

C3-halogenation of cytisine generates potent and efficacious nicotinic receptor agonists

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Abstract

Neuronal nicotinic acetylcholine receptors subserve predominantly modulatory roles in the brain, making them attractive therapeutic targets. Natural products provide key leads in the quest for nicotinic receptor subtype-selective compounds. Cytisine, found in Leguminosae spp., binds with high affinity to $\alpha 4\beta 2^*$ nicotinic receptors. We have compared the effect of C3 and C5 halogenation of cytisine and methylcytisine (MCy) on their interaction with native rat nicotinic receptors. 3-Bromocytisine (3-BrCy) and 3-iodocytisine (3-ICy) exhibited increased binding affinity (especially at $\alpha 7$ nicotinic receptors; $K_i \sim 0.1 \mu\text{M}$) and functional potency, whereas C5-halogenation was detrimental. 3-BrCy and 3-ICy were more potent than cytisine at evoking [³H]dopamine release from striatal slices ($\text{EC}_{50} \sim 11 \text{ nM}$), [³H]noradrenaline release from hippocampal slices ($\text{EC}_{50} \sim 250 \text{ nM}$), increases in intracellular Ca^{2+} in PC12 cells and inward currents in *Xenopus* oocytes expressing human $\alpha 3\beta 4$ nicotinic receptor ($\text{EC}_{50} \sim 2 \mu\text{M}$). These compounds were also more efficacious than cytisine. C3-halogenation of cytisine is proposed to stabilize the open conformation of the nicotinic receptor but does not enhance subtype selectivity.

Keywords: Nicotinic acetylcholine receptor; Dopamine release; Noradrenaline release; [³H]epibatidine binding; [³H]methyllycaconitine binding; *Xenopus* oocyte

1. Introduction

Nicotinic acetylcholine receptors (nicotinic receptors) in the central nervous system (CNS) are ligand gated ion channels that exert a predominantly modulatory influence on transmitter release, synaptic efficacy and neuronal function (Dajas-Bailador and Wonnacott, 2004). These subtle effects on brain function have made nicotinic receptors attractive therapeutic targets for a diverse range of disorders, including Alzheimer's disease, Parkinson's disease, attention deficit hyperactivity disorder and pain (Hogg and Bertrand, 2004; Cassels et al., 2005). The pentameric nicotinic receptors are assembled from a portfolio of at least 6 α ($\alpha 2$ – $\alpha 7$) and 3 β ($\beta 2$ – $\beta 4$) subunits expressed in

mammalian neurones, resulting in a variety of nicotinic receptor subtypes that differ in their pharmacological and biophysical properties or cellular regulation (Gotti and Clementi, 2004). Most abundant in the CNS is the $\alpha 4\beta 2^*$ subtype (where * denotes the possible inclusion of additional, unspecified subunits; Lukas et al., 1999). $\alpha 4\beta 2^*$ nicotinic receptors are characterised by high affinity binding of agonist radioligands. Also relatively widespread is the homomeric $\alpha 7$ nicotinic receptor, of particular interest because of its high relative permeability to Ca^{2+} and rapid desensitization (Séguéla et al., 1993; Fucile, 2004). $\alpha 3\beta 4^*$ nicotinic receptors mediate synaptic transmission at autonomic ganglia, and represent a minority population of nicotinic receptors in the brain.

The development of suitably selective and potent ligands for discriminating nicotinic receptor subtypes, for therapeutic utility with minimal side effects and for research purposes, remains a

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challenge. Nature has yielded many nicotinic receptor ligands and natural products have provided starting templates for drug design (Jensen et al., 2005; Cassels et al., 2005). One naturally occurring compound is cytisine (Fig. 1), present in many plants of the Leguminosae family, including *Laburnum* and *Sophora* spp. Cytisine is a conformationally restricted quinolizidine alkaloid that binds with high affinity to predominantly $\alpha 4\beta 2^*$ nicotinic receptors (Pabreza et al., 1991; Flores et al., 1992). Functionally cytisine is a nicotinic receptor agonist with greater potency at heteromeric rather than at $\alpha 7$ nicotinic receptors. However, its agonist efficacy is highly dependent on the type of β subunit present, and cytisine is a partial agonist at $\beta 2^*$ nicotinic receptors (Papke and Heinemann, 1994). This property has been exploited to use cytisine as an aid for smoking cessation (Tabex[®]) and to develop a novel synthetic ligand for the same purpose (Coe et al., 2005a,b).

Structure activity studies based on cytisine have examined halogenated and *N*-substituted derivatives for binding affinity and agonist activity at recombinant neuronal nicotinic receptors heterologously expressed in cell lines or *Xenopus* oocytes (Houlihan et al., 2001; Slater et al., 2003; Fitch et al., 2005). The common finding from these studies is that C5 substitution decreases binding affinity and potency, whereas activity is retained or improved following C3 substitution. This was also true for an extensive series of substituted phenyl ring replacements of carbocyclic analogues of cytisine (Coe et al., 2005b). *N*-methylcytisine (another natural product also known as caulophylline) showed diminished potency and efficacy at all nicotinic receptor subtypes tested but its activity could be improved by C3 bromination (Slater et al., 2003). The *N,N*-dimethylated, quaternary amine version also displayed lower binding affinity (Fitch et al., 2005) and aliphatic or alicyclic *N*-substitutions generated nicotinic receptor antagonists (Carbonelle et al., 2003).

The aim of the present work was to compare C3 and C5 halogenated derivatives of cytisine and *N*-methylcytisine for activity at native brain nicotinic receptors. [³H]Dopamine release from rat striatal preparations in vitro is governed by presynaptic $\beta 2^*$ nicotinic receptors on dopaminergic terminals, comprising $\alpha 4\beta 2^*$ and $\alpha 6\beta 2\beta 3^*$ subtypes (Champtiaux et al., 2003; Salminen et al., 2004), whereas nicotinic receptor-evoked [³H]noradrenaline release from rat hippocampal preparations displays a different pharmacology consistent with the involvement of $\beta 4^*$ nicotinic receptors (Luo et al., 1998). Compounds

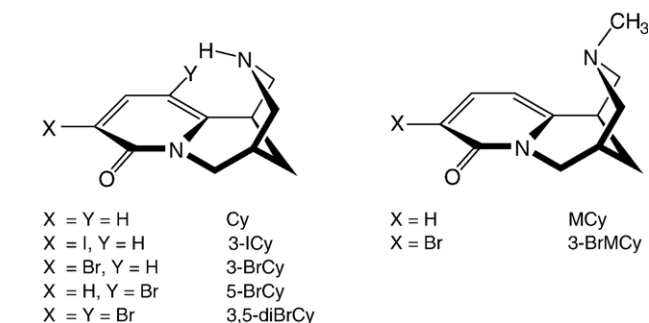


Fig. 1. Structures of cytisine derivatives.

were also compared for activity at native nicotinic receptors, predominantly $\beta 4^*$, expressed in PC12 cells, and human $\alpha 3\beta 4$ nicotinic receptor heterologously expressed in *Xenopus* oocytes. As well as increasing potency in all assays, C3-halogenation also improved efficacy with respect to [³H]dopamine release.

2. Materials and methods

2.1. Competition binding assays

P2 membranes were prepared from whole rat brain (minus cerebellum) by differential centrifugation as described previously (Davies et al., 1999). The washed pellet was resuspended in 50 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.01% sodium azide (2.5 ml/g original weight) and stored in 5 ml aliquots at -20°C . Protein was determined using bovine serum albumin as standard (Bradford, 1976). Competition binding assays were performed as described previously (Sharples et al., 2000, 2002). Rat brain P2 membranes (0.08 mg/ml for [³H]epibatidine binding and 0.80 mg/ml for [³H]methyllycaconitine ([³H]MLA) binding) were incubated with serial dilutions of cytisine or derivatives and [³H]epibatidine (1.0 nM) or [³H]MLA (2.0 nM) in a total volume of 1.0 or 0.25 ml, respectively. For [³H]epibatidine binding the assay buffer contained 20 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 20 mM Tris, 0.1 mM PMSF, and 0.01% sodium azide, pH 7.4. For [³H]MLA binding Tris/phosphate buffer, pH 7.4, (40 mM K₂HPO₄, 10 mM KH₂PO₄, 1 mM EDTA, 0.1 mM PMSF, 200 mM Tris, 0.01% sodium azide, 0.1% (w/v) bovine serum albumin) was used (Davies et al., 1999). Nonspecific binding was defined in the presence of 1 mM (–)-nicotine. Incubations were carried out at room temperature for 60 min for [³H]epibatidine or 120 min for [³H]MLA, followed by 30 min at 4°C . Bound radioligand was separated by rapid filtration on Whatman GFA/E filter paper (presoaked overnight in 0.3% polyethylenimine in PBS, pH 7.4), using a Brandel cell harvester. Filters were counted for radioactivity in a PerkinElmer Tri-Carb liquid scintillation counter 1500 (counting efficiency 45%).

2.1.1. Data analysis

IC₅₀ values were derived from competition binding data by fitting data points to the Hill equation using a nonlinear least-squares curve fitting facility of Sigma Plot 2.0 for Windows (SPSS Inc., Chicago, IL):

$$\% \text{ Bound} = 100\% / (1 + ([\text{Ligand}] / \text{IC}_{50})^{nH})$$

where *nH* is the Hill number, [Ligand] is the concentration of the competing ligand, and IC₅₀ is the concentration of competing ligand that displaces 50% of specific radioligand bound. *K_i* values were calculated from IC₅₀ values (Cheng and Prusoff, 1973), assuming *K_d* values of 266 pM and 1 nM for [³H]epibatidine and [³H]MLA, respectively, determined in corresponding saturation binding assays.

2.2. Transmitter release

The measurement of [^3H]dopamine release from rat striatal slices and [^3H]noradrenaline release from hippocampal slices was based on the methods described by Anderson et al. (2000) and Puttfarcken et al. (2000), respectively. Rats (250–350 g) were killed by cervical dislocation, and the striatum and hippocampus were rapidly dissected. Striatal and hippocampal slices (0.25 mm) were prepared using a McIlwain tissue chopper, washed three times with Krebs-bicarbonate buffer (118 mM NaCl; 2.4 mM KCl; 2.4 mM CaCl_2 ; 1.2 mM MgSO_4 ; 1.2 mM KH_2PO_4 ; 25 mM Na_2HCO_3 ; 10 mM glucose; oxygenated with 95% O_2 /5% CO_2 ; pH 7.4), and loaded with [^3H]dopamine or [^3H]noradrenaline (50 and 70 nM, respectively) for 30 min at 37 °C. After four washes with Krebs buffer plus nomifensine (0.5 μM), slices were dispersed into 96-well Multiscreen filter plates (Millipore, Bedford, MA). Striata or hippocampi from two animals were sufficient for one 96-well plate. Buffer was removed using a vacuum filtration unit (Millipore), leaving tissue slices on well filters. Subsequently, 70 μl of Krebs buffer containing nomifensine, with or without antagonist, was added to each well and allowed to incubate for 5 min at 37 °C in an atmosphere of 95% O_2 and 5% CO_2 . Basal release was collected via vacuum filtration into a 96-well Optiplate (PerkinElmer Life Sciences). Then 70 μl of buffer (with or without agonists and/or antagonists) was added to each well, and allowed to incubate for 5 min at 37 °C. Stimulated release was collected by rapid filtration into another 96-well Optiplate. Microscint-40 (170 μl ; PerkinElmer) was added to each well and radioactivity was counted using a scintillation counter (Wallac microBeta Trilux 1450, PerkinElmer Life Sciences, Espoo, Finland, counting efficiency 31%). To estimate radioactivity remaining in the slices at the end of the experiment, filters were removed from the 96-well plate and counted for radioactivity in a PerkinElmer Life Sciences Tri-Carb 1500 liquid scintillation counter (counting efficiency, 45%). Total radioactivity present in the slices at the start of the stimulation was calculated as the sum of dpm tritium released plus dpm tritium radioactivity present in slices at the end of experiment, after correction for counting efficiency. Data are presented as fractional release for each well [stimulated release/(basal release+stimulated release+remaining radioactivity in the tissue)–basal release/(stimulated release+remaining radioactivity)].

2.3. Cell culture and calcium fluorimetry

Rat pheochromocytoma cells (PC12 cells) were maintained in 75 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum, 190 U/ml penicillin, 0.2 mg/ml streptomycin and 2 mM L-glutamine. The cells were incubated at 37 °C in 5% CO_2 -enriched, humidified atmosphere, until confluent. Cells were dissociated by trituration and plated at 50,000 cells/ cm^2 on poly-L-lysine-coated 96-well plates.

Ca^{2+} -mediated increases in fluo-3 fluorescence in PC12 cells were monitored as described previously (Dajas-Bailador et al., 2002). Briefly, the medium was removed from 75% to 80%

confluent cultures and then PC12 cells were washed twice with Tyrode's salt solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl_2 , 1.8 mM CaCl_2 , 0.2 mM NaH_2PO_4 , 12 mM NaHCO_3 , 5.5 mM glucose, pH 7.4) and incubated with 10 μM Fluo-3 AM and 0.02% pluronic acid for 1 h at room temperature in the dark. Cells were washed twice with Tyrode's salt solution, before adding 80 μl of buffer to each well, with or without antagonists. After 10 min at room temperature in the dark, changes in fluorescence (excitation, 485 nm; emission, 538 nm) were measured using a Fluoroskan Ascent fluorescent plate reader (Labsystems, Helsinki, Finland). Basal fluorescence levels were monitored for 5 s before addition of 20 μl of stimulus (nicotine or cytosine analog) and changes in fluorescence were monitored for a further 20 s. To normalize Fluo-3 signals, responses from each well were calibrated by determination of the maximum and minimum fluorescence values by addition of 1% Triton X-100 (F_{max}) followed 350 mM MnCl_2 (F_{min}). Data were calculated as a percentage of $F_{\text{max}} - F_{\text{min}}$. Values are expressed as a percentage of the response to 100 μM nicotine included in all experiments, and are the mean of at least 3 independent experiments with at least 8 replicates per experiment.

2.4. Expression of human $\alpha 3\beta 4$ nicotinic receptor in *Xenopus* oocytes and two electrode voltage clamp recording

Xenopus toads were kept and euthanized in accordance with Animals (Scientific Procedures) Act, 1986, UK. Stage V and VI *Xenopus* oocytes were prepared as previously described (Houlihan et al., 2001). The human $\alpha 3$ (supplied by RJ Lukas, Barrow Institute, Arizona) and $\beta 4$ (supplied by J Lindstrom, Pennsylvania University, Pennsylvania) nicotinic receptor subunits, ligated into the pcDNA3.1 expression vector, were dissolved in distilled water at approximately equal concentrations of 1 $\mu\text{g}/\text{ml}$ (spectrophotometric plus agarose gel electrophoresis determinations). Mixtures of these solutions at 1:1 $\alpha 3/\beta 4$ ratios were injected into the nuclei of oocytes in a volume of 18.4 nl/oocyte, using a variable volume automatic oocyte microinjector (Drummond Broomall, PA, USA). The total amount of nicotinic receptor subunit expression vector injected per oocyte was kept constant (1 ng). After injection the oocytes were incubated at 19 °C in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.3 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 0.82 mM MgSO_4 , 15 mM HEPES, and 50 mg/l neomycin (pH 7.6 with NaOH). Experiments were performed between 2 and 5 days after injection.

Oocytes were impaled by two agarose-cushioned microelectrodes filled with 3 M KCl (0.5–2.5 M Ω) and voltage clamped using a modified Geneclamp 500B amplifier (Axon Instruments, Union City, CA, U.S.A.) according to the methods described by Houlihan et al. (2001). Oocytes were continually supplied with fresh Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2 , 10 mM HEPES, pH 7.2) in a 60 μl bath, using a gravity-driven perfusion system at a rate of 5 ml/min. Acetylcholine was applied by gravity perfusion using a manually activated valve and for a period sufficient (approx. 10–15 s) to obtain a stable plateau response (at low concentrations) or the beginning of a sag after a peak (at higher concentrations). Between each

successive drug application, the cell was superfused with Ringer solution for 4 min to allow drug clearance and prevent receptor desensitization. Concentration–response curves for activation by acetylcholine or by cytisine analogs were constructed by normalizing to 1 mM acetylcholine, which is about the maximal response to acetylcholine at $\alpha 3\beta 4$ nicotinic receptors (Fig. 7). Normalized data points were used to generate EC_{50} , E_{max} and $nHill$ estimates (Houlihan et al., 2001). To construct antagonist concentration–response curves, the responses elicited by the co-application of acetylcholine and increasing concentrations of either 3,5-diBrCy or *N*-methylcytisine were normalized to the responses evoked by acetylcholine alone. The concentration of acetylcholine used in these studies was 100 μM (close to acetylcholine EC_{50}). Constant responses to acetylcholine were obtained before the co-application of acetylcholine and drug. In these studies, human $\alpha 3\beta 4$ nicotinic receptors were not pre-incubated with the test drugs prior to co-application with acetylcholine to avoid cross-desensitization of the receptors that might have arisen from the agonist effects of these compounds.

Data analysis: Agonist concentration–response data were fitted by nonlinear regression (Prism 3.01, GraphPad, USA) to the equation:

$$I = I_{max}/[1 + (EC_{50}/X)^{nH}]$$

Where I_{max} is the maximum observed response, X is the agonist or antagonist concentration, EC_{50} is the agonist concentration that produces 50% of the maximum response and nH is the Hill coefficient.

2.5. Materials

Adult male Sprague–Dawley rats were obtained from the University of Bath Animal House breeding colony. *Xenopus laevis* toads were purchased from Blades Biological (Kent, UK). [7,8- 3H]Dopamine (44 Ci/mmol), [7,8- 3H]noradrenaline (35 Ci/mmol) and [3H]epibatidine, (54 Ci/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). [3H]Methyllycaconitine (43.7 Ci/mmol), α -conotoxin MII and cytisine were obtained from Tocris Cookson (Bristol, UK). (–)-Nicotine hydrogen tartrate, mecamylamine, dihydro- β -erythroidine, nomifensine, pargyline, bovine serum albumin, tissue culture media and serum were from Sigma Chemical Co. (Poole, Dorset, UK). Fluo-3 AM and pluronic acid were from Molecular Probes (Eugene, OR). All other chemicals used were of analytical grade and obtained from standard commercial sources. Plasticware for tissue culture was from Apogent (Roskilde, Denmark).

Cytisine derivatives were synthesized as previously described (Houlihan et al., 2001; Slater et al., 2003). Briefly, cytisine was purified from the seeds of the Mexican plant *Sophora secundiflora* using standard methodology. *N*-methylcytisine was similarly purified from seeds of the Chilean *Sophora macrocarpa*. Bromination of cytisine or *N*-methylcytisine with a slight excess of molecular bromine in acetic acid led to the formation of a mixture of products containing a small amount of 3,5-dibromocytisine (3,5-diBrCy) or 3,5-dibromo-*N*-methylcytisine (3,5-diBrMCy) and mainly 3- and 5-bromocytisine (3-BrCy and 5-BrCy, respectively) or 3- and 5-bromo-*N*-methylcytisine (3-BrMCy and 5-BrMCy, respectively). These were separated by column chromatography on silica gel, crystallized to homogeneity, and characterized by 1H and ^{13}C NMR and HREIMS. Definitive structure assignments were based on 1H – 1H COSY experiments. 3-iodo-cytisine (3-ICy) was prepared similarly using iodine monochloride and was also fully characterized. Structures are shown in Fig. 1.

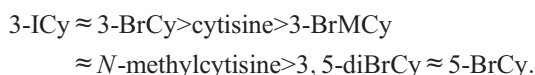
3-BrCy and 5-BrCy, respectively) or 3- and 5-bromo-*N*-methylcytisine (3-BrMCy and 5-BrMCy, respectively). These were separated by column chromatography on silica gel, crystallized to homogeneity, and characterized by 1H and ^{13}C NMR and HREIMS. Definitive structure assignments were based on 1H – 1H COSY experiments. 3-iodo-cytisine (3-ICy) was prepared similarly using iodine monochloride and was also fully characterized. Structures are shown in Fig. 1.

3. Results

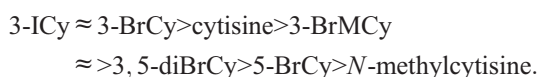
3.1. Radioligand binding studies

Cytisine, *N*-methylcytisine and their halogenated isosteres (Fig. 1) were evaluated for their binding affinities at $\alpha 7$ and non- $\alpha 7$ neuronal nicotinic receptors by measuring the displacement of [3H]epibatidine (non- $\alpha 7$) or [3H]MLA ($\alpha 7$) binding to rat brain P2 membranes (Fig. 2). The $pIC_{50} \pm S.E.M.$ values for these compounds are summarized in Table 1.

Cytisine displaced [3H]epibatidine binding with a shallow curve, consistent with binding to 2 sites, with K_i values of ~ 0.6 and 47 nM, as previously reported (Whiteaker et al., 2000). Halogenation at the C3 position to give 3-BrCy and 3-ICy resulted in more potent ligands with respect to [3H]epibatidine binding ($K_i = 0.21$ and 0.16 nM, respectively). The binding data for these compounds were best fitted by a single site Hill plot (Fig. 2A). Bromination at C5, or C5 and C3 positions, resulted in markedly lower potencies compared with the parent compound ($K_i = 307$ and 250 nM, respectively). The natural product *N*-methylcytisine also exhibited decreased activity, relative to cytisine itself ($K_i = 75$ nM), and C3 bromination of *N*-methylcytisine did not increase its potency ($K_i = 68$ nM). The rank of order of potency for inhibition of [3H]epibatidine binding is:



At $\alpha 7$ nicotinic receptors a similar order of potency was observed (Fig. 2B). Cytisine appeared to bind to a single site with $K_i = 30.4 \mu M$. 3-BrCy and 3-ICy were more than 2 orders of magnitude more potent at displacing [3H]MLA ($K_i = 0.11$ and 0.12 μM , respectively). *N*-methylcytisine was devoid of activity at this site but C3 bromination of this compound produced an activity comparable with that of cytisine itself ($K_i = 47 \mu M$). Bromination at the C5 position decreased affinity, compared with cytisine ($K_i = 103 \mu M$) but this was recovered by dual bromination at C3 and C5 ($K_i = 43 \mu M$). To summarize, the rank order of potency for inhibition of [3H]MLA binding is:



3.2. Nicotinic modulation of transmitter release from rat brain slices

The cytisine derivatives were compared for their abilities to evoke the release of [3H]dopamine from striatal slices and

Table 1
Potencies of cytisine and its derivatives at rat brain binding sites and in transmitter release assays

Ligand	$[^3\text{H}]$ epibatidine binding site		$[^3\text{H}]$ MLA binding site		$[^3\text{H}]$ dopamine release from striatal slices pEC ₅₀ ±S.E. M. (M)	$[^3\text{H}]$ noradrenaline release from hippocampal slices pEC ₅₀ ±S.E. M. (M)
	pIC ₅₀ ±S.E.M. (M)	K _i (nM)	pIC ₅₀ ±S.E.M. (M)	K _i (nM)		
Cytisine	-9.05 -7.16	0.60 47	-4.20±0.18	30403	-6.56±0.12	-5.13±0.06
3-BrCy	-9.51±0.05	0.208	-6.63±0.15	112	-7.93±0.12	-6.53±0.18
3-ICy	-9.61±0.05	0.165	-6.62±0.25	115	-7.95±0.14	-6.66±0.11
N-methylcytisine	-6.95±0.07	75	-	-	-4.84±0.06	≫-4
3-BrMCy	-6.99±0.08	68	-4.013±0.04	46765	-5.34±0.18	~-4
3,5-diBrCy	-6.43±0.06	250	-4.046±0.02	43343	-5.82±0.15	~-4
5-BrCy	-6.34±0.03	307	-3.670±0.03	103020	-4.75±0.08	~-4
Nicotine	-	-	-	-	-6.86±0.11	-5.29±0.13

$[^3\text{H}]$ noradrenaline from hippocampal slices. Nicotine was also included as a reference agonist. With respect to $[^3\text{H}]$ dopamine release (Fig. 3A), cytisine displayed its well-known partial agonist effect, with maximum release that is approximately 50% of that seen with nicotine. The EC₅₀ values of nicotine and cytisine were comparable (0.14 and 0.27 μM, respectively; Table 1). 3-BrCy and 3-ICy were markedly more potent at evoking the release of $[^3\text{H}]$ dopamine (EC₅₀=11.7 and 11.2 nM, respectively). Notably these compounds showed higher efficacy than cytisine, approaching that of nicotine (Fig. 3A). The other derivatives examined were all less potent than cytisine: N-methylcytisine and 5-BrCy were the weakest compounds (EC₅₀=14.5 and 17.8 μM, respectively). Additional C3 bromination of these compounds improved their potency (EC₅₀ for 3-BrMCy=4.6 μM; EC₅₀ for 3,5-diBrCy=1.5 μM).

With respect to eliciting $[^3\text{H}]$ noradrenaline release from hippocampal slices, all compounds were about 50-fold less potent than in the $[^3\text{H}]$ dopamine release assay (Fig. 3B). Cytisine was comparable with nicotine in both potency (EC₅₀=7.4 and 5.5 μM, respectively) and efficacy. Again the C3-halogenated derivatives 3-BrCy and 3-ICy were most potent (EC₅₀=0.29 and 0.22 μM, respectively), whereas the other brominated derivatives had little activity (EC₅₀ ~100 μM) and N-methylcytisine was essentially inactive.

Subtype-selective antagonists were used to explore the nicotinic receptor subtypes activated by cytisine and its C3 derivatives. Dihydro-β-erythroidine (DHβE) has been useful for identifying β2* nicotinic receptor. However the competitive nature of its antagonism can be problematic as inhibition will be inversely related to the agonist concentration. Therefore concentration-response curves for nicotine- and cytisine-evoked $[^3\text{H}]$ dopamine release were undertaken in the presence and absence of DHβE (Fig. 4A, B). The antagonist shifted the concentration-response curve to the right; 10 μM DHβE was sufficient to completely block $[^3\text{H}]$ dopamine release evoked by agonist concentrations approaching the maximal response. Using these conditions, $[^3\text{H}]$ dopamine release evoked by 50 nM 3-BrCy and 3-ICy was fully abolished (Fig. 4D).

Similar experiments with the α6/α3β2* selective antagonist αconotoxin-MII (200 nM) gave about 30% inhibition of nicotine-evoked $[^3\text{H}]$ dopamine release, and this was insurmountable with increasing agonist concentration (Fig. 4C). This apparently uncompetitive mode of action may reflect the relatively high concentration of αconotoxin-MII used to achieve maximum blockade, or its slow binding and un-binding kinetics, or the large size of the toxin, relative to agonist molecules, such that it has additional contact points on the receptor that make it more resistant to dissociation in the presence of agonist. $[^3\text{H}]$ dopamine release elicited by 50 nM 3-BrCy and 3-ICy was also inhibited by 200 nM αconotoxin-MII, by 28.2% and 37.7%, respectively (Fig. 4D). A maximally effective concentration of cytisine (1 μM) was used to evoke $[^3\text{H}]$ dopamine release in this experiment, giving responses of similar magnitude to those elicited by the sub-maximal concentration (50 nM) of C3-halogenated derivatives (see Fig. 3A), in order to obtain more reliable estimates of partial inhibition. Cytisine-evoked $[^3\text{H}]$ dopamine release was much more sensitive to αconotoxin-MII, which gave 62.1% inhibition (Fig. 4D), consistent with cytisine's partial agonism at αconotoxin-MII-insensitive, α4β2* nicotinic receptors (Grady et al., 2002).

The actions of these antagonists were very different with respect to nicotinic receptor-evoked $[^3\text{H}]$ noradrenaline release. Neither DHβE (Fig. 5A, B) nor αconotoxin-MII (Fig. 5C) had any significant effect on nicotine- or cytisine-evoked $[^3\text{H}]$ noradrenaline release, consistent with the lack of involvement of β2* nicotinic receptors (including α6/α3β2* nicotinic receptors). The lack of effect of increasing concentrations of DHβE on $[^3\text{H}]$ noradrenaline release elicited by 10 μM 3-BrCy and 3-ICy is shown in Fig. 5D. Importantly, the responses to these novel agonists were almost totally prevented by 10 μM mecamylamine (Fig. 5D), confirming that these compounds are acting via nicotinic receptors.

3.3. Nicotinic receptor-evoked Ca²⁺ responses in PC12 cells

Cytisine, 3-BrCy and 3-ICy were examined over the concentration range 1–100 μM for their ability to increase

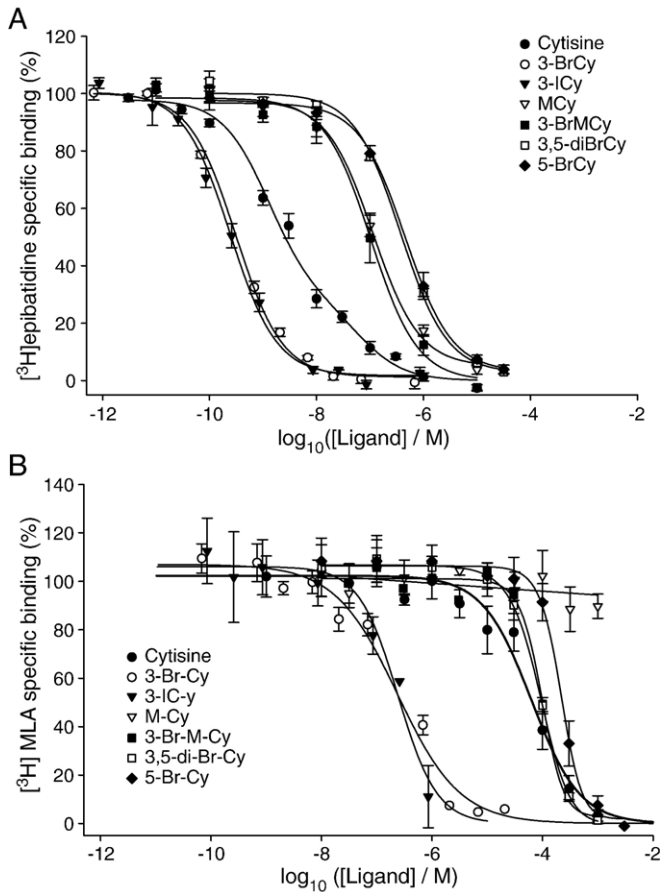


Fig. 2. Competition by cytosine and cytosine derivatives for (A) $[^3\text{H}]$ epibatidine and (B) $[^3\text{H}]$ MLA binding to rat brain P2 membranes. Membranes were incubated with serial dilutions of unlabeled ligands and (A) 1 nM $[^3\text{H}]$ epibatidine for 1 h at room temperature or (B) 2 nM $[^3\text{H}]$ MLA for 2 h at room temperature, in the presence and absence of 1 mM nicotine to define non-specific binding. Bound radioligand was separated by filtration through glass fiber filters and quantitated by scintillation spectrometry. Each point represents the mean \pm S.E.M. of at least three separate experiments, each conducted in triplicate. Data points are fitted to a single site Hill Equation (or a two site Hill equation for inhibition of $[^3\text{H}]$ epibatidine binding by cytosine) as described in the Materials and Methods.

intracellular Ca^{2+} in PC12 cells (Fig. 6). Each compound produced a concentration-dependent increase in fluorescence: the C3-halogenated compounds were about 10 times more potent. Responses to each drug concentration were largely eliminated in the presence of 10 μM mecamylamine.

3.4. Two-electrode voltage clamp recordings from *Xenopus* oocytes expressing human $\alpha 3\beta 4$ nicotinic receptors

Marked differences were observed in the potency and relative efficacy displayed by cytosine and its *N*-methylated and bromo-isosteres at human $\alpha 3\beta 4$ nicotinic receptors (Fig. 7A; parameters estimated from concentration–response data are summarized in Table 2). Cytosine, 3-BrCy, 5-BrCy and 3-BrMCy evoked inward currents in oocytes expressing $\alpha 3\beta 4$ nicotinic receptors, while currents evoked by 3,5-diBrCy were too small to be reliably measured. *N*-methylcytosine did not evoke any inward currents, even at concentrations higher than

1 mM. The concentration–response data for cytosine, 3-BrCy, 5-BrCy and 3-BrMCy were best fitted to a single Hill equation (Fig. 7A). The most potent agonist at the human $\alpha 3\beta 4$ nicotinic receptor was 3-BrCy ($\text{EC}_{50} = 2.1 \mu\text{M}$), which was about 50-fold more potent than cytosine or 5-BrCy. Interestingly, cytosine was a partial agonist at human $\alpha 3\beta 4$ nicotinic receptors. 3-BrMCy showed intermediate potency but very low efficacy, as did 5-BrCy. In contrast, 3-BrCy was fully efficacious and comparable to acetylcholine, with regard to the maximum response. The least effective agonists 3,5-diBrCy and *N*-methylcytosine were antagonists of acetylcholine-evoked currents, with estimated IC_{50} values of 115 ± 9 and $687 \pm 50 \mu\text{M}$, respectively (Fig. 7B).

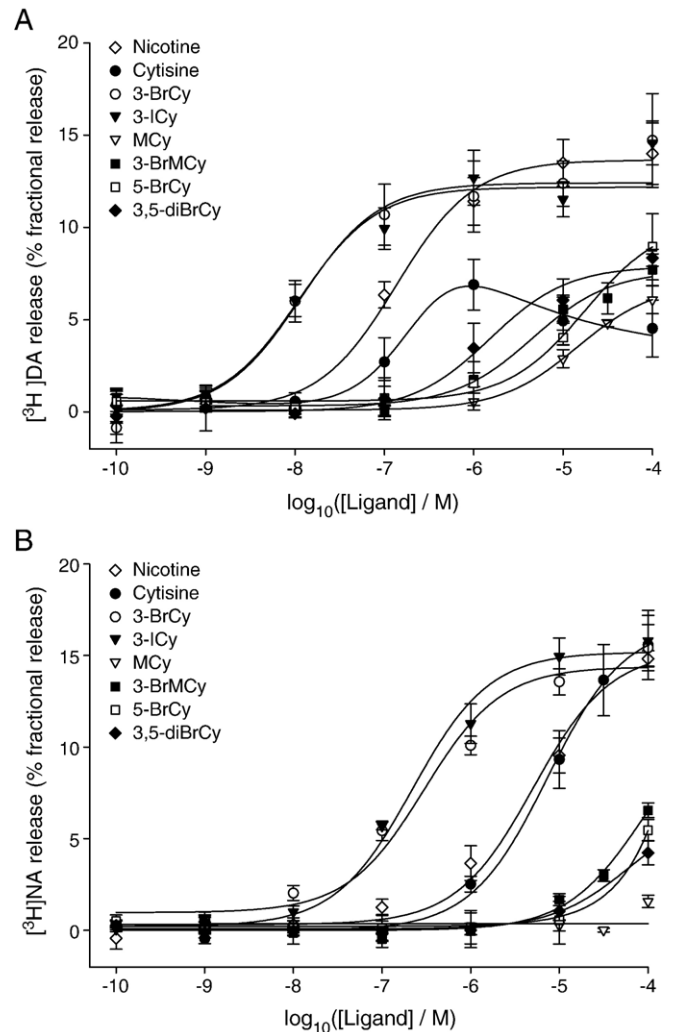


Fig. 3. Release of (A) $[^3\text{H}]$ dopamine from rat striatal slices and (B) $[^3\text{H}]$ noradrenaline from rat hippocampal slices, evoked by cytosine and its derivatives. Slices were preloaded with tritiated transmitter, washed and dispensed into 96-well filter plates. Basal release was collected by filtration after 5 min incubation with Krebs buffer. Test drugs were added in fresh buffer and stimulated release was collected after a further 5 min incubation period. Stimulated release is calculated as a fraction of the total radioactivity in the tissue at the point of stimulation as described in the Materials and Methods. Each point represents the mean \pm S.E.M. of at least three separate experiments, each conducted with 8 replicates. Data points are fitted to the Hill Equation as described in the Materials and Methods.

4. Discussion

We have compared the effect of C3 and C5 halogenation of cytosine and *N*-methylcytosine on binding affinity and functional potency at native rat nicotinic receptors. These studies confirm that C3-halogenation enhances affinity (especially at $\alpha 7$ nicotinic receptors) whereas C5-halogenation is detrimental. Cytosine elicited [^3H]dopamine release from striatal preparations with characteristic low efficacy relative to nicotine; 3-BrCy and 3-ICy were more potent and more efficacious. All compounds were markedly less potent at evoking [^3H]noradrenaline release from hippocampal slices: only cytosine and the C3-halogenated derivatives showed substantial activity. This was consistent with the potency of the compounds in eliciting Ca^{2+} increases in rat PC12 cells and currents from human $\alpha 3\beta 4$ nicotinic receptors expressed in *Xenopus* oocytes.

Previous studies have demonstrated that C3-substitution of cytosine is well tolerated, and halogenation at this position enhances biological activity, whereas C5-substitution decreases

activity (Houlihan et al., 2001; Slater et al., 2003; Coe et al., 2005a,b; Fitch et al., 2005). These studies utilized heterologously expressed defined nicotinic receptor subtypes. Here we have focused mainly on native rat nicotinic receptors, which are likely to have more complex and more heterogeneous subunit arrangements. Heterogeneity was evident in the inhibition of [^3H]epibatidine binding to rat brain membranes by cytosine, which best fitted a two site model as discussed for mouse brain (Whiteaker et al., 2000). Although the C3-halogenated derivatives were substantially more potent than cytosine itself in competing for [^3H]epibatidine-labelled sites, the binding curves for these derivatives fit a single site model. This suggests that C3-substitution produces less discriminating compounds. Indeed, C3-halogenation resulted in a greater increase in binding affinity (compared with cytosine) at $\alpha 7$ nicotinic receptors defined by [^3H]MLA binding, with K_i values almost 300 times lower than that for the parent compound. This compares with ~ 10 -fold decreases in K_i values for 3-BrCy and 3-ICy binding to [^3H]epibatidine sites, relative to cytosine (Table 1).

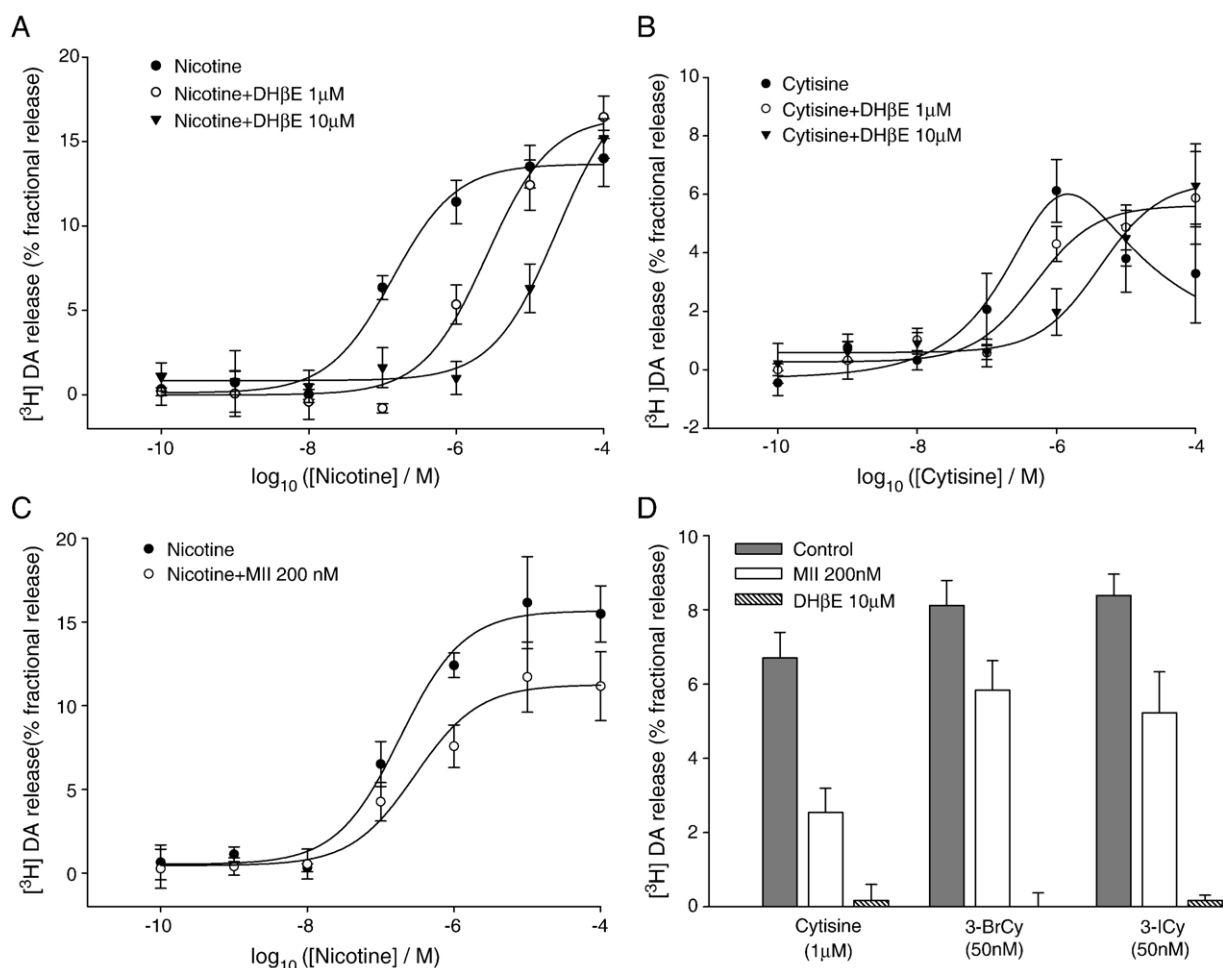


Fig. 4. Pharmacological characterization of nicotinic receptor-evoked [^3H]dopamine release from rat striatal slices. Concentration–response curves for [^3H] dopamine release evoked by nicotine (A,C) or cytosine (B) were conducted in the presence and absence of DHβE (1, 10 μM ; A,B) or α conotoxin-MII (MII, 200 nM; C). [^3H] dopamine release evoked by cytosine (1 μM) or 3-BrCy or 3-ICy (50 nM) was compared in the presence and absence of 10 μM DHβE or 200 nM α conotoxin-MII (D). Stimulated release is calculated as a fraction of the total radioactivity in the tissue at the point of stimulation as described in the Materials and Methods. Each point represents the mean \pm S.E.M. of at least three separate experiments, each conducted with 8 replicates. Data points in A, B and C are fitted to the Hill Equation as described in the Materials and Methods.

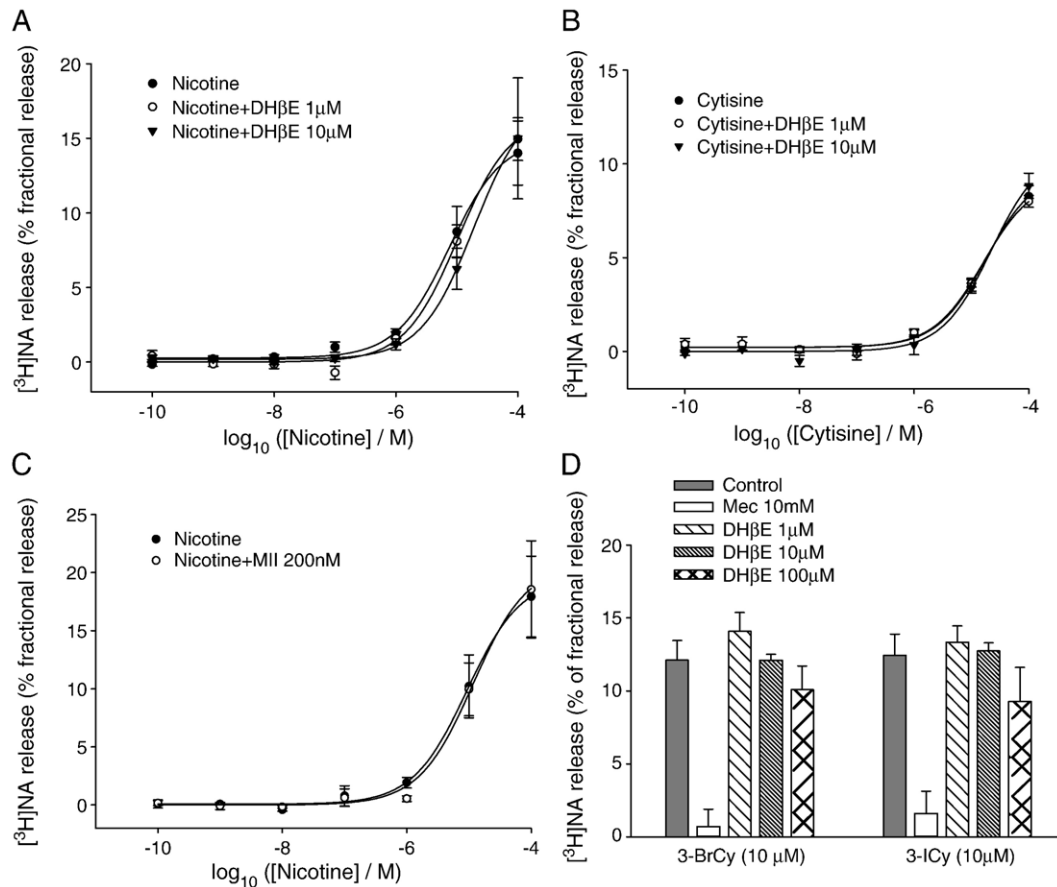


Fig. 5. Pharmacological characterization of nicotinic receptor-evoked [^3H]noradrenaline release from rat hippocampal slices. Concentration–response curves for [^3H]noradrenaline release evoked by nicotine (A, C) or cytisine (B) were conducted in the presence and absence of DH β E (1, 10 μM ; A, B) or α conotoxin-MII (MII, 200 nM; C). [^3H]noradrenaline release evoked by 3-BrCy or 3-ICy (10 μM) was compared in the presence and absence of mecamylamine (Mec, 10 μM) or DH β E (1, 10, 100 μM) (D). Stimulated release is calculated as a fraction of the total radioactivity in the tissue at the point of stimulation as described in the Methods. Each point represents the mean \pm S.E.M. of at least three separate experiments, each conducted with 8 replicates. Data points in A, B and C are fitted to the Hill Equation as described in the Materials and Methods.

At [^3H]epibatidine binding sites *N*-methylcytisine was somewhat more potent than 5-BrCy, and their affinities were not improved by additional C3-bromination. This contrasts with the interactions at $\alpha 7$ nicotinic receptors, where *N*-methylcytisine was inactive, 5-BrCy exhibited weak activity, and the affinity of both compounds was improved by additional C3 bromination. Together these results suggest that the $\alpha 7$ nicotinic receptor binding pocket can interact more favorably with C3 substituents.

The functional activity of these compounds was assessed with respect to the presynaptic nicotinic modulation of [^3H]dopamine and [^3H]noradrenaline release, from striatal and hippocampal slices, respectively. These transmitters are regulated by distinct populations of presynaptic nicotinic receptor subtypes. Striatal [^3H]dopamine release is modulated by $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nicotinic receptors present on dopaminergic terminals (Champtiaux et al., 2003; Salminen et al., 2004). Cytisine is well documented as a partial agonist in this preparation (Grady et al., 2002), reflecting its low efficacy at $\alpha 4\beta 2^*$ nicotinic receptors (Papke and Heinemann, 1994; Coe et al., 2005a). The less potent C5-brominated derivatives retained the low efficacy of the parent compound. However, 3-BrCy and

3-ICy gained both potency and efficacy. This can be attributed to a greater activation of $\alpha 4\beta 2^*$ nicotinic receptors, because the $\alpha 6/\alpha 3\beta 2^*$ -selective antagonist α conotoxin-MII inhibited a lower proportion of the response to the 3-substituted derivatives, compared with the response to cytisine (Fig. 4). Interestingly, the low efficacy of cytisine that was observed at heterologously expressed $\alpha 3\beta 4$ nicotinic receptors was also overcome by bromination at C3 (Fig. 7A) and the insignificant efficacy of *N*-methylcytisine at these receptors increased to a measurable value (Table 2). Hence substitution at C3 may favor interaction with a subsite contributed by the α subunit to the binding pocket that may be crucial for affinity and/or efficacy. The effect of a bromine or iodine atom at this position shows that partial agonism (the ability to bind to the nicotinic receptor with a reduced probability of channel activation) cannot be simply due to the semi-rigid tricyclic skeleton that is common to all cytisinoids. Modeling studies suggest that cytisine can adopt different orientations in the agonist binding site (Costa et al., 2003; Zapata-Torres, unpublished results), and the open state of the nicotinic receptor is presumably stabilized when the ligand is anchored by more than the usually accepted cation— π , hydrogen bond acceptor and aromatic interactions.

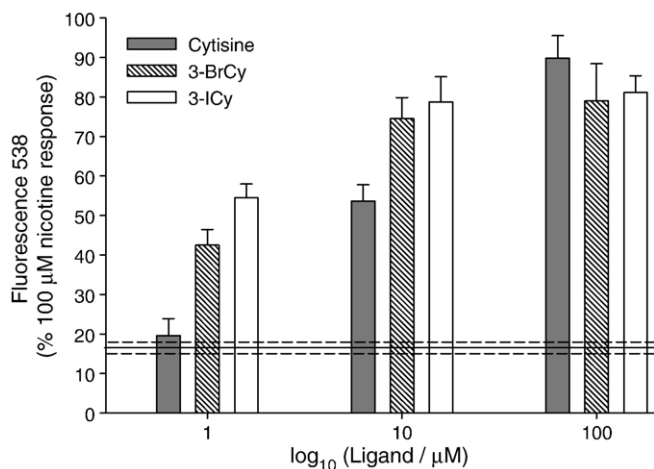


Fig. 6. Cytisine and cytosine derivatives evoked increases in intracellular Ca^{2+} in PC12 cells. PC12 cells were loaded with Fluo-3 AM and stimulated with cytosine, 3-BrCy or 3-ICy, in the presence or absence of 10 μM mecamylamine. Nicotine (100 μM) was used as a control. Fluorescence was monitored for 20 s after agonist application. Values at the 20 s timepoint are expressed as a percentage of the nicotine control response. Data represent the mean \pm S.E.M. of at least 3 independent experiments, each conducted with 8 replicates. Values for all compounds, tested at each concentration, in the presence of mecamylamine are combined for clarity; and represented as a dashed line, fine dashed lines depict the S.E.M.

The lower potency of all the compounds for evoking $[\text{}^3\text{H}]$ noradrenaline release from hippocampal slices, the high efficacy of cytosine that matched that of nicotine and the 3-halogenated derivatives in this preparation (Fig. 3B), and the insensitivity to DH β E and α conotoxin-MII (Fig. 5) are compatible with regulation by different nicotinic receptor subtype(s) that lack the β 2 subunit. α 3 β 4* nicotinic receptors on noradrenergic terminals have been proposed (Clarke and Reuben, 1996; Luo et al., 1998; Barik and Wonnacott, 2006). The enhanced potency of 3-BrCy and 3-ICy is maintained. The ability of 3-BrCy, 3-ICy and cytosine to elicit increases in intracellular Ca^{2+} in PC12 cells is comparable with the stimulation of $[\text{}^3\text{H}]$ noradrenaline release. PC12 cells express α 3, α 5, β 2, β 3, β 4 and α 7 nicotinic receptor subunits (Rogers et al., 1992): although α 3 β 4* nicotinic receptors predominate, the precise subunit composition is likely to differ from that of brain noradrenergic neurons (Léna et al., 1999).

The rank order of potency of cytosine and its derivatives at human α 3 β 4 nicotinic receptors expressed in *Xenopus* oocytes was comparable to that derived for both $[\text{}^3\text{H}]$ dopamine and $[\text{}^3\text{H}]$ noradrenaline release assays. 3-BrCy was the most potent compound, followed by cytosine and 3-BrMCy. 3,5-diBrCy and *N*-methylcytosine were shown to be weak antagonists of human α 3 β 4 nicotinic receptors, with little agonist activity; *N*-methylcytosine also had negligible agonist activity in the $[\text{}^3\text{H}]$ noradrenaline release assay. Interestingly, the efficacy of cytosine was lower with respect to activation of human α 3 β 4 nicotinic receptors than observed for $[\text{}^3\text{H}]$ noradrenaline release. These discrepancies may reflect species differences in α 3 β 4 nicotinic receptors or the more complex subunit composition of native nicotinic receptors (including α 3 β 4* nicotinic receptors) responsible for $[\text{}^3\text{H}]$ noradrenaline release.

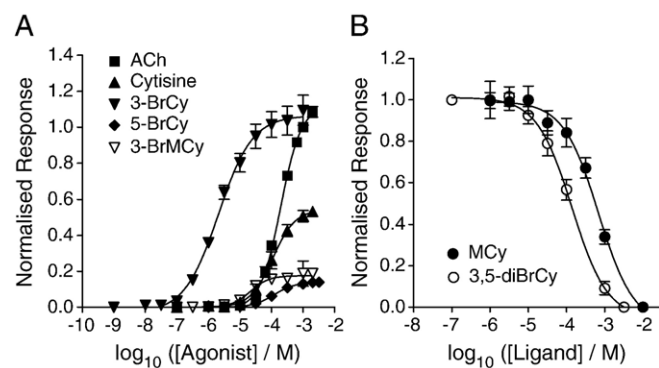


Fig. 7. Interaction of cytosine and cytosine derivatives with human α 3 β 4 nicotinic receptors expressed in *Xenopus* oocytes. Whole cell currents in response to agonist application were measured by two-electrode voltage clamp. Concentration–response curves for activation of whole cell currents by acetylcholine, cytosine, 3-BrCy, 3Br-*N*-methylcytosine and 5-BrCy (A) or inhibition of responses to 100 μM acetylcholine by *N*-methylcytosine and 3,5-diBrCy (B). Each point represents data from 5 to 7 cells, normalized to the amplitude of responses to 1 mM acetylcholine measured in parallel. The data are fitted to a single component Hill curve as described in the Materials and Methods.

These studies have confirmed and extended previous observations that halogenation of cytosine at the C3 position results in a striking increase in potency. This is in contrast to the relative lack of effect of halogenation of nicotine at the analogous position, with respect to hydrogen bond formation (Dukat et al., 1999), and the modest effects of eliminating chlorine from the chloropyridine ring of epibatidine (Spang et al., 2000; see Slater et al., 2003). The 5-iodo analogue of A-85380, however, does exhibit improved potency and selectivity for nicotinic receptor subtypes compared with the parent compound (Mukhin et al., 2000). These apparent discrepancies could be due to the different orientations in which nicotine and cytosine are presumed to bind to nicotinic receptors, as suggested originally by Barlow and Johnson (1989) on the basis of the X-ray structures of the alkaloids, and supported recently by docking studies (Costa et al., 2003). Thus, substitution at C3 of cytosine might enhance binding and efficacy by anchoring the pharmacophore in an active orientation, while the corresponding substitution at C5 of

Table 2

Potency and efficacy of cytosine and its derivatives at human α 3 β 4 nAChR expressed in heterologously in *Xenopus* oocytes

Agonist	pEC ₅₀ \pm S.E.M. (M)	E _{max} \pm S.E.M.	nH \pm S.E.M.
Acetylcholine	-3.71 \pm 0.01	1.00 \pm 0.01	1.30 \pm 0.01
Cytosine	-3.97 \pm 0.05	0.54 \pm 0.01	1.20 \pm 0.02
3-BrCy	-5.65 \pm 0.04	1.00 \pm 0.01	0.81 \pm 0.03
5-BrCy	-3.92 \pm 0.01	0.14 \pm 0.01	1.30 \pm 0.02
3,5-diBrCy	ND	\ll 0.01	ND
<i>N</i> -methylcytosine	NA	0	—
3-BrMCy	-4.67 \pm 0.02	0.18 \pm 0.02	1.50 \pm 0.10

pEC₅₀, E_{max} and nHill values represent the mean \pm S.E.M. of 5–7 independent experiments.

ND, not determined; currents were too small to allow curve fitting.

NA, no agonist activity, even at concentrations greater than 1 mM.

nicotine (and also of cytosine) might leave the substituent in a position where such an interaction is not favored.

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