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Nitrogen substitution modifies the activity of cytisine on neuronal nicotinic receptor subtypes

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Abstract

Cytisine very potently binds and activates the α3β4 and α7 nicotinic subtypes, but only partially agonises the α4β2 subtype. Although with a lower affinity than cytisine, new cytisine derivatives with different substituents on the basic nitrogen (CC1–CC8) bind to both the heteromeric and homomeric subtypes, with higher affinity for brain [^{3}H]epibatidine receptors. The cytisine derivatives were tested on the Ca^{2+} flux of native or transfected cell lines expressing the rat α7, or human α3β4 or α4β2 subtypes using Ca^{2+} dynamics in conjunction with a fluorescent image plate reader. None elicited any response at doses of up to 30–100 μM, but all inhibited agonist-induced responses. Compounds CC5 and CC7 were also electrophysiologically tested on oocyte-expressed rat α4β2, α3β4 and α7 subtypes. CC5 competitively antagonised the α4β2 and α3β4 subtypes with similar potency, whereas CC7 only partially agonised them with maximum responses of respectively 3% and 11% of those of 1 mM acetylcholine. Neither compound induced any current in the oocyte-expressed α7 subtype, and both weakly inhibited acetylcholine-induced currents. Adding chemical groups of a different class or size to the basic nitrogen of cytisine leads to compounds that lose full agonist activity on the α3β4 and α7 subtypes.

Keywords: Cytisine; Nicotinic receptor, neuronal; Agonist; Antagonist; Partial agonist; Binding; FLIPR; Electrophysiology

1. Introduction

Neuronal nicotinic acetylcholine receptors are widely expressed in the vertebrate nervous system, where they act as postsynaptic receptors exciting neurons, or as presynaptic receptors modulating the release of many neurotransmitters (Jones et al., 1999; Wonnacott, 1997). They are known to be involved in normal central nervous system (CNS) functions such as learning, memory consolidation, arousal and locomotor activity, as well as in pathological conditions such as degenerative Alzheimer's and Parkinson's diseases, Tourette's syndrome and schizophrenia. The receptors form a family of acetylcholine-gated cation channels consisting of different subtypes, each of which has a specific anatomical distribution in the central and peripheral nervous systems. This heterogeneity is based on the 12 genes cloned so far, which codify for the corresponding nicotinic receptor subunits α2–α10, β2–β4, and by the fact that subtypes can be generated by the homopentameric or heteropentameric assembly of up to four different subunits (Lindstrom, 2000; Clementi et al., 2000b). These receptor subtypes are not only permeable to Na^+ and K^+ but also to Ca^{2+} ions and, depending on the nicotinic receptor subtype, Ca^{2+} can carry from 2% to 10% of the agonist elicited current (Lindstrom, 2000).

The observation that specific nicotinic receptor subtypes may be involved in different physiological and pathological

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conditions has underlined the importance of finding subtype-specific compounds to be used for therapeutic purposes reviewed in Clementi et al. (2000a). Many papers have reported the results of studies with nicotinic receptor agonists such as epibatidine, acetylcholine or nicotine derivatives reviewed in Holliday et al. (1997); but much less is known about cytisine derivatives (Houlihan et al., 2001; Imming et al., 2001). Cytisine has a high affinity for many nicotinic receptor subtypes and can discriminate among them (Hall et al., 1993) because it is a full and potent agonist of α7-containing subtypes, a good receptor agonist of β4-containing subtypes, but only a weak and partial agonist of β2-containing subtypes (Amar et al., 1993; Covernton et al., 1994; Chavez-Noriega et al., 1997; Papke and Heinemann, 1994). Cytisine has all of the classical in vivo pharmacological activities of nicotinic agonists, including the stimulation of evoked neurotransmitter release (Clarke and Reuben, 1996; Kristufek et al., 1999), antinociceptive activity making it a potent acute analgesic (Seale et al., 1998; Rao et al., 1996), and the modulation of locomotor activity at high doses (Museo and Wise, 1995).

Although the stability of the cytisine–iron complex has not been defined, cytisine appears to inhibit free hydroxyl radical formation in the Fenton reaction, probably as a result of iron chelation. This property, and the fact that cytisine has been shown to reduce 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopamine depletion in vivo, suggests that it may be particularly useful in treating Parkinson's disease by chelating iron ions and thus preventing cell death (Ferger et al., 1998).

In the search for new subtype-selective drugs exploiting these different properties, we have synthesised and characterised eight cytisine derivatives CC1 to CC8; see formulas in Fig. 1. They have a common cytisine skeleton, but the amine function is substituted by residues that increase the lipophilicity of the original compound and can modify their receptor binding capability.

The N-substituents are aliphatic or alicyclic moieties in CC4, CC6, CC7 and CC8, chloroheteroaryls in CC1, CC2 and CC3, and arylalkyl in CC5. It is also worth noting that CC4 is the first example of a set of compounds, whose synthesis is in progress, possessing two cytisine residues joined by a polymethylene chain of increasing length. The different sizes of the molecules could make them capable of discriminating the nicotinic receptor subtypes by preventing molecule entrance to the binding pockets or allowing their interaction with accessory binding sites.

CC8 was designed as a possible prodrug: it contains a very lipophilic and cumbersome tricyclo [3.3.1.1 3,7]decane moiety which improves the crossing of the blood–brain barrier, and should prevent the rapid hydrolysis of the ester function, thus avoiding or reducing the direct effect of free cytisine on peripheral receptors.

In order to define the selectivity and relative affinity of the compounds in comparison with reference nicotinic agonists and antagonists, we tested them in binding studies

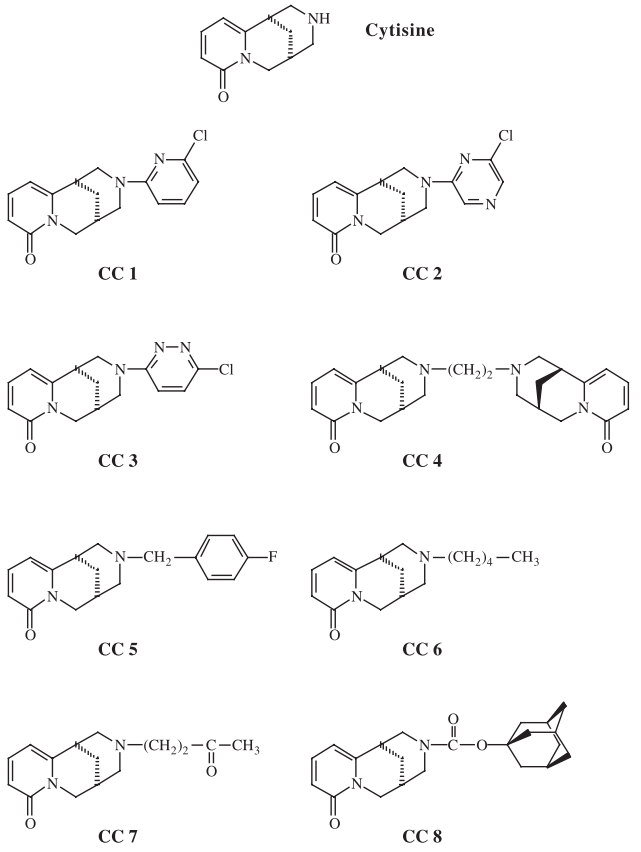

Fig. 1. Chemical structures of cytisine and cytisine derivatives CC1–CC8.

of the major neuronal nicotinic receptor subtypes present in the rat central and peripheral nervous systems; furthermore, we assessed their functional profile on native and heterologously expressed nicotinic receptor subtypes using as indicator the intracellular Ca^{2+} increase detected by fluorescent imaging plate reader (FLIPR), and finally characterised the two most interesting compounds by means of electrophysiological studies of the same rat nicotinic receptor subtypes, reconstituted in *Xenopus* oocytes.

2. Materials and methods

2.1. Compounds synthesis

The synthesis of 1,2-bis*N*-cytisinylethane (CC 4), *N*-4-fluorobenzyl cytisine (CC 5), *N*-pentylcytisine (CC 6) and *N*-3-oxobutylcytisine (CC 7) has been described by Canu Boido and Sparatore, 1999. The remaining compounds were prepared as follows.

2.1.1. N-[2-6-Chloropyridyl]cytisine, CC 1

0.44 g (3 mmol) of 2,6-dichloropyridine and 0.4 ml of triethylamine were added to a solution of cytisine (0.57 g; 3 mmol) in 2 ml of dimethylformamide. The mixture was heated in a sealed tube at 120 °C for 4 h. After cooling, 3 ml

of 1 N sodium hydroxide solution was added; the precipitate was collected, washed several times with water and dried: 0.40 g (42% yield), after crystallisation from ether melted at 198–203 °C. Analysis (C,H,N) for $C_{16}H_{16}ClN_3O + 0.75\ H_2O$.

2.1.2. N-[2-6-Chloropyrazinyl]cytisine, CC 2

Cytisine (0.57 g; 3 mmol), 2,6-dichloropyrazine (0.45 g; 3 mmol) and triethylamine (0.4 ml) in 2 ml of dimethylformamide were heated in a sealed tube at 140 °C for 3 h. After alkalinization as above, extraction with dichloromethane yielded a waxy solid that, after washing with ether, gave 0.66 g (72% yield) of crystals melting at 158–161 °C. Analysis (C,H,N) for $C_{15}\ H_{15}\ ClN_4O + 0.25\ H_2O$.

2.1.3. N-[3-Chloropyridazinyl]cytisine, CC 3

Cytisine (0.57 g; 3 mmol), 3,6-dichloropyridazine (0.45 g; 3 mmol) and triethylamine (0.4 ml) in 2 ml of dimethylformamide were heated in a sealed tube at 140 °C for 6 h. Three milliliters of 1 N sodium hydroxide solution was added to the reaction mixture, which was then heated on a boiling water bath. The precipitate was collected and washed several times with water; after drying, 0.28 g 30% yield of crystals melting at 219–221 °C was obtained. Analysis (C,H,N) for $C_{15}\ H_{15}\ ClN_4O + 0.5\ H_2O$.

2.1.4. N-1-Adamantyloxycarbonyl cytisine, CC 8

0.59 g (3 mmol) of 1-adamantylfluoroformate and 0.6 ml of triethylamine were added to a solution of cytisine (0.57 g; 3 mmol) in warm, anhydrous toluene (15 ml), which was then refluxed under nitrogen for 12 h. The solvent was removed under reduced pressure and the residue partitioned between water and dichloromethane. After elimination of the solvent, the residue was crystallised from acetone/dry ether to obtain 0.62 g (56% yield) of crystals melting at 234–237 °C. Analysis C,H,N for $C_{22}H_{28}N_2O_3$.

2.2. [^{3}H]Epibatidine and [^{125}I]αBungarotoxin binding to membranes

The neuronal tissues were obtained from adult rat brain without cerebellum, and rat superior cervical ganglia. The animals were housed and cared for in accordance with the guidelines laid down by the European Communities Council Directive (86/609/EEC of 24 November 1986). The tissues were first homogenised using an UltraTurrax brain or Potter homogeniser superior cervical ganglion, in an excess of 50 mM Na phosphate pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA and 2 mM phenylmethylsulfonylfluoride, and then centrifuged and rinsed twice; centrifugation 20 min at 30 000 × *g*; rinse in 50 mM Na phosphate pH 7.4, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA and 2 mM phenylmethylsulfonylfluoride before final suspension in a buffer 50 mM Tris–HCl pH 7, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ containing 20 μg ml^{-1} of leupeptin, bestatin, pepstatin A and aprotinin protease inhibitors.

Saturation experiments were performed by incubating the brain and superior cervical ganglion homogenates overnight with 0.01–5 nM [^{3}H]epibatidine (Amersham, specific activity: 54.6 Ci mmol^{-1}) at 4 °C, or with 0.01–10 nM [^{125}I]αBungarotoxin (Amersham, specific activity 200–243 Ci mmol^{-1}) at 20 °C. In order to prevent the binding of [^{3}H]epibatidine to the αBungarotoxin-binding receptors (that binds epibatidine with low affinity (Gerzanich et al., 1995; Balestra et al., 2000), the membranes were preincubated with 2 μM αBungarotoxin and then with [^{3}H]epibatidine. In the case of [^{125}I]αBungarotoxin, 2 mg ml^{-1} bovine serum albumin was added to the suspension buffer. Specific radioligand binding was defined as total binding minus nonspecific binding determined in the presence of 100 nM cold epibatidine or 1 μM cold αBungarotoxin, respectively. The inhibition of radioligand binding by cytisine, nicotine, acetylcholine, dihydro-β-erythroidine, αBungarotoxin, *d*-tubocurarine, decamethonium, *N*,*N*,*N*-trimethyl-1-4-*trans*-stilbenoxy-2-propylammonium iodide (F3) and CC1–CC8 was measured by preincubating increasing doses (10 pM–5 mM) for 30 min at room temperature, followed by overnight incubation with [^{3}H]epibatidine at a final concentration of 40 pM for brain and 150 pM for superior cervical ganglion or 1 nM [^{125}I]αBungarotoxin at the same temperatures as those used for the saturation experiments. These ligand concentrations were used for the competition binding experiments because they are in the range of the K_d values of the ligands for the different subtypes.

The experimental data obtained from the three saturation and three competition binding experiments were analysed by means of a nonlinear least square procedure using the LIGAND program according to Munson and Rodbard, 1980. The binding parameters were calculated by simultaneously fitting three independent saturation experiments and the K_i values were determined by fitting the data of three independent competition experiments. The errors in the K_d and K_i values of the simultaneous fits were calculated using the LIGAND software, and expressed as percent of coefficient of variation (CV) corresponding to the standard error divided by the parameter value. All of the figures were computer-generated using Prism 2.0 software GraphPad, San Diego, CA, USA. These fits were made according to theoretical equations of one-site-specific binding nonlinear regression for the saturation curves where the error bars of the specific binding curves are standard deviations of individual differences between the total and nonspecific values for each data point, linear regression for the Scatchard representations, and specific binding inhibition nonlinear regression for the inhibition curves, using the equation described by Gaddum, 1937:

$$\%\ \text{of}\ B_{max} = 100 \times \frac{(K_d + [\text{radioligand}])}{K_d\left(1 + \frac{[\text{inhib}]}{K\text{inhib}}\right) + [\text{radioligand}]}$$

2.3. Cell culture

IMR32 cells were grown at 37 °C with 5% CO_2 in minimal essential medium supplemented with 10% fetal bovine serum, 3 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and plated on poly-D-lysine-coated 96-well plates at a density of 5×10^4 cells/well. These cells expressed both αBungarotoxin-binding receptors containing the α7 subunit and the epibatidine-binding receptors containing α3β4 subunits (Balestra et al., 2000). For this assay, cells were preincubated with 1 μM αBungarotoxin in order to block the α7-containing receptors.

GH4C1 cells stably expressing the rat α7 subtype were generated as previously described (Virginio et al., 2002). These α7-GH4C1 cells were grown at 37 °C with 5% CO_2 in Ham's/F10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum and 1 mM glutamine, and plated on poly-D-lysine-coated 96-well plates at a density of 1×10^5 cells/well.

Human α4β2-embryonic kidney cells (HEK293) cells were generated as described for the α7 expressing cells and grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 400 μg/ml geneticin and 200 μg/ml hygromicin B, and plated on poly-D-lysine coated 96-well at a density of 5 × 10 4 cells/well.

2.4. FLIPR measurements of $[Ca^{2+}]_i$

Twenty-four hours after plating, the cells were washed with Hanks buffered salt solution (HBSS) containing: 20 mM HEPES, pH 7.4, 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO3, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4 0.81 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.5 mM glucose. They were then loaded for 30–60 min with the calcium-sensitive fluorescent dye Fluo-4 AM final concentration 2 μM in HBSS also containing 0.02% pluronic acid and 5 mM probenecid; the unincorporated dye was removed from the cells by washing with HBSS.

Cell fluorescence was determined before drug additions, and monitored λ_{Ex} = 488 nm, λ_{Em} 516 nm in the FLIPR immediately following exposure to increasing concentration of agonists as described by Kuntzweiler et al. (1998) and Schroeder and Neagle (1996). In order to discriminate the agonist and antagonist activity of the cytisine derivatives in the same experiment, a FLIPR protocol including two additions was established. The cytisine derivative solutions first addition were added at different concentrations 10 min before the standard agonist second addition, which was always used at an EC_{max} concentration of 5 μM nicotine for α7-GH4C1 cells, 10 or 5 nM epibatidine for α4β2-HEK293 and IMR32 cells, respectively. Responses were measured as peak minus basal fluorescence intensity, plotted against time, and the maximum values recorded after the first and second addition were measured and fitted for EC_{50} and IC_{50} calculations, respectively. The data are expressed as mean ± S.E. of three determinations. Curve-fitting and parameter estimation were carried out using LIGAND programme.

2.5. Electrophysiological recordings and data analysis

Mature female specimens of *Xenopus laevis* obtained from AmRep Breda, The Netherlands, were anesthetised by submersion in 0.1% 3-aminobenzoic acid ethyl ester, and their ovarian lobes were surgically removed. The oocytes were defolliculated manually after treatment with collagenase type I (1.5 mg/ml calcium-free Barth's solution) for 1.5 h at room temperature. The cDNAs of rat nicotinic receptor subunits ligated into the pSM plasmid vector containing the SV40 viral promotor were a kind gift from Dr. J. Patrick Baylor College of Medicine, Houston, TX, USA.

Plasmids coding for the α and β subunits of nAChRs, dissolved in H_2O at a 1:1 molar ratio, were co-injected into the nuclei of stage V and VI oocytes within 8 h of harvesting using a Drummond microinjector. Approximately 1 ng of each plasmid containing α and β cDNA, 2 ng for α7, was injected in a total injection volume of 10–15 nl per oocyte. After injection, the oocytes were incubated at 19 °C in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $CaNO_{32}$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES and 50 mg l^{-1} neomycin pH 7.6 with NaOH. The oocyte experiments were performed after 2–5 days of incubation.

The experiments were performed as previously described Zwart et al., 1995; Zwart and Vijverberg, 1997. The oocytes were placed in a silicon tube Ø3 mm that was continuously perfused with saline solution 115 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.2 with NaOH at a rate of ~ 20 ml min^{-1}. Aliquots of concentrated stock solutions of acetylcholine and cytisine in distilled H_2O, CC5 and CC7 in dimethyl sulfoxide were added to the saline immediately before the experiments. The drugs were applied alone or in co-application, by switching between control and drug-containing saline using a servomotor-operated valve. Agonist and drug applications were alternated with 5 min of agonist-free saline superfusion in order to allow the receptors to recover from desensitization. Membrane currents were low-pass filtered 8-pole Bessel; −3 dB at 300 Hz, digitised 12 bits; 1024 samples/5 s record, and stored on disk for off-line computer analysis. The data are expressed as the mean values ± S.D. of *n* oocytes.

Concentration–effect curves were fitted to the Hill equation:

$$i/i_{max} = 1/\{1 + EC_{50}/[agonist]^{nH}\} \quad (1)$$

$$i/i_{max} = 1/\{1 + [antagonist]/IC_{50}^{nH}\} \quad (2)$$

The concentration–effect curves were obtained from three separate oocytes. The curves drawn in Figs. 3B and

4A–B represent the mean concentration–effect curves calculated using the mean of the parameters obtained by fitting the concentration–effect curves to the data obtained from single oocytes. Curve fitting was performed using Jandel Sigmaplot 3.0 software SPSS, Chicago, IL, USA.

2.6. Source of compounds

[^{125}I]αBungarotoxin and [^{3}H]epibatidine were purchased from Amersham Pharmacia Buckinghamshire, UK, and acetylcholine, nicotine, cytisine, dihydro-β-erythroidine, αBungarotoxin, decamethonium *d*-tubocurarine, EDTA, EGTA, phenylmethylsulfonylfluoride, Tween 20, neomycin, dimethyl sulfoxide, protease inhibitors, poly-D-lysine and probenecid from Sigma, St. Louis, MO, USA.

Cell culture media, fetal bovine and horse serum, glutamine, penicillin/streptomycin were purchased from Life Technologies, Milano, Italy. Fluo-4 AM, pluronic acid from Molecular Probes, Leiden, The Netherlands. Bovine serum albumin was purchased from Boehringer Mannheim Germany, and ± -epibatidine from Biomol Plymouth Meeting, PA, USA. The previously described F3 (Gotti et al., 1998) was synthesised at the University of Milan Italy. All of the other salts and reagents were from Merck Darmstadt, Germany or Mallinckrodt Baker, Deventer, The Netherlands.

3. Results

3.1. Pharmacological characterisation of native receptors by radioligand binding

In order to study the pharmacological profile of the native nicotinic receptors present in brain and sympathetic ganglia, we used as ligands [^{3}H]epibatidine and [^{125}I]αBungarotoxin. [^{3}H]epibatidine bound to rat brain membrane with a K_d of 37 pM (CV = 19%) and a B_{max} of 118.4 ± 22 fmol mg^{-1} of protein (mean ± S.D.) and the results are reported in Table 1.

The specific binding to superior cervical ganglion membrane homogenate was saturable with a K_d of 143 pM (CV = 8%) and a B_{max} of 225 ± 50 fmol mg^{-1} of protein (Table 1). The nonspecific binding in both tissues was 2–15% of total binding and the Scatchard plot of specific binding was linear.

The specific binding of [^{125}I]αBungarotoxin to rat brain membranes was saturable with a K_d of 0.51 nM (CV = 9%) and a B_{max} of 49.3 ± 26.4 fmol mg^{-1} of protein (Table 1); the nonspecific binding was 3–23% of total binding.

In order to define the pharmacological profile of the native nicotinic receptors further, we performed binding studies using classical nicotinic agonists and antagonists at different concentrations, and evaluated their potency in inhibiting the equilibrium binding of 40 pM [^{3}H] epibatidine to brain or 150 pM [^{3}H]epibatidine to superior cervical ganglion membranes, and that of 1 nM[^{125} I]α Bungarotoxin to brain membranes. The K_i values of the inhibition curves were calculated by simultaneously fitting the data from two to three separate experiments and are reported in Table 1. The cytisine, acetylcholine and nicotine agonists had the highest affinity for the [^{3}H]epibatidine brain receptors, intermediate affinity for the [^{3}H]epibatidine superior cervical ganglion receptors, and the lowest affinity for the brain [^{125}I]αBungarotoxin receptors (see Table 1).

Table 1
Affinity of reference agonists and antagonists for native nicotinic receptor subtypes

	Brain, [^{3}H]-Epi labelled	SCG, [^{3}H]-Epi labelled	Brain, [^{125}I]-αBgtx labelled
K_d (nM)	0.037 (19)	0.143 (8)	0.512 (9)
K_i (nM)			
Cytisine	0.55 (20) 137 (47)	122 (10)	9.31 (14)
Nicotine	7.2 (21)	299 (14)	469 (33)
ACh	29.1 (21)	520 (40)	2180 (24)
K_i (μM)			
DhβE	1 (7)	55.9 (14)	12.1 (12)
αBgtx	>30	>>50	0.000274 (11)
d-Tc	30 (14)	21 (12)	9.31 (14)
DM	28 (16)	647 (21)	83.7 (18)
F3	30.4 (27)	10.2 (21)	0.054 (33)

The K_d and K_i values were derived from three [^{3}H]epibatidine saturation and competition binding experiments on rat brain and SCG membranes, and three [^{125}I]-αBungarotoxin saturation and competition binding experiments on rat brain membranes. The curves were fitted using a nonlinear least squares analysis program and the *F* test (Munson and Rodbard, 1980). The numbers in brackets represent the %CV. DHβE: dihydro-*β*-erythroidine; αBgtx: αBungarotoxin; *d*-Tc: *d*-tubocurarine; DM: decamethonium; F3: *N*,*N*,*N*-trimethyl-1-4-*trans*-stilbenoxy-2-propylammonium iodide; Epi: epibatidine.

Cytisine had two classes of affinity for brain [^{3}H]epibatidine receptors, the majority of these brain sites (87%) bound [^{3}H]epibatidine with an affinity of 0.55 nM (CV = 20%), and the rest 13% bound [^{3}H]epibatidine with an affinity of 137 nM (CV = 47%). This biphasic profile was not observed for nicotine or acetylcholine.

The K_i values of dihydro-β-erythroidine, αBungarotoxin, *d*-tubocurarine, decamethonium and F3 antagonists for the subtypes present in the neuronal membranes are shown in Table 1. Dihydroβ-erythroidine had the lowest K_i value for the brain [^{3}H]epibatidine receptors, whereas αBungarotoxin and F3 had nanomolar affinity for the brain [^{125}I]α-Bungarotoxin receptors and micromolar affinity for the brain and ganglionic [^{3}H]epibatidine receptors.

3.2. Pharmacological characterisation of cytisine derivatives by binding studies

The inhibition curves of the binding of the cytisine derivatives to native membrane receptors are shown in Fig. 2A–C. All of the curves were best fitted by a one-site model; the calculated K_i values are shown in Table 2. Only CC7 had a similar potency to that of cytisine; the other compounds

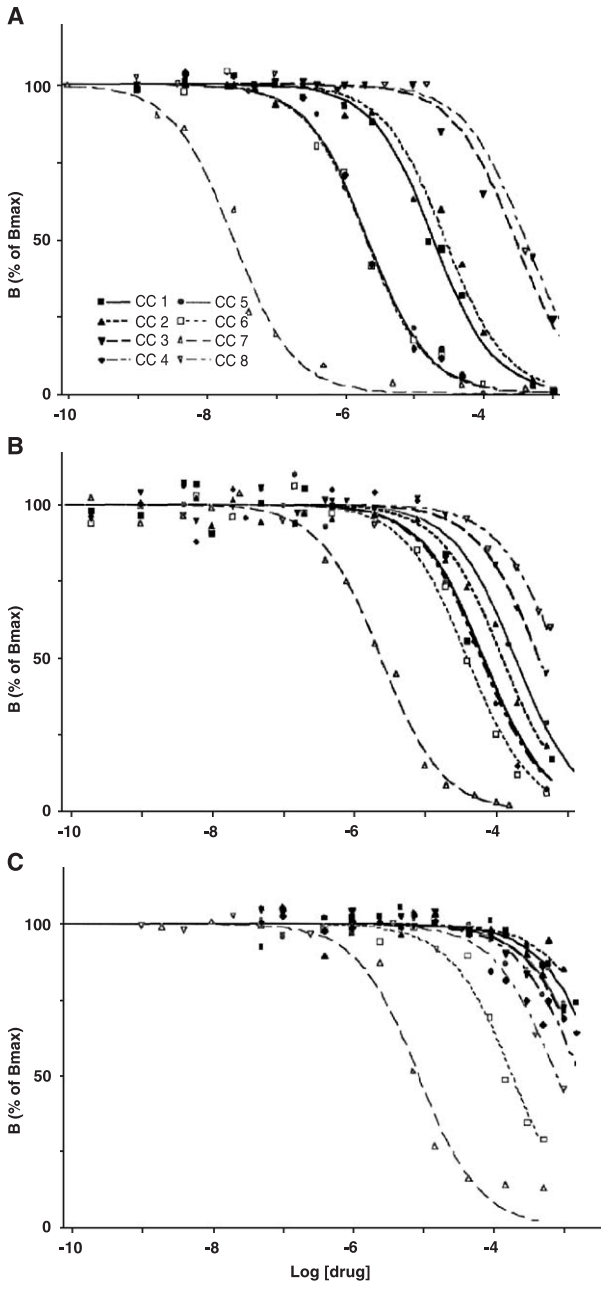

Fig. 2. (A) CC1–CC8 inhibition of the binding of [^{3}H]epibatidine to rat brain membrane. The membrane-bound receptors were incubated for 30 min at 20 °C with the indicated concentrations of compounds CC1–CC8. [^{3}H]epibatidine at a final concentration of 40 pM was then added and left overnight at 4 °C. (B) CC1–CC8 inhibition of the binding of [^{3}H]epibatidine to superior cervical ganglion homogenate. The superior cervical ganglion bound receptors were incubated for 30 min at 20 °C with the indicated concentrations of compounds CC1–CC8. [^{3}H]epibatidine at a final concentration of 150 pM was then added and left overnight at 4 °C. (C) CC1–CC8 inhibition of the binding of [^{125}I]-αBungarotoxin to rat brain membranes. The rat membranes were incubated for 30 min at 20 °C with the indicated concentrations of compounds CC1–CC8. [^{125}I]αBungarotoxin at a final concentration of 1 nM was then added and left overnight at 20 °C. All of the curves in A, B and C were obtained from the data of three separate experiments, using Prism 2.0 software.

Table 2
K_i of CC1-CC8 compounds on native nicotinic receptor subtypes

	Brain, [^{3}H]-Epi labelled	SCG, [^{3}H]-Epi labelled	Brain, [^{125}I]-αBgtx labelled
K_d (nM)	0.037 (19)	0.143 (8)	0.512 (9)
K_i (μM)			
CC1	1.20 (30)	56.9 (13)	>100
CC2	2.13 (21)	70.7 (14)	>100
CC3	12.5 (22)	>100	64.1 (39)
CC4	0.392 (22)	58.8 (15)	36.7 (22)
CC5	0.330 (21)	79.9 (12)	78.7 (50)
CC6	0.322 (22)	20.2 (8)	39.7 (20)
CC7	0.0087 (24)	0.552 (27)	1.24 (22)
CC8	16.8 (15)	>100	>100

The K_d and K_i values were derived from [^{3}H]epibatidine saturation and competition binding experiments on rat brain and SCG membranes, and [^{125}I]αBungarotoxin saturation and competition binding experiments on rat brain membranes, as described in Table 1 and Materials and methods. The numbers in brackets represent the %CV. Abbreviations as in Table 1.

had much higher K_i values for all three subtypes. Taking into account the %CV, brain [^{3}H]epibatidine receptors showed a rank order of potency of CC7>CC6>CC5>CC4>CC1>CC2>CC3>CC8, the ganglionic [^{3}H]epibatidine receptors a rank order of CC7>CC6>CC1>CC4>CC2 ≥ CC5>CC3 ≥ CC8, and the brain [^{125}I]αBungarotoxin receptors a rank order of CC7>CC4 ≥ CC6 ≥ CC3 ≥ CC5 ≥ CC2 ≥ CC1 ≥ CC8.

CC5 had the highest K_i ratio between the ganglionic and brain [^{3}H]epibatidine receptors 240-fold, thus suggesting a good degree of selectivity towards the tested heteromeric nicotinic receptor subtypes.

3.3. Functional assessment of the effects of cytisine derivatives using Ca^{2+} dynamics and FLIPR

The above reported binding data indicate the affinity of cytisine derivatives on the receptor subtypes but do not give any indication on their functional activity. Therefore, given that the published data show that cytisine has different agonist potency and efficacy depending on the subtype tested, we screened all of the cytisine derivatives using the FLIPR technique which exploits the Ca^{2+} transport activity of nicotinic receptors.

Application of the nicotinic agonists nicotine, cytisine and epibatidine to HEK-293 cells expressing the brain α4β2 receptors, to the IMR32 cells expressing the ganglionic α3β4 receptors and to the GH4Cl cells expressing the α7 receptors induced an increase in intracellular fluorescence due to Ca^{2+} influx. This agonist-induced fluorescent signal was abolished when the cells were preincubated with the nicotinic receptor antagonist dihydro-β-erythroidine for the α4β2-HEK and α3β4-IMR32 and methyllycaconitine for α7 GH4Cl cells.

Concentration–effect curves were constructed for the agonists, and the derived EC_{50} values are reported in Table 3.

Table 3
Functional assessment of the potency of agonists and cytisine-derivatives using calcium dynamics and FLIPR

	α4β2 transfected	α3β4 IMR32	α7 transfected
EC_{50}			
Cytisine (μM)	>100	10.7 ± 2	1.3 ± 0.05
Nicotine (μM)	3.1 ± 0.4	8.7 ± 2.3	1.2 ± 0.02
Epibatidine (nM)	8.5 ± 1	1.95 ± 1	29 ± 1
IC_{50} (μM)			
CC1	0.5 ± 0.05	18.6 ± 4.1	>30
CC2	3.89 ± 3	>100	>30
CC3	6.92 ± 0.48	>100	>30
CC4	0.33 ± 0.16	15.5 ± 1.5	>30
CC5	0.28 ± 0.08	2.19 ± 0.4	>30
CC6	0.15 ± 0.09	7.24 ± 1	>30
CC7	0.13 ± 0.04	>100	>30
CC8	3.39 ± 1	1.29 ± 0.4	>30

The reported values are the mean ± S.E.M. of three separate experiments obtained from dose–response curves performed using increasing concentrations of the compounds and an EC_{max} concentration of agonists (5 μM nicotine for α7-GH4C1 cells, 10 nM epibatidine for α4β2-HEK293 cells and 5 nM epibatidine for IMR32 cells).

Both nicotine and epibatidine were full agonists with similar efficacy but different potency on all three expressed subtypes, whereas cytisine was a full agonist for the α3β4 and α7 expressed subtypes but only a partial agonist of the α4β2 subtype.

The cytisine derivatives were tested in this functional assay and dose–response curves were performed using the two-addition protocol (see Materials and methods). When added alone, none of the cytisine derivatives induced Ca^{2+} influx in any of the tested cell lines but they inhibited the epibatidine-induced response in α3β4-IMR cells and α4β2-HEK or the nicotine-induced response in α7-GH4Cl cells in a dose-dependent manner (see results in Table 3).

These compounds were more potent in inhibiting agonist-induced responses in the HEK-293 cells expressing the brain α4β2 receptors, and except for CC7, had IC_{50} values that were very similar to the K_i values determined by binding studies. When tested on the ganglionic α3β4 receptors, CC1, CC2, CC3, CC4 and CC6 had IC_{50} values that were very similar to their K_i values, whereas CC5, CC7 and CC8 showed more than 35-fold differences between their IC_{50} values and the K_i values determined by binding studies.

In the case of the α7 subtype the cytisine derivatives did not induce any Ca^{2+} influx and, at concentrations higher than 30 μM, inhibited nicotine-induced currents.

3.4. Electrophysiological recordings

The CC5 and CC7 derivatives were chosen for electrophysiological testing on oocytes expressing rat α3β4, α4β2 and α7 nicotinic receptor subtypes because the binding experiments showed that CC7 had the best affinities for all of the tested native neuronal nicotinic receptor subtypes, and CC5 the highest K_i ratio between the brain and ganglionic [^{3}H]epibatidine receptors.

3.4.1. Effect of CC5 on rat α3β4, α4β2 and α7 subtypes

The nicotinic agonists acetylcholine and cytisine produced concentration-dependent inward currents in voltage clamped oocytes expressing different nicotinic receptor subunit combination. The data obtained from three separate full dose–response curves are reported in Table 4.

The oocytes α3β4 or α4β2 nicotinic receptors, which responded with large inward currents upon superfusion with 1 mM acetylcholine Fig. 3, did not respond to superfusion with 1, 10 or 100 μM of CC5 (not shown), thus demonstrating that CC5 is not an α3β4 or α4β2 nicotinic receptor agonist. In order to investigate whether CC5 inhibits α3β4 and α4β2 receptors, 10 μM of CC5 was applied during ion currents induced by 10 μM acetylcholine on α3β4 receptors or 3 μM acetylcholine on α4β2 receptors. These concentrations of acetylcholine are equally effective, $\sim EC_{10}$, on both nicotinic receptor subtypes. The 10 μM acetylcholine-induced ion currents mediated by α3β4 receptors were inhibited by 39.4 ± 4.2% (n = 3) and the 3 μM acetylcholine-induced ion currents mediated by α4β2 receptors were inhibited by 65.5 ± 3.8% (n = 3) Fig. 3A. The percentage of inhibition was calculated as the current amplitude at the end of CC5 application divided by the maximum current obtained after CC5 washout. Complete concentration–effect curves were obtained in triplicate using the same method of applying various concentrations of CC5 during responses to 10 μM acetylcholine for α3β4 receptors and 3 μM acetylcholine for α4β2 receptors (see Fig. 3B). Data fitting (Eq. (2)) yielded mean IC_{50} and nH estimates of 19 ± 2 μM and 0.95 ± 0.07 for α3β4 receptors, and 6 ± 1 μM and 0.83 ± 0.05 for α4β2 receptors. In order to determine whether the inhibitory effect of CC5 on α3β4 and α4β2 receptors was competitive or not, we measured the effect of 100 μM CC5 on the ion currents induced by a low and a high concentration of acetylcholine. The results are shown in Fig. 3C. The inhibition by 100 μM CC5 was

Table 4
Comparison of the potency and efficacy of acetylcholine and cytisine on oocyte-expressed subtypes

	α4β2	α3β4	α7
Acetylcholine			
EC_{50} (μM)	66 ± 29	60 ± 10	167 ± 35
E_{max} (%)	105 ± 2	113 ± 12	111 ± 16
nH	1.1 ± 0.1	1.8 ± 0.4	1.5 ± 0.3
Cytisine			
EC_{50} (μM)	29.5 ± 0.5	67 ± 5	120 ± 11
E_{max} (%)	10.8 ± 0.8	77 ± 6	99 ± 3
nH	1 ± 0.1	1 ± 0.1	1.2 ± 0.1

The values were obtained from the dose-response curves and are the mean values ± S.D. of the data obtained from three separate oocytes.
The $\%E_{max}$ represents %efficacy in comparison with 1 mM acetylcholine.

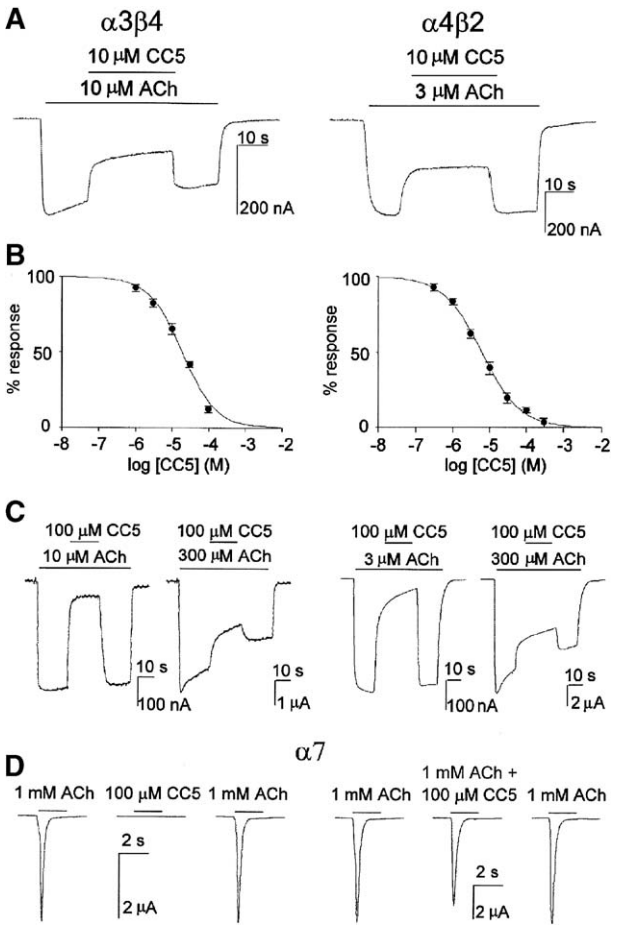

Fig. 3. Effects of CC5 on oocyte-expressed α3β4, α4β2 and α7 nicotinic receptors. (A) Effects of 10 μM of CC5 on the ACh-induced ion currents mediated by α3β4 (left) and α4β2 receptors (right). (B) Concentration–effect curves of CC5 for the inhibition of the 10 μM acetylcholine-induced ion current mediated by α3β4 receptors (left), and of the 3 μM acetylcholine-induced ion current mediated by α4β2 receptors (right). Data fitting yielded mean IC_{50} and nH estimates of 19 ± 2 μM and 0.95 ± 0.07 for α3β4 receptors, and 6 ± 1 μM and 0.83 ± 0.05 for α4β2 receptors. (C) Left traces: Inhibition by 100 μM CC5 of the α3β4 receptor-mediated ion current induced by 10 and 300 μM acetylcholine. Right traces: Inhibition by 100 μM CC5 of the α4β2 receptor-mediated ion current induced by 3 and 300 μM acetylcholine. For both subunit combinations, the amount of inhibition was smaller when the ion currents were induced by the higher acetylcholine concentration. This surmountability of the CC5 effect indicates that the CC5-induced inhibition of the α3β4 and α4β2 receptor-mediated ion currents is a competitive effect. (D) Effects of CC5 on α7 receptors. Left traces: Oocytes responded with large inward currents upon the superfusion of 1 mM acetylcholine but did not respond to the superfusion of 100 μM CC5, thus demonstrating that CC5 is not an α7 nicotinic receptor agonist. Right traces: Co-application of 100 μM CC5 and 1 mM acetylcholine reduced the peak amplitude of the acetylcholine-induced ion current in a reversible manner.

less on the ion currents induced by a high acetylcholine concentration than on those induced by a low concentration, thus demonstrating that the inhibition by CC5 was surmountable and suggesting that the inhibitory effects were due to competitive interactions of CC5 with α3β4 and α4β2 receptors.

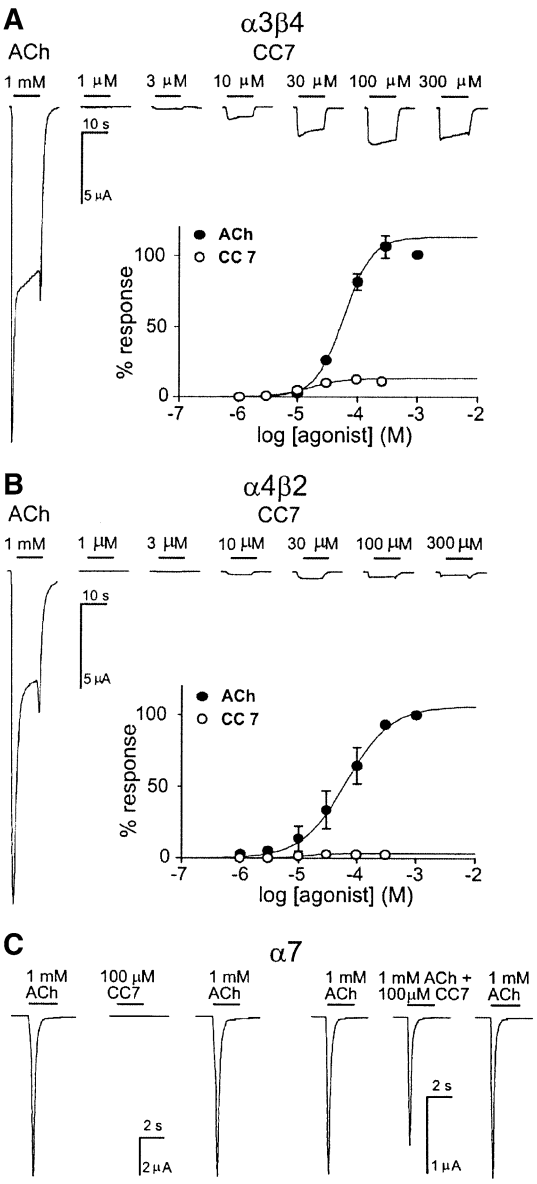

Fig. 4. Effects of CC7 on oocyte-expressed α3β4, α4β2 and α7 receptors. (A) The α3β4 receptor-mediated inward currents induced by a near maximum concentration of 1 mM acetylcholine and by various concentrations of CC7. All the inward current amplitudes have been normalized to the control inward current induced by 1 mM acetylcholine. Inset: Mean concentration–effect curves of acetylcholine and CC7 demonstrating that CC7 is a partial agonist of α3β4 receptors. (B) The α4β2 receptor-mediated inward currents induced by a near maximum concentration of 1 mM acetylcholine and by various concentrations of CC7. All the inward current amplitudes have been normalized to the control inward current induced by 1 mM acetylcholine. Inset: Mean concentration–effect curves of acetylcholine and CC7 demonstrating that CC7 is a partial agonist of the α4β2 receptors. Fitting the data relating to the α4β2 receptors yielded mean EC_{50}, nH and E_{max} estimates of respectively 12 ± 2 μM, 2.05 ± 0.45 and 3.2 ± 0.6% for CC7. Fitting the data relating to the α3β4 receptors yielded mean EC_{50}, nH and E_{max} estimates of respectively 15.8 ± 2.6 μM, 1.50 ± 0.22 and 12.9 ± 2.3% for CC7. (C) Effects of CC7 on α7 receptors. Left traces: Oocytes responded with large inward currents upon the superfusion of 1 mM acetylcholine, but did not respond to the superfusion of 100 μM CC7, thus demonstrating that CC7 is not an α7 nicotinic receptor agonist. Right traces: Co-application of 100 μM CC7 and 1 mM acetylcholine reduced the peak amplitude of the acetylcholine-induced ion current in a reversible manner.

The oocytes expressing α7 receptors did not respond with a detectable inward current to superfusion with 100 μM CC5 (Fig. 3D, left panel; $n=3$), thus indicating that CC5 is not an α7 nicotinic receptor agonist. The co-application of 100 μM CC5 with 100 μM acetylcholine (a concentration less than the EC_{50}) or the nearly maximum effective concentration of 1 mM acetylcholine-induced inward currents that were respectively $18 \pm 3\%$ and $13.6 \pm 4.3\%$ smaller than the control responses, which indicates that CC5 is a weak inhibitor of homooligomeric α7 receptors (Fig. 3D, right panel; $n=3$).

3.4.2. Effects of CC7 on rat α3β4, α4β2 and α7 subtypes

The oocytes expressing α3β4 or α4β2 receptors responded with large inward currents upon superfusion with 1 mM acetylcholine but with very reduced currents after superfusion with CC7 (Fig. 4A and B), thus indicating that CC7 is only a partial agonist for both nicotinic receptor subtypes. Complete concentration–effect curves for acetylcholine and CC7 were obtained in triplicate. In order to correct for differences in expression between oocytes, the acetylcholine- and CC7-induced inward current amplitudes were normalized to that of the 1 mM acetylcholine-induced control inward current. The mean concentration–effect curves of acetylcholine and CC7 for α3β4 and α4β2 receptors are shown in the insets of Fig. 4A and B. Fitting the data (Eq. (1)) relating to the α3β4 receptors yielded mean EC_{50}, nH and E_{max} estimates of respectively 15.8 ± 2.6 μM, 1.50 ± 0.22 and $12.9 \pm 2.3\%$ for CC7. It was concluded that CC7 is a partial agonist of α3β4 receptors, with a maximum response that is 11.4% that of acetylcholine. Fitting the data (Eq. (1)) relating to the α4β2 receptors yielded mean EC_{50}, nH and E_{max} estimates of respectively 12 ± 2 μM, 2.05 ± 0.45 and $3.2 \pm 0.6\%$ for CC7. It was concluded that CC7 is a partial agonist of α4β2 receptors, with an efficacy of 3.0% in comparison with acetylcholine. The data obtained at the highest agonist concentrations for α3β4 were excluded from the curve fitting because this concentration blocked open nicotinic receptor channels.

As in the case of CC5, 100 μM CC7 did not induce any detectable inward current on oocyte-expressed α7 nicotinic receptors (left panel, Fig. 4C; $n=3$), thus indicating that CC7 is not an α7 nicotinic receptor agonist. The co-application of the nearly maximum effective concentration of 1 mM acetylcholine with 100 μM CC7 induced inward currents that were $20.1 \pm 6.3\%$ smaller than the control responses, which showed that CC7 is a weak inhibitor of homooligomeric α7 receptors (right panel, Fig. 4C; $n=3$).

4. Discussion

Neuronal nicotinic receptors are involved in a number of physiological and pathological nervous system processes, and nicotinic ligands are potentially useful as cognitive function enhancers, anxiolytics and analgesics, as well as in the treatment of neurological disorders such as Parkinson's and Alzheimer's disease, and the study and treatment of addiction (Gotti et al., 1997; Clementi et al., 2000b). The search for new and possibly selective ligands has continuously increased over recent years (Holladay et al., 1997; Lloyd and Williams, 2000). We chose cytisine to develop novel nicotinic compounds for two main reasons. First of all, it has differential potency and efficacy depending on the nicotinic receptor subtype: it is a potent agonist of α7 homomeric receptors and the most efficacious agonist of oocyte-expressed α3β4 nicotinic receptor subtypes, but is less efficacious on α3β2 receptors (Covernton et al., 1994). Secondly, surprisingly few cytisine derivatives have been reported in the literature (Barlow and Mcleod, 1969; Schwarz et al., 2000, Houlihan et al., 2001; Imming et al., 2001).

The main findings of our studies are: (i) adding different substituents to the nitrogen group of cytisine decreases the affinity of the derivatives for the nicotinic receptor subtypes, although, like cytisine itself, they still have a higher affinity for brain α4β2-containing receptors; (ii) functional studies assessing Ca^{2+} flux using the FLIPR technique showed that all of the cytisine derivatives lost the cytisine agonist profile and became antagonists (competitive, noncompetitive or channel blocking) or partial agonists with very low intrinsic activity; and (iii) electrophysiological studies of the effects of CC5 and CC7 on oocyte-expressed subtypes confirmed their loss of full agonist effects on α7 and α3β4 subtypes.

4.1. Affinity for receptor subtypes

To investigate whether the cytisine derivatives have selective activity towards the different neuronal nicotinic subtypes in ganglia and the CNS, we studied their inhibition of the binding of [^{3}H]epibatidine (which binds the heteromeric β2 and/or β4-containing receptors) and [^{125}I] αBungarotoxin (which binds the α7-containing receptors) to membranes obtained from rat brain and superior cervical ganglion.

In a preliminary study of classical nicotinic agonists and antagonists, we found that the number and pharmacological profile of brain [^{3}H]epibatidine and [^{125}I]αBungarotoxin receptors closely agree with the published data concerning the same native subtypes (Dávila-García et al., 1997; Gnädisch et al., 1999; Gotti et al., 1997; Quick et al., 1996) or the transfected rat α4β2 and α7 subtypes (Parker et al., 1998; Quick et al., 1996).

Studies in KO mice and rat striatum have shown that, in addition to α4- and β2-containing receptors, rodent brains also have receptors containing α3, α6 and β4 subunits (Zoli et al., 1998, 2002; Whiteaker et al., 2002; Champtiaux et al., 2002). This may explain the lower cytisine affinity in 13% of the receptors, which we found in rat brain and others have found in mouse brain (Marks et al., 1998; Whiteaker et al., 2000; Champtiaux et al., 2002).

The affinity of [^{3}H]epibatidine for rat superior cervical ganglion nicotinic receptors is very similar to that found in mouse superior cervical ganglion (Del Signore et al., 2002),

but lower than that for the heterologously expressed rat α3β4 subtype in oocytes or mammalian cell lines (Parker et al., 1998; Xiao et al., 1998). This could be due to the fact that the presence of subunits, other than α3 and β4, in native superior cervical ganglion receptors may play a role in defining ligand affinity, as we have previously shown in mouse superior cervical ganglion (Del Signore et al., 2002).

Binding studies showed that all of the cytisine derivatives had a higher affinity for brain [^{3}H]epibatidine receptors, although their K_i values were higher than those of cytisine; CC7, CC6, CC5 and CC4 had nanomolar affinities for the brain [^{3}H]epibatidine receptors and, except for CC7, micromolar affinities for ganglionic [^{3}H]epibatidine receptors and brain [^{125}I]αBungarotoxin receptors. CC7 had the highest affinity for all three subtypes, and CC5 had the highest ratio between the affinities of brain and ganglionic[^{3}H]epibatidine receptors (higher than that of all of the tested antagonists).

The simple non-bulky aliphatic substituent in CC7 led to a small reduction in receptor affinity in comparison with cytisine. The fact that CC7 has a higher affinity than CC6 could be due to the shorter length of the chain, but the possibility of its partial conversion to cytisine through a "β-elimination" reaction during overnight incubation should not be overlooked.

In the remaining cases, the bulkiness of the N-substituent greatly decreased the overall affinity of the compounds, although the three receptor subtypes were differently affected depending on the nature of the substituents. As a consequence of the position of the chlorine atom on the chloroheteroaryl residues, CC1 and CC2 had a higher affinity than CC3 for ganglionic and α4β2-containing subtypes, but the reverse was true for the α7-containing subtype.

CC4 was designed with two cytisine units connected by a polymethylene chain because a number of nicotinic ligands, such as hexamethonium, decamethonium, *d*-tubocurarine, alkane- and azaalkanediguanidium, have two ammonium or similar cationic heads joined by a chain whose elongation can change their subtype selectivity (Paton and Zaimis, 1949; Villarroya et al., 1996). The presence of the second cytisine moiety in CC4 decreased its binding affinity for all of the subtypes.

Finally, as expected, CC8 had the poorest affinity for all of the subtypes. Due to the overwhelming steric hindrance exerted by the adamantane moiety on the carbamate function, it is very unlikely that even partial hydrolysis giving rise to cytisine would take place under the working conditions of the binding assays.

4.2. Functional studies

4.2.1. FLIPR assay

We carried out functional studies of Ca^{2+} fluxes by FLIPR, and of ionic currents by electrophysiology. The increase in intracellular Ca^{2+} due to nicotinic receptor activation showed that none of the compounds have full agonist activity, but all of them blocked the response to nicotinic agonists in all receptor subtypes. As also suggested by the electrophysiological results with CC7, the drawback of this assay is that it is not possible to discriminate whether the blocking is due to an antagonist (competitive, noncompetitive or channel blocking) effect, or to the fact that the cytisine compounds are agonists with such a low intrinsic activity that they induce a nondetectable ion flux that can block the subsequent effect of the agonist and/or desensitise the nicotinic receptor. In the case of the CC5 compound, the blocking effect was due to a competitive antagonistic effect, as clearly demonstrated by electrophysiological techniques. The IC_{50} values for the α4β2 subtype were very close to the affinity values determined by binding studies, whereas there was no strict correlation between the affinity and potency of CC5, CC7 and CC8 for the α3β4 subtype.

In the case of CC5 and CC8, the K_i values where much higher than the IC_{50} values, whereas the opposite was true for CC7. We can only explain this by the fact that, like muscle nicotinic receptors, neuronal nicotinic receptors rapidly undergo transitions to various interconvertible conformational states (closed, open or desensitised) characterised by different affinities for nicotinic agonists or antagonists as reviewed by Changeux and Edelstein (1998).

4.2.2. Electrophysiology

Electrophysiological studies of the CC5 and CC7 compounds confirmed their loss of full agonist activity on the α3β4 and α7 subtypes. CC5 had no agonist activity but was a competitive antagonist against rat α4β2 and α3β4 receptors. As also found in the functional FLIPR test, it also lost the selectivity toward the α4β2 subtype shown by the binding studies.

The CC5 IC_{50} values for the α3β4 and α4β2 subtypes determined by electrophysiological and FLIPR techniques are not identical. This could be because of the difference in the technique and/or receptor species (human with FLIPER and rat in oocytes) and/or heterologous expression system, as has previously been shown by Lewis et al. (1997).

CC7 has partial agonist activity on both the α4β2 and α3β4 subtypes. Its effect on the α4β2 subtype is qualitatively similar to that of cytisine, as described by Papke and Heinemann (1994) and determined by us, but its effect on the α3β4 subtype is different. Cytisine is the most potent agonist of the rat α3β4 subtype (Covernton et al., 1994): we found that it is as potent as acetylcholine and gave 77% of the response obtained with 1 mM acetylcholine, whereas the maximum response to CC7 was only 11%.

The effect of cytisine on the same subtypes, and the observed difference in the potency of cytisine reported in this study, could be due to the fact that Coverton et al. (1994) measured the potency ratio of cytisine and acetylcholine at low agonist concentrations, whereas we derived the EC_{50} from the complete dose–response curve.

These electrophysiological results suggest that the inclusion of an alkyl group in CC7 generated a compound that is

still capable of binding and activating central α4β2 receptors, but has lost the potent stimulatory effect of cytisine on α3β4-containing and α7 receptors. Furthermore, this structural modification reduces the hydrophilic of the compound, and increases its lipophilic nature thus increasing its ability to cross the blood/brain barrier and perhaps making it even more useful for in vivo studies.

In conclusion, we show that modifying the nitrogen of cytisine leads to compounds that are more lipophilic than cytisine, retain the high affinity for the α4β2 subtype, but have lost its full agonist effects of cytisine on α3β4 and α7 subtypes.

The structural requirements necessary to activate nicotinic receptors seem to be more stringent than those required for binding; even doubling the number of cytisine molecules (as in the case of CC4) led to a compound with antagonist activity. However, we do not know whether the specific and high affinity binding of the molecules is affected by the conformation of the receptors (open, closed or desensitised).

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