 'in vitro' studies clearly demonstrate that the two compounds inhibit the contractile responses in vagus nerve-stomach and phrenic nerve-hemidiaphragm preparations with opposite selectivities: F3 had greater neuromuscular blocking activity (IC_{50} of 14.43 μM) than MG624 (IC_{50} 485 μM).

Given the fact that MG624 has a selective effect on α7-containing receptors and only affects muscle-type AChR at very high concentrations it could be a very useful tool not only for the *in vitro* study of the α7 subtype, but also and especially for *in vivo* studies because of its good therapeutic index (LD

50 = 30 mg/kg *versus* 0.5 mg/kg to obtain long-lasting ganglioplegic activity) (Mantegazza & Tommasini, 1955). Its therapeutic index could be even better if the α7 subtype is considered as its target, towards which its affinity is 307 times higher than towards the α3β4 subtype. The α7 receptor has recently aroused great interest since it has been found to be present in discrete and important areas of the brain, such as the hippocampus and caudate and motor cortex (Rubboli *et al*., 1994); furthermore, *in vitro* experiments suggest that its Ca^{2+} permeability may lead to pleiotropic effects on neuronal communication and development (Role & Berg, 1996), and it has also been suggested that it may be involved in several brain pathologies (reviewed in Gotti *et al*., 1997a). However, its role in brain function is not yet well known partly because of the lack of appropriate tools for studying it.

Although other selective α7 antagonists are currently available and act at lower concentrations than MG624, such as the *Bungarus* and *Conus* α-toxins, and methyllycaconitine (reviewed in Gotti *et al*., 1997a), we believe that they could be rather toxic, expensive and more difficult to use in *in vivo* studies.

The nicotinic agonists so far known are not selective and even the recently discovered nicotinic ligands (Epi, ABT 418, A-35380 and GTS 21) have a lower affinity for the α7 than for the α4β2 subtype (reviewed in Gotti *et al*., 1997a). The only α7 agonist so far reported is the very recently discovered anabaseine (Kem *et al*., 1997), a toxin that has a K_i of 58 nM in inhibiting the binding of ^{125}I-αBgtx to the α7 receptor present in rat brain membranes, a value that is very similar to that found for MG624 and F3; the drawback of this agonist is that it also inhibited the binding of ^{125}I-αBgtx to the muscle-type AChR and the binding of ^{3}H-methylcarbamylcholine to the rat α4β2 subtype with very similar potency.

For all of these reasons, MG 624 and F3 could be very important for understanding the function of the α7 receptor *in vivo*. It is also conceivable that they could be used for clinical purposes and possibly for other pathologies in which experimental data suggest that α7 may be involved, such as the control of nicotine addiction, the control of cell proliferation in some lung tumours (Codignola *et al*., 1994; Quik *et al*., 1994) or the control of brain cell excitability (Marks *et al*., 1989).

Chemical characteristics

In our compounds, three functional groups seem to be important to explain their affinity and selectivity towards neuronal nicotinic AChR subtypes: (i) the styryl substituent; (ii) the alkyl bridge connecting the quaternary ammonium group to the phenoxy oxygen; (iii) the ammonium head. It is very interesting to observe that the styryl moiety at the *para* position of the phenyl ring confers a preferential predisposition for neuronal nicotinic AChRs, (especially for the α7 subtype), although it is not sufficient (F2 has a rather low affinity for neuronal nicotinic AChRs). Our results obtained using a small number of compounds allow a tentative suggestion that a *trans* conjugated, approximately coplanar structure might favor receptor-bonding interactions with the α7 subtype, involving Van der Waals and hydrophobic bonding and possibly charge-transfer complexing.

The 4-oxystilbene derivative F3 can be considered an open chain congener of compounds II (Figure 1), formally obtainable by the cleavage of the 3,4 bond of the azacycle. The binding data for compounds II indicate that some of them possess nanomolar affinity for the α4β2 subtype. On the contrary, the open chain congener N,N,N-trimethyl-1-(4-*trans*-stilbenoxy)-2-propylammonium, F3, has completely lost the high affinity towards the α4β2 subtype but has acquired nanomolar affinity for the α7 subtype and low affinity for the receptor containing the β4 subunit. Moreover, the enantiomers of this compound, F3A and F3B, show little stereoselective binding. Two structural variations of F3 were considered: (i) the alteration of the quaternary ammonium head and the removal of the methyl group from the alkyl bridge (see MG 624); (ii) the reverse position of the ammonium head and the stilbenoxy residue (see F2). In the first case, a favourable influence of the N,N,N-triethyl ammonium moiety on affinity can be observed. The differences in K_i values between MG 624 and F3 may be due to better exploitation of the binding pocket as a result of the ammonium volume. A rather low affinity for neuronal nicotinic AChRs is shown by F2, which carries the methyl group near the oxygen. This result leads to the hypothesis that the methyl group has an unfavourable effect on the modulation of the sterical fit to the binding site of the receptor. In this regard, it is supposed that the methyl group interferes with a hypothetical region of steric sensitivity or with free access to the H-bond donor group.

These results clearly show that the 4-oxystilbene moiety, an ammonium head and an alkyl bridge with a methylene group close to the phenoxy oxygen are necessary requirements for achieving neuronal nicotinic AChR α7 subtype selectivity.

In conclusion, we have found that two compound belonging to oxystilbene derivates show a strong selectivity towards neuronal nicotinic receptors, particularly the α7 subtype. We suggest that further investigations of this class of compounds could be important for finding compounds with neuronal nicotinic AChR selectivity, and for understanding the structure of the ligand binding site of these receptors.

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