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Halogenated cytisine derivatives as agonists at human neuronal nicotinic acetylcholine receptor subtypes

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Abstract

Cytisine (cy) is a potent and competitive partial agonist at α4 subunit-containing nicotinic acetylcholine (nACh) receptors while at homomeric α7-nACh receptors it behaves as a full agonist with a relatively lower potency. In the present study, we assessed the effects of bromination or iodination of the pyridone ring of cy and *N*-methylcytisine (*N*-Me-cy) on the effects of these compounds on recombinant human (h) α7, hα4β2 and hα4β4 nACh receptors expressed in clonal cell lines and *Xenopus* oocytes. Halogenation at C(3) of cy or *N*-Me-cy usually brings about a marked increase in both affinity and efficacy at hα7, hα4β2 and hα4β4 nACh, the extent of which depends on whether the halogen is bromine or iodine, and upon receptor subtype. The effects of halogenation at C(5) are strongly influenced by the specific halogen substituent so that bromination causes a decrease in both affinity and efficacy while iodination decreases affinity but its effects on efficacy range from a decrease (hα7, hα4β4 nACh receptors) to a marked increase (hα4β2 nACh receptors). Based on these findings, which differ from those showing that neither the affinity nor efficacy of nicotine, 3-(2-azetidinylmethoxy)-pyridine or epibatidine are greatly affected by halogenation, dehalogenation or halogen exchange at equivalent positions, we suggest that cy, *N*-Me-cy and their halo-isosteres bind to neuronal nACh receptors in a different orientation allowing the halogen atom to interact with a hydrophobic halogen-accepting region within the predominantly hydrophobic agonist-binding pocket of the receptors.

Keywords: Cytisine; Halocytisines; Neuronal nicotinic receptors; α7 Nicotinic receptors; α4β2 Nicotinic receptors; α4β4 Nicotinic receptors

1. Introduction

Mounting evidence indicates that many disorders of the central nervous system (CNS), including Alzheimer's and Parkinson's diseases, some epilepsies, anxiety and Tourette syndrome may benefit from the modulation of nicotinic acetylcholine (nACh) receptor function. Although only a few naturally occurring nACh receptor subunit combinations and stoichiometries have been identified to date, the presence of at least nine different α subunits and three β subunits in avian, rodent or human CNS suggests that many nACh receptor subtypes may exist (Lukas et al., 1999). Some, like the homomeric α7 nACh receptor and the α4β2 combination, seem to be quite abundant in different brain regions, while the existence and distribution of others is less well established. A common feature is the sparse knowledge of their pharmacology, in part due to a lack of selective receptor subtype ligands. The discovery of more selective nACh receptor agonists and antagonists is a prerequisite for an adequate understanding of CNS nicotinic function and for the development of useful nicotinic analgesics, drugs for the treatment of cognitive decline, or other agents potentially useful in the management of a broad range of mental conditions.

Cytisine (cy) is an alkaloid with a strongly restricted conformation and well-established nicotinic agonist

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pharmacology, exhibiting low nanomolar affinity at α4β2 nACh receptors and low micromolar affinity at the α7 subtype, which has been used as a template to infer active conformations of flexible nicotinic drugs (Bencherif et al., 1998). Considering the high affinity of cy for nACh receptors and its semi-rigid structure, it is surprising that so little is known about the pharmacology of cy derivatives. *N*-Methylcytisine (caulophylline; *N*-Me-cy) and its methiodide were shown three decades ago to be less potent nicotinic agonists than cy itself at peripheral receptors (Barlow and McLeod, 1969). A large variety of *N*-substituted cy has been prepared and assayed in a number of tests, but the cholinergic receptor pharmacology of these cy derivatives has not been specifically addressed (e.g. Canu Boido and Sparatore, 1999). Many ring-substituted and *N*-protected cy derivatives were reported a couple of years ago with the aim of developing compounds for PET studies of nACh receptors (Marrière et al., 2000), but no pharmacological results were published for these substances. More recently, we reported that bromination of the pyridone ring of cy at C(3), which is adjacent to the hydrogen bond accepting carbonyl group, increases both the potency and efficacy of cy at recombinant human neuronal nACh receptors, the extent of which is significantly influenced by receptor subtype (Houlihan et al., 2001a). This finding prompted us to propose that the binding site of neuronal nACh receptors may have a halogen-accepting hydrophobic pocket near the hydrogen bond donor moiety believed to interact with the carbonyl oxygen of cy, which when occupied by a halogen atom favours hydrogen bonding between cy and the receptor binding site, ultimately leading to higher agonist affinity and efficacy. Because in this proposal the size of the halogen atom and its electronic properties should have a significant influence on agonist affinity and efficacy, we have investigated the agonist effects of other 3-Br-cy (i.e. 3-bromo-*N*-methyl-cytisine, 3-Br-*N*-Me-cy) as well as 3-iodocytisine (3-I-cy) on recombinant hα7, hα4β2 and hα4β4 nACh receptors expressed in *Xenopus* oocytes and clonal cell lines. Furthermore, to define more precisely the structural–functional architecture of the putative halogen atom-accommodating hydrophobic region of the receptor binding site we have also examined the agonist effects of 5-bromocytisine (5-Br-cy), 5-bromo-*N*-methylcytisine (5-Br-*N*-Me-cy) and 3,5-dibromocytisine (3,5-diBr-cy).

2. Methods

2.1. Chemistry

Cy was purified from the seeds of the Mexican plant *Sophora secundiflora* using standard methodology. *N*-Me-cy was similarly purified from seeds of the Chilean *Sophora macrocarpa*. The direct dihalogenation of cy and *N*-Me-cy was first described in the literature over a hundred years ago (Partheil, 1894), and monohalogenated cy were prepared recently *via* *N*-protected intermediates (Marrière et al., 2000) or by direct halogenation with *N*-halosuccinimides (Imming et al., 2001). We have found that treatment of cy with bromine (Houlihan et al., 2001b) or iodine monochloride in acetic acid affords similar results. Briefly, bromination of cy 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-diBr-cy) and mainly 3- and 5-bromo-cy (3-Br-cy and 5-Br-cy, respectively). These were separated by column chromatography on silica gel, crystallized to homogeneity, and characterized by ^{1}H and ^{13}C NMR and HREIMS. Definitive structure assignments were based on ^{1}H-^{1}H COSY experiments. 3-Iodocytisine (3-I-cy) and 5-iodocytisine (5-I-cy) were prepared similarly using iodine monochloride and were also fully characterized. 3-Bromo-*N*-methylcytisine (3-Br-*N*-Me-cy) and 5-bromo-*N*-methylcytisine (5-Br-*N*-Me-cy) were prepared in the same way as the non-*N*-methylated cy analogues. Structures are shown in Fig. 1.

2.2. Ligand binding assays

Established cultures of the SH-SY5Y-hα7 clonal cell line (Peng and Lukas, 1998), which overexpress the human (h) α7 nACh receptor, were used for [^{125}I]α-bungarotoxin (α-BgTx) binding assays. SH-EP1-hα4β2

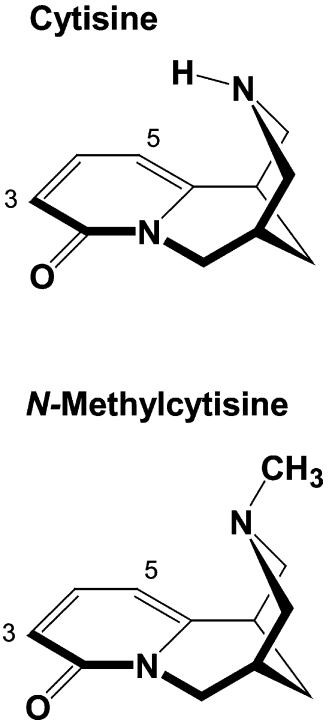

Fig. 1. Structures of cytisine and *N*-methylcytisine. Positions C(3) and C(5) are numbered.

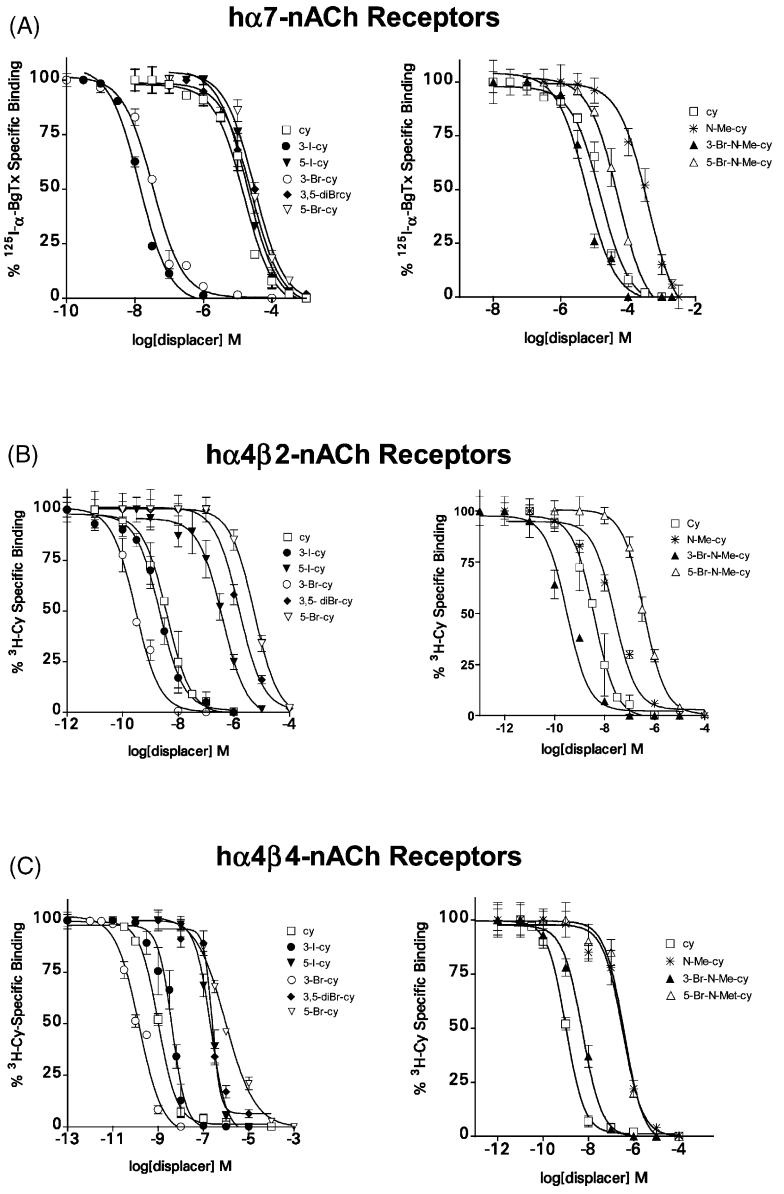

Fig. 2. Competition for [^{125}I]α-BgTx in hα7- (A) or [^{3}H]cy binding sites in hα4β2- (B) or α4β4- (C) nACh receptors by unlabelled cy, *N*-Me-cy and their halo-isosteres. Homogenates were incubated with 8–9 concentrations of drugs before addition of 1 nM [^{125}I]α-BgTx or [^{3}H]cy. Curves are representative of 3–5 determinations for each drug. Where no error bars are seen, they are smaller than the symbols. Data represent the mean ± SEM of at least four experiments, carried out in triplicate.

(Pacheco et al., 2001; Peng et al., 1999) and SH-EP1-hα4β4 (Eaton et al., 2000) clonal cell lines that express hα4β2 and hα4β4 nACh receptors, respectively, were assayed with [^{3}H]cytisine ([^{3}H]cy). Membrane homogenates for all clonal cell lines were prepared and utilized in binding assays using methods described previously (Houlihan et al., 2001b) to give a final protein concentration in the assay tubes of approximately 30–50 μg per assay tube. Equilibrium competition binding studies were performed in a final volume of 250 μl binding saline (in mM: 140 NaCl, 1 EGTA, 10 HEPES, pH 7.4 for [^{125}I]α-BgTx binding and 120 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 50 Tris, pH 7.0 for [^{3}H]cy binding). For [^{125}I]α-BgTx binding assays, preparations were incubated for 90 min at room temperature (21 °C) and non-specific binding was determined with 10 μM nicotine. In [^{3}H]cy binding studies, incubations were carried out at 4 °C for 75 min and 10 μM nicotine was used to define non-specific binding. For [^{125}I]α-BgTx or [^{3}H]cy saturation binding studies the same solutions and total membrane protein were used, but the concentration of radioligand varied from 0.05–3 nM. Bound and free fractions were separated by rapid vacuum filtration through Whatman GF/C filters presoaked in binding saline with 0.1% polyethyleneimine ([^{3}H]cy). Radioactivity was determined with a γ counter or by liquid scintillation,

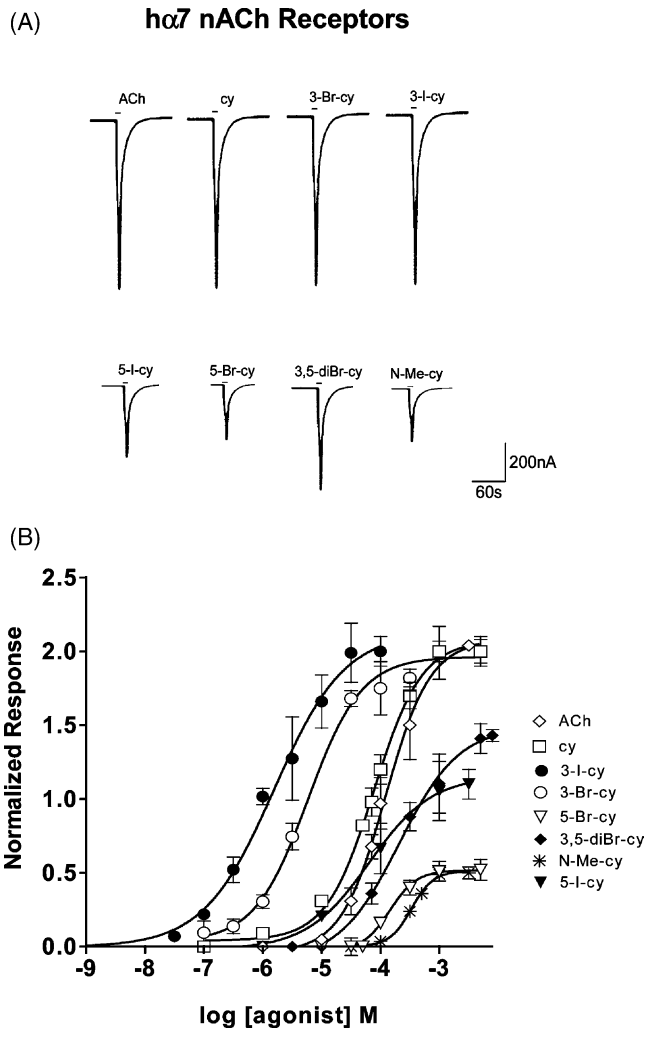

Fig. 3. Agonist effects of cy, *N*-Me-cy and halogenated derivatives on hα7 nACh receptors expressed in *Xenopus* oocytes. (A) Representative traces showing whole-cell currents elicited by application of approximate EC_{50} concentrations of ACh (100 μM), cy (80 μM), 3-Br-cy (5 μM), 3-I-cy (2 μM) or 2 mM 5-Br-cy, 5-I-cy, 3,5-diBr-cy or *N*-Me-cy onto oocytes expressing recombinant hα7 nACh receptors. Oocytes were voltage clamped at −60 mV. (B) DRC and efficacy for the agonist effects of ACh, cy, *N*-Me-cy and halo-cytisines. The data were normalized to the responses elicited by EC_{50} ACh (100 μM) and then fitted using a single Hill equation. Data points represent the mean ± SEM of 4–6 experiments.

as appropriate.

2.3. Whole-cell recording

Xenopus laevis oocytes at developmental stages V and VI were prepared and injected with hα7 or combinations of hα4 + hβ2 or hβ4 in vitro transcribed cRNAs as reported previously (Houlihan et al., 2001b). Whole-cell currents were measured 3–5 days post-injection by a two-electrode voltage clamp (GeneClamp 500, Axon Instruments, USA) using agarose-cushioned electrodes containing 3 M KCl. Oocytes were continually supplied with fresh Ringer solution (in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES, pH 7.2) in a 100 μl bath, using a gravity-driven perfusion system at a rate of 4 ml/min. Modified Ringer solution ($CaCl_2$ replaced by $BaCl_2$) was used when recording from oocytes expressing hα7 nACh receptors. Drugs were applied by gravity perfusion using a manually activated valve. Agonists were applied 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 perfused with Ringer solution for 3 min to allow drug clearance and prevent receptor desensitization. Dose–response curves (DRC) for agonists were constructed by normalizing to the maximal response to the agonist and used to generate EC_{50} and nHill estimates (Houlihan et al., 2001b). For comparison of relative agonist efficacy, the agonist responses for each oocyte expressing hα4β2 or hα4β4 nACh receptors were normalized to the response elicited by 30 μM ACh alone (the ACh $EC_{25–30}$ concentration in hα4β4 and low-affinity hα4β2 nACh receptors, and EC_{100} in high-affinity hα4β2 nACh receptors; see Figs. 4(B) and 5(B)). The agonist responses of oocytes expressing hα7 nACh receptors were normalized to the responses elicited by 100 μM ACh (the ACh EC_{50} concentration in hα7 nACh receptors; see Fig. 3(B)).

2.4. Data analysis

Functional concentration–response data were fitted by non-linear 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 the agonist or antagonist concentration, EC_{50} the agonist concentration that produces 50% of the maximum response and nH is the Hill coefficient. Biphasic agonist concentration–response data were fitted to the sum of two empirical Hill equations comparable to those used previously (Buisson and Bertrand, 2001; Covernton and Connolly, 2000; Houlihan et al., 2001b).

Radioligand displacement data were similarly fitted to the equation:

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

where IC_{50} is the concentration of unlabelled ligand that causes 50% of inhibition of specific radioligand binding. The binding parameters (K_d and B_{max}) of [^{125}I]a-BgTx or [^{3}H]cy were determined from saturation binding isotherm data using the equation $Y = B_{max}X/K_d + X$, wherein B_{max} is the maximal binding, K_d the apparent equilibrium dissociation binding constant, X the concentration of ligand and Y is the binding. The K_i value of the test compounds was determined using the equation of Cheng and Prusoff, $K_i = EC_{50}/1 + [X]/K_d$.

Potency (EC_{50}) values are presented as geometric

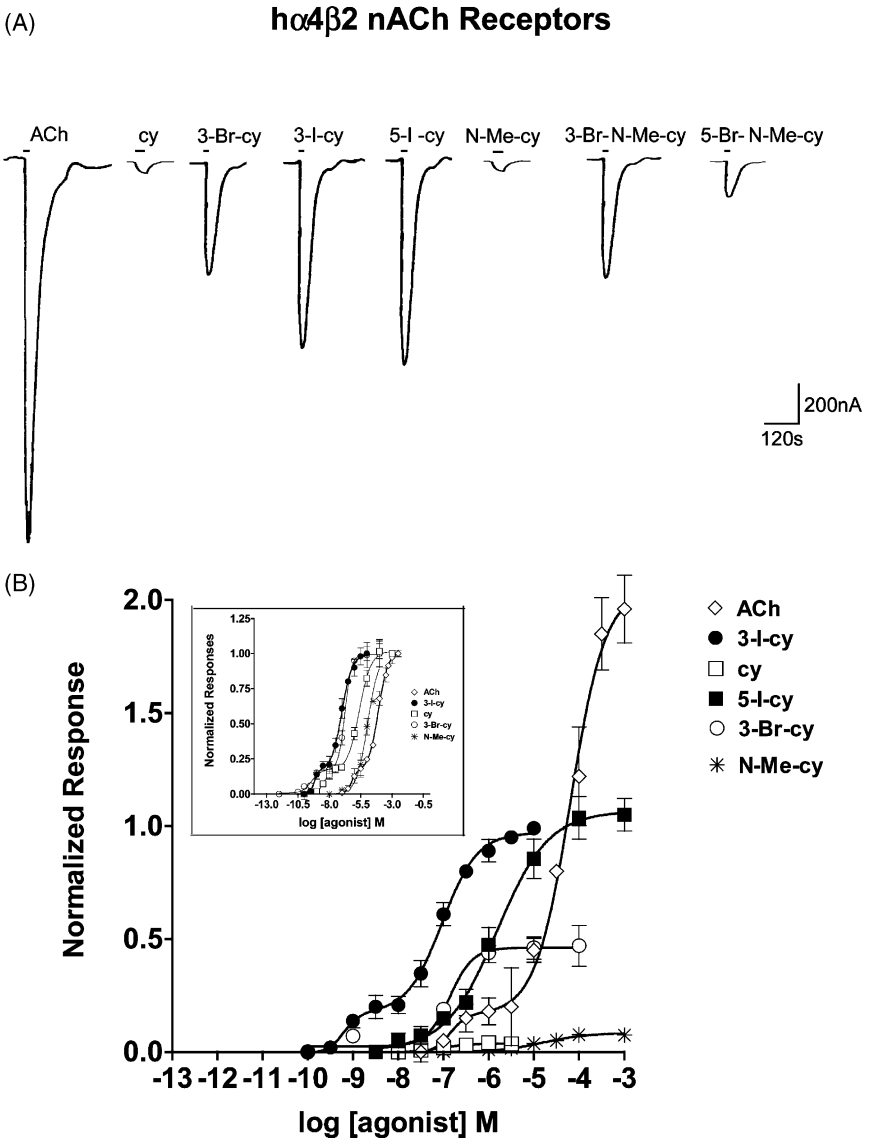

Fig. 4. Agonist effects of cy, *N*-Me-cy and their halogenated derivatives on hα4β2 nACh receptors expressed in *Xenopus* oocytes. (A) Representative traces showing whole-cell currents elicited by application of approximate EC_{50} ACh (30 μM) and 1 mM cy, *N*-Me-cy, 3-Br-cy, 3-Br-*N*-Me-cy or 5-Br-*N*-Me-cy. Neither 5-Br-cy nor 3,5-diBr-cy elicited activated currents when applied on their own to oocytes expressing hα4β2 nACh receptors, even at concentrations as high as 1 mM. Voltage clamp was at −60 mV. (B) Concentration–response curve and relative efficacies for the agonist effects of cy and derivatives in comparison to ACh. Note that 3-I-cy and ACh yielded concentration–response data that were best fitted with the sum of two Hill equations. 5-I-cy was best fitted with a single Hill equation. The data were normalized to the responses elicited by 30 μM ACh (EC_{100} for the high-affinity component and approximately $EC_{20–25}$ for the low-affinity component). Data points represent the mean ± SEM of 4–6 experiments. Inset shows the concentration–response curve of agonists yielding biphasic data. The data were normalized to the maximal effects of the agonists in order to estimate the ratio of the high- and low-affinity components.

means (+ standard error of the mean (+SEM), −SEM). Hill coefficient (nH), efficacy, K_d and B_{max} values are expressed as arithmetic mean ± SEM. Results are derived from at least three independent experiments carried out using different batches of clonal cell cultures or oocytes. Where appropriate, one-way Anova or Student's *t*-test for unpaired data were used and values of $P \leq 0.05$ were regarded as significant.

3. Results

3.1. Radioligand binding studies

The effects of cy and its halogenated isosteres on the binding of [^{3}H]cy to hα4β2- or hα4β4-nACh receptors, and on [^{125}I]α-BgTx binding to hα7-nACh receptors were examined using membrane fractions from SH-EP1-

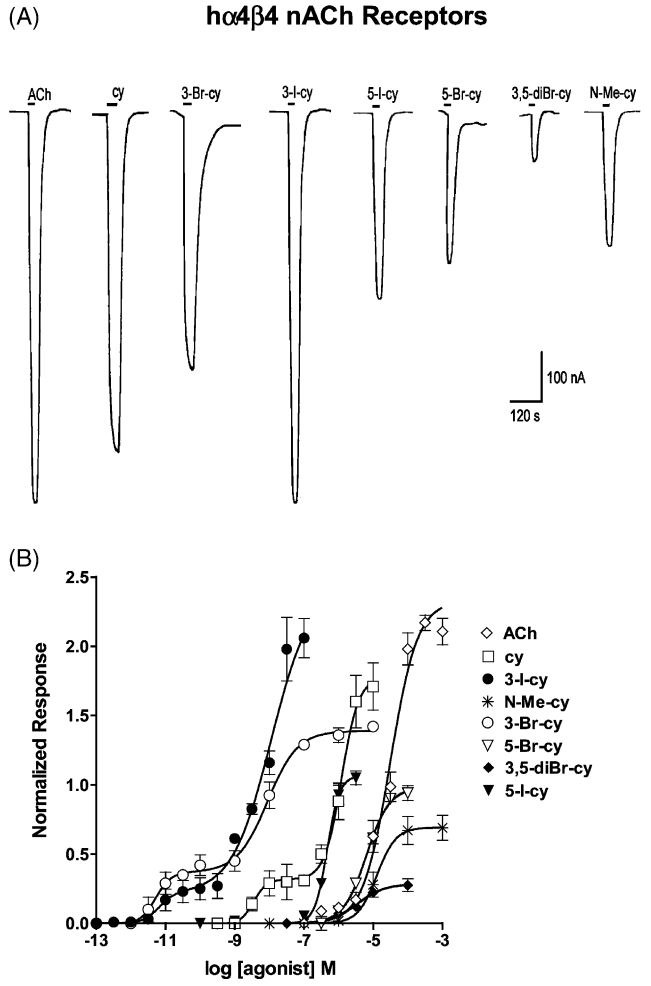

Fig. 5. Agonist effects of cy, *N*-Me-cy and their halogenated isosteres on hα4β4 nACh receptors expressed in *Xenopus* oocytes. (A) Representative traces showing whole-cell currents elicited by application of EC_{50} ACh (30 μM) and EC_{max} concentrations of cy, *N*-Me-cy and their halogenated derivatives onto oocytes expressing hα4β4 nACh receptors. Oocytes were voltage-clamped at −60 mV. (B) Concentration–response curve and relative efficacies for the agonist effects of cy and 3-I-cy in comparison to ACh. The curves for cy, 3-Br-cy and 3-I-cy were best fitted with two Hill equations ($p < 0.5$) while ACh was fitted to a single Hill equation. The data were normalized to the responses elicited by 30 μM ACh (the approximate EC_{50}). Data represent the mean ± SEM of 4–5 experiments. Inset shows the concentration–response curve of agonists yielding biphasic data. The data were normalized to the maximal effects of the agonists in order to estimate the ratio of the high- and low-affinity components.

hα4β4, SH-EP1-hα4β2 cells or SH-SY5Y-hα7 cells, respectively. The K_d for [^{125}I]α-BgTx (1 ± 0.2 nM) binding to hα7-nACh receptors and [^{3}H]cy binding to hα4β4- (0.1 ± 0.02 nM) and hα4β2- (0.43 ± 0.08 nM) nACh receptors were comparable to previously published data (Houlihan et al., 2000, 2001a,b; Monteggia et al., 1995). Fig. 2 shows that specific binding of both [^{125}I]α-BgTx to hα7 nACh receptors and [^{3}H]cy to hα4β2 or hα4β4 nACh receptors was fully displaced by cy and its 3-, 5- and 3,5-halogenated isosteres in a concentration-dependent and monophasic manner. Estimated K_i values are summarized in Table 1. Halogenation at C(3) of the pyridone ring of cy caused significant increases in the affinity of cy for hα7, hα4β2 and hα4β4 nACh receptors ($P < 0.05$), the extent of which was influenced by receptor subtype. For example, the affinity of 3-Br-cy for hα7 nACh receptors was about 500-fold higher than that of cy, while its affinity for hα4β2 and hα4β4 nACh receptors relative to that of cy was about 14- and 8-fold higher, respectively. The specific halogen atom present at C(3) also influenced the affinity of cy for hα7, hα4β2 and hα4β4 nACh receptors. 3-I-Cy was about 1200-fold more potent than cy at inhibiting ^{125}I-α-BgTx binding to hα7-nACh receptors, but 3-I-cy had potency comparable to cy at hα4β2 nACh receptors and had ~3.5-fold less potency than cy at α4β4-nACh receptors ($P < 0.05$). Nevertheless, 3-I-cy was less potent than 3-Br-cy as an inhibitor of [^{3}H]cy binding to hα4β2- or hα4β4 receptors. Both bromination and iodination at C(5) markedly reduced the affinity of cy for the α4 subunit-containing nACh receptor subtpes, but the effects of iodination at this position were less marked than those of bromination. Thus, although 5-I-cy was much less potent than cy, it was more potent than 5-Br-cy at inhibiting binding of [^{3}H]cy to hα4β2 and hα4β4 nACh receptors (Fig. 2; Table 1; $P < 0.05$). On the other hand, bromination or iodination at C(5) caused no significant difference at $P < 0.05$ in [^{125}I]α-BgTx binding displacement compared to cy, as the respective K_i values were not significantly different from each other (Fig. 2; Table 1). Bromination of cy at both C(3) and C(5) yielded 3,5-diBr-cy, which was less potent than cy or the 3-halocytisines. 3,5-DiBr-cy was slightly less potent than 5-Br-cy at hα7 nACh receptors, but more potent than 5-Br-cy at both hα4β2 and hα4β4 nACh receptors.

To determine whether halogenation at C(3) or C(5) also affects the affinity of *N*-Me-cy, we tested the effect of bromination at C(3) or C(5) on the potency of this compound. As shown in Fig. 2, *N*-Me-cy was significantly less potent ($P < 0.05$) than cy at inhibiting binding of either [^{125}I]α-BgTx to hα7 nACh receptors or [^{3}H]cy to hα4β2 or hα4β4 nACh receptors. The affinity of *N*-Me-cy for neuronal nACh receptors, like that of cy, was significantly receptor subtype-selective ($P < 0.05$). In comparison to the affinity of cy, the affinity of *N*-Me-cy for hα4β4 nACh receptors was about 320-fold lower, while its affinity for hα7 and hα4β2 nACh receptors was about 23-fold and 6-fold lower, respectively. As for cy, bromination at C(3) caused significant increases ($P < 0.05$) in the affinity of *N*-Me-cy for hα7 (32-fold increase), hα4β2 (80-fold increase) or hα4β4 (58-fold increase) nACh receptors, thus making 3-Br-*N*-Me-cy as potent as cy at hα7 nACh receptors or more potent than cy at hα4β2 nACh receptors. Bromination at C(5) produced an isomer (5-Br-*N*-Me-cy) that was significantly ($P < 0.05$) more potent than *N*-Me-cy at inhibiting [^{125}I]α-BgTx binding to hα7 nACh receptors, while its

Table 1
Comparisons of binding affinities for cytisine, *N*-methylcytisine and their halogenated derivatives at hα7, hα4β2 and hα4β4 nACh receptors

Drug	K_i		
	hα7 (μM)	hα4β2 (nM)	hα4β4 (nM)
Cy	8 ± 1	1.2 ± 0.06	0.091 ± 0.007
3-Br-cy	0.016 ± 0.001	0.088 ± 0.005	0.012 ± 0.008
3,5-diBr-cy	1 ± 1	500 ± 40 μM	24 ± 2
5-Br-cy	10 ± 1	2000 ± 10	70 ± 3
3-I-cy	0.007 ± 0.00003	0.7 ± 0.03	0.34 ± 0.03
5-I-cy	8 ± 2	10 ± 3	19 ± 1
N-Me-cy	191 ± 2	7.6 ± 0.4	29 ± 4
3-Br-*N*-Me-cy	6 ± 0.5	0.095 ± 2	0.5 ± 0.02
5-Br-*N*-Me-cy	25 ± 12	100 ± 1	35 ± 1

Data represent the mean ± SEM of four experiments, each with triplicate samples. The radioligand concentration in all displacement studies was 1 nM and the equilibrium dissociation constants (K_d) used to estimate K_i values were 1 nM for [^{125}I]α-BgTx binding to hα7 nACh receptors, 0.43 nM for the binding of [^{3}H]cy to hα4β2 nACh receptors and 0.1 nM for the binding of [^{3}H]cy to hα4β4 nACh receptors. K_d values were determined as indicated in Section 2.

affinity for hα4β2 or hα4β4 nACh receptors, respectively, was lower than or comparable to that of the parent compound.

To summarize, the rank order of potency for [^{125}I]α-BgTx binding blockade at hα7 nACh receptors by cy and halogenated cy is: 3-I-cy > 3-Br-cy > 3-Br-*N*-Me-cy > 5-I-cy ≈ cy > 5-Br-cy > 3,5-diBr-cy > 5-Br-*N*-Me-cy ≫ *N*-Me-cy The rank order of potency for blockade of [^{3}H]cy binding to hα4β2 nACh receptors is: 3-Br-cy ≈ 3-Br-*N*-Me-cy > 3-I-cy > cy > *N*-Me-cy ≫ 5-I-cy ≈ 5-Br-*N*-Me-Cy > 3,5-diBr-cy > Br-cy, whereas at hα4β4 nACh receptors, the rank order is: 3-Br-cy > cy > 3-I-cy ~ 3-Br-*N*-Me-cy > 5-I-cy ≈ 3,5-diBr-cy ~ *N*-Me-cy > 5-Br-*N*-Me-cy > 5-Br-cy.

3.2. Functional effects on hα7 nACh receptors

As shown in Fig. 3, cy and its halogenated derivatives induced inward currents in oocytes expressing hα7 nAChR, but only cy, 3-Br-cy and 3-I-cy behaved as full agonists of hα7 nACh receptors. Halogenation at C(5) decreased efficacy, but the extent of the decrease was influenced by the halogen atom present. Thus, the relative efficacy of 5-Br-cy was about 1/4 of that of cy, while the efficacy of 5-I-cy was about 1/2 of that of the full agonists. Interestingly, bromination at both C(3) and C(5) partially compensated the loss of efficacy relative to that of 5-Br-cy: the efficacy of 3,5-diBr-cy was about 3-fold higher than that of 5-Br-cy. *N*-Me-cy displayed the same low efficacy as 5-Br-cy. A summary of the values of relative efficacy is given in Table 2. The rank order of relative efficacy at hα7 nACh receptors was ACh = 3-Br-cy = 3-I-cy = cy > 3,5-diBr-cy > 5-I-cy > 5-Br-cy ≈ *N*-Me-cy.

The concentration–response data for the functional effects of cy and its analogues were best fitted to a single Hill equation (Fig. 3(B); $P < 0.001$), which is comparable to previous findings (Houlihan et al., 2001b). 3-I-cy, with an EC_{50} of 1.5 μM, was significantly more potent than cy (EC_{50} 83 μM; $P < 0.05$) or 3-Br-cy (EC_{50} 5 μM). 5-Br-cy was about 2-fold less potent than cy, although 3,5-diBr-cy was as potent as cy. The least potent agonist tested was *N*-Me-cy (EC_{50} 340 μM), which is in accord with the radioligand binding data. The rank order of potency for agonist effects of cytisine and derivatives on hα7 nACh receptors was 3-I-cy > 3-Br-cy > 5-I-cy > cy ≈ 3,5-diBr-cy > 5-Br-cy > *N*-Me-cy

3.3. Functional effects on hα4β2 and hα4β4 nACh receptors

Receptor subtype and the specific halogen substituent affected the relative functional efficacy and potency of both cy and *N*-Me-cy (Figs. 4–5; data are summarized in Tables 2 and 3). For example, although the relative functional efficacy of cy at hα4β2 receptors was only 1/25th of that of ACh, cy behaved as an almost full agonist at hα4β4 nACh receptors. Bromination at C(3) caused a significant ($P < 0.05$) increase in the efficacy of cy at hα4β2 nACh receptors: 3-Br-cy was about 10-fold more efficacious than cy. In contrast, 3-Br-cy was slightly less efficacious than cy at hα4β4 nACh receptors. Iodination at C(3) also caused a significant ($P < 0.05$) increase in efficacy at hα4β2 nACh receptors, 3-I-cy attaining a relative efficacy about half of that of ACh and double of that of 3-Br-cy. 3-I-cy was a full agonist at hα4β4 nACh receptors. 5-Br-cy and 3,5-diBr-cy did not evoke currents through hα4β2 receptors, even at concentrations as high as 1 mM, but they were agonists at hα4β4 nACh receptors, albeit less efficacious than the full agonists ACh and 3-I-cy (Fig. 5). 5-I-cy, unlike 5-Br-cy, activated currents in both hα4 subunit-contain-

Table 2
Comparison of relative efficacies and ratios of high- and low-affinity concentration–response curve components of cytisine, *N*-methylcytisine and their halogenated derivatives

Drug	hα7	hα4β2	hα4β4
Cy	2.1 ± 0.05	0.04 ± 0.002	1.8 ± 0.06
High-affinity		0.17 ± 0.03	0.22 ± 0.04
Low-affinity		0.83 ± 0.04	0.78 ± 0.04
3-Br-cy	1.97 ± 0.04	0.45 ± 0.01	1.4 ± 0.03
High-affinity		0.17 ± 0.03	0.23 ± 0.08
Low-affinity		0.83 ± 0.04	0.77 ± 0.09
3,5-diBr-cy	1.44 ± 0.07	NAE	0.29 ± 0.03
5-Br-cy	0.51 ± 0.02	NAE	0.98 ± 0.003
3-I-cy	2.13 ± 0.09	0.94 ± 0.01	2.1 ± 0.3
High-affinity		0.18 ± 0.03	0.27 ± 0.09
Low-affinity		0.82 ± 0.04	0.73 ± 0.09
5-I-cy	1.15 ± 0.03	1.07 ± 0.02	1.06 ± 0.02
N-Me-cy	0.51 ± 0.01	0.084 ± 0.008	0.7 ± 0.006
3-Br-*N*-Me-cy	ND	0.5 ± 0.05	ND
5-Br-*N*-Me-cy	ND	0.21 ± 0.07	ND

Relative efficacy (I_{max}) values correspond to the maximal responses elicited by agonists normalized to the EC_{50} ACh response. Ratios of high- and low-affinity components were determined by normalizing the responses of agonists yielding biphasic DRC to their maximal effects and fitting the data to the sum of two Hill equations as described in Section 2. Data represent the mean ± SEM of at least four experiments. Key: ND, not determined.

Table 3
Comparisons of functional potencies of cytisine, *N*-methylcytisine and their halogenated derivatives at hα7, hα4β2 and hα4β4 nACh receptors

	EC_{50}		
Drug	hα7	hα4β2	hα4β4
Cy	83 μM (60, 40)		
High-affinity		5 nM (2, 8)	4 nM (1, 8)
Low-affinity		2 μM (0.9, 3.8)	1 μM (0.8, 1.2)
3-Br-cy	5.3 μM (3.6, 7.8)		
High-affinity		0.4 nM (0.1, 1.7)	5 pM (2,11)
Low-affinity		0.2 μM (0.1, 0.3)	10 nM (5, 12)
3,5-diBr-cy	87 μM (61, 98)	NAE	4 μM (1, 8)
5-Br-cy	197 μM (142, 2400)	NAE	6 μM (4, 10)
3-I-cy	1.5 μM (0.9, 2)		
High-affinity		0.8 nM (0.1, 2)	7 pM (6, 8)
Low-affinity		86 nM (63, 126)	8 nM (6, 13)
5-I-cy	64 μM (46, 95)	1.5 μM (1, 2)	0.47 μM (0.3, 0.6)
N-Me-cy	340 μM (298, 360)	13 μM (7, 18)	13 μM (11, 14)

Data were analysed as illustrated in Figs. 3–5 to determine EC_{50} values for activation of hα7, hα4β2 or hα4β4 nACh receptors expressed heterologously in *Xenopus* oocytes. DRC data for hα4β2 or hα4β4 nACh receptors were best fitted ($P < 0.0.5$) to the sum of two Hill equations. EC_{50} are the geometric means (−SEM, +SEM) from at least four oocytes from different batches. Key: NAE, no agonist activity.

ing nACh receptors, displaying similar relative efficacies at both receptor subtypes about 1/2 of that of ACh. The relative efficacy of *N*-Me-cy at hα4β2 nACh receptors was almost as low as that of cy, while at hα4β4 nACh receptors its efficacy was about 35% of that of ACh. Because 5-Br-cy did not activate hα4β2 nACh receptors, we tested whether 5-Br-*N*-Me-cy behaved in a similar fashion at this receptor type. As shown in Fig. 4 5-Br-*N*-Me-cy was a partial agonist at hα4β2 nACh receptors with a relative efficacy higher than that of cy or *N*-Me-cy. 3-Br-*N*-Me-cy also behaved as a partial agonist at hα4β2 nACh receptors with a relative efficacy 10-fold higher than that of cy or *N*-Me-cy. The rank order of relative efficacies at hα4β2 receptors was as follows: ACh > 5-I-cy ≈ 3-I-cy ≫ 3-Br-cy ≈ 3-Br-*N*-Me-cy > 5-Br-*N*-Me-cy > *N*-Me-cy ≈ cy > 5-Br-cy = 3,5-diBr-cy = 0. At hα4β4 nACh receptors the rank order of relative efficacies was: 3-I-cy ≈ cy > 3-Br-cy > 5-I-cy ≈ 5-Br-cy > *N*-Me-cy > 3,5-diBr-cy.

The concentration–response data for cy and the 3-hal-

ogenated cytisine derivatives at both hα4β2 and hα4β4 nACh receptors were best fitted to the sum of two independent Hill equations ($P < 0.05$) yielding agonist concentration–response curves with high-affinity (low nanomolar range) and low-affinity (low micromolar range) components (Figs. 4(B) and 5(B)). The ratios of the high-affinity and low-affinity components were determined from concentration–response data obtained by normalizing the agonist data to their maximal effects and were approximately 20–30 and 70–80%, respectively (Figs. 4(B) and 5(B); Table 2), which is comparable to data published previously for ACh and 3-Br-cy (Houlihan et al., 2001a,b). 5-I-cy yielded monophasic concentration–response data at both receptor subtypes, and its EC_{50} at both receptors were in the low micromolar–high nanomolar range. *N*-Me-cy, 5-Br-cy and 3,5-diBr-cy yielded monophasic concentration–response data at hα4β4 nACh receptors and were the least potent of the agonists tested with EC_{50} significantly ($P < 0.05$) higher than those of 5-I-cy. In summary, the rank order of agonist potency taking into account only the EC_{50} values of drugs yielding monophasic concentration–response curves and EC_{50} values of the low-affinity component of those agonists producing biphasic concentration–response curves are as follows. Hα4β2 nACh receptor: 3-I-cy > 3-Br-cy > 5-I-cy > cy > *N*-Me-cy. Hα4β4 nACh receptor: 3-I-cy > 3-Br-cy > 5-I-cy > cy > 3,5-diBr-cy > 5-Br-cy > *N*-Me-cy. The rank order of agonist potency taking into account only EC_{50} values of drugs yielding monophasic concentration–response curves and EC_{50} values of the high-affinity component of those agonists producing biphasic concentration–response curves are as follows. Hα4β2 nACh receptor: 3-Br-cy > 3-I-cy > cy > 5-I-cy > *N*-Me-cy. Hα4β4 nACh receptor: 3-Br-cy ≈ 3-I-cy > cy > 5-I-cy ≫ 3,5-diBr-cy > 5-Br-cy > *N*-Me-cy.

3.4. Correlations

For each nACh receptor subtype evaluated, plots of agonist potency (EC_{50}) versus binding affinity (K_i) showed good correlations between these parameters (r^2 values of 0.96 for hα7 nACh receptors, 0.95 for hα4β2, and 0.89 for hα4β4 nACh receptors when including high-affinity components of ligands inducing biphasic concentration–response curves for α4β2 or α4β4 nACh receptors (Table 4). Correlation coefficients were lower (0.14 and 0.63, respectively, for hα4β2 and hα4β4 nAChR receptors) when including low-affinity components instead (Table 4), even though this brought slopes for these relationships closer together (0.51 ± 0.05 for hα7 nACh receptors; 0.22 ± 0.31 for hα4β2 nAChR receptors compared to 1.26 ± 0.16 when including high-affinity components, and 0.68 ± 0.23 for hα4β4 nACh receptors compared to 1.64 ± 0.26 when including high-affinity components). This helped to indicate that hα7

Table 4
Correlation between binding affinity (K_i) and agonist potency (EC_{50})

hα7 nACh Receptor	hα4β2 nACh Receptor		hα4β4 nACh Receptor	
	K_i vs. h-a EC_{50}	K_i vs. l-a EC_{50}	K_i vs. h-a EC_{50}	K_i vs. l-a EC_{50}
r^2 0.96	0.95	0.14	0.89	0.63
Slope 0.54 ± 0.05	0.24 ± 0.1	1.26 ± 0.16	0.68 ± 0.23	1.64 ± 0.26

Correlation coefficients (r^2) and slopes were determined from plots of agonist potency (EC_{50}) vs. binding affinity (K_i). Key: h-a, high-affinity and l-a, low-affinity.

nACh receptors do not display higher affinity sites for functional interaction with 3-Br-cy, 3-I-cy, or cy, yet that high-affinity functional interactions with these ligands better match binding affinities for hα4β2 and hα4β4 nACh receptors.

4. Discussion

The rigidity of the cy molecule makes it an attractive template for structure–activity studies. Halogenation of cy would be expected to increase its lipophilicity, thus improving its absorption and its ability to pass the blood-brain barrier, but knowledge of the nACh receptor pharmacophore is too limited to speculate any further regarding the effect of cy derivatization upon its pharmacology (Glennon and Dukat, 2000; Tønder et al., 2001). Nevertheless, since the demonstration that epibatidine, which is chlorinated at C(2), the carbon atom next to the hydrogen bond accepting nitrogen on its pyridine ring, is a potent nicotinic agonist analgesic (Qian et al., 1993), a large number of congruently 6-halogenated nicotine (Dukat et al., 1999) and 3-(2-azetidinylmethoxy)pyridine (Holladay et al., 1998), and 2-halo-dechloroepibatidine (Carroll et al., 2001) analogues have been synthesized and assayed at nACh receptors. However, the presence of halogen atoms at C(6) in nicotine, or the substitution of chlorine for hydrogen or other halogens at C(2) in epibatidine does not appear to increase affinity for neuronal nACh receptors to the same extent that bromination or iodination at C(3) does in cy or *N*-Me-cy. It should be stressed that C(3) of cy is the position next to the hydrogen bond accepting carbonyl group of its pyridone system, and may thus be considered broadly comparable to C(6) or C(2) in pyridine derivatives such as nicotine, epibatidine and the 3-(2-azetidinylmethoxy) pyridines. The profound changes in affinity observed for 3-Br-cy and 3-I-cy are therefore unexpected.

Bromination or iodination of cy at C(3) causes remarkable increases in the radioligand competition affinity of cy for the binding site of recombinant hα7 nACh receptors. Bromination at C(3) causes smaller increases at hα4β2 and hα4β4 nACh receptors, while iodination at this position has little effect on the affinity for these receptors. Imming et al. (2001) reported a similar pattern of change on native rat, presumably α7 and α4β2, nACh receptors. Bromination and iodination at C(3) also causes significant changes in the functional potency of cy at hα7, hα4β2 and hα4β4 nACh receptors that parallel the pattern of change observed in the radioligand binding studies. At hα4-containing heteromeric nACh receptors, 3-halogenated cy derivatives were more efficacious than the parent compounds, which indicates that halogenation at C(3) favours receptor activation. The importance of C(3) for the agonist effects of cy on nACh receptors is further emphasized by the remarkable increase in the affinity (and efficacy, at least at hα4β2 nACh receptors) of *N*-Me-cy, which is generally less potent and efficacious than cy, upon bromination at this position. The favourable effects of halogenation of cy at C(3) on affinity and efficacy, and the more modest changes in the cases of the nicotine and epibatidine analogues, suggest that cy and its isosteres may bind to these receptors in a different orientation to the pyridine derivatives. Interestingly, such an insight was published more than a decade ago on the basis of a comparison of the crystal structures of nicotine and cy (Barlow and Johnson, 1989), in spite of the obvious conformational flexibility of the nicotine molecule. In this model the pyridone ring of cy is tilted relative to the charged nitrogen atom at an angle similar to that of the pyridine ring in nicotine, while the carbonyl group in the pyridone ring of cy is on the side of the ring opposite to the pyridine nitrogen in nicotine. The most obvious implication of this is that the pyridine nitrogen of nicotine and the pyridone oxygen of cy interact with different domains within the receptor agonist-binding site. This possibility is consistent with the finding that neither halogenation at C(6) of nicotine (Dukat et al., 1999) nor substitution of chlorine in epibatidine for other halogen atoms (Carroll et al., 2001) cause significant changes in affinity. Additional support for this hypothesis comes from the replacement of chlorine in the epibatidine enantiomers for hydrogen, which causes only modest decreases in their potencies at reconstituted rat α7, and larger decreases at α4β2 nACh receptors, with little change in their efficacies relative to ACh (Spang et al., 2000). Bromination of nicotine at the alternative C(5) position reduces affinity for [^{3}H]nicotine binding sites in whole rat brain membranes, and causes loss of potency and/or efficacy at α4β2 nACh receptors, as a stimulator of striatal [^{3}H]dopamine release or of $^{86}Rb^+$ efflux from mouse thalamic synaptosomes, as well as reduced potency in several in vivo models (Cosford et al., 1996; Dukat et al., 2002), indicating that 3-Br-cy and 3-I-cy also differ from 5-bromonicotine in their nACh receptor pharmacology.

Which property of the halogen substituent is important for receptor affinity? The findings of this study and previous studies of 3- and 5-Br-cy (Houlihan et al., 2001b; Imming et al., 2001), 6-halogenated nicotine (Dukat et al., 1999) and 2-halo-dechloroepibatidine (Carroll et al., 2001) analogues suggest that both the electronic properties and the size of the halogen atom influence the affinity of the parent compound for nACh receptors, but these effects follow different trends at different receptor subtypes. Thus, the size of the substituent may be a limiting factor at α4 subunit-containing receptors. For example, 3-I-cy (Imming et al., 2001; this study) and 2-I-dechloroepibatidine (Carroll et al., 2001) are either equipotent with or less potent than their brominated counterparts, and the present results indicate that the affinity of 3-I-cy at α4β4 nACh receptors is less than that of cy. It

might be surmised, therefore, that the putative halogen-binding hydrophobic region that accommodates the halogen substituent is not bulk-tolerant. Halogen atoms bigger than Br may not fit well into the hypothetical hydrophobic halogen-binding pocket of the agonist-binding region of α4 subunit-containing receptors, offsetting the effect of their electronic properties on ligand affinity and efficacy. As the agonist-binding site of nAChRs is lined with aromatic and other hydrophobic residues (Galzi and Changeux, 1995), it is difficult to suggest a precise location for this putative halogen-binding region, although the docking studies of Le Novère et al. (2002) would seem to indicate that in the case of epibatidine the chlorine atom approaches Leu-108 (histidine in the α4 subunit) and Gln-116 (phenylalanine in the β4 subunit) of the 'complementary component' of the agonist-binding cavity. The dominant interaction of agonists with nACh receptors is almost certainly a cation–π interaction with a specific tryptophan residue (Zhong et al., 1998), while the pyridine nitrogen of epibatidine may interact with the tyrosine residue four or five places beyond the cystine bridge of the 'principal component' (Grutter et al., 2000; Le Novère et al., 2002). If the carbonyl oxygen of cytisine derivatives also interacts with this aminoacid residue, the C(3) halogen atom might occupy a similar position to the epibatidine chlorine atom. On the other hand, a C(5) substituent on the cytisine scaffold in a similar position would probably clash with the wall of the agonist-binding site. These speculations, however, would have to be supported by additional experimental and modelling studies on our compounds.

The structural requirements for affinity and efficacy are somehow different. Thus, bromination at C(3) produces cy or *N*-Me-cy derivatives more efficacious and more potent than the parent compounds. However, iodination at C(3), which has either no effect or decreases affinity relative to bromination at C(3), yields isosteres that are more efficacious than cy and 3-Br-cy. Thus, unlike affinity, the effects of halogenation at C(3) on efficacy may not be so sensitive to the size of the halogen atom. It is interesting that the same pattern is observed among 5-halogenated cy isosteres. Both 5-Br-cy and 5-I-cy are less potent than cy but their relative efficacies are quite different. 5-Br-cy has reduced efficacy relative to cy but 5-I-cy has either the same (e.g. at hα7 nACh receptors) or higher (e.g. at hα4β2 nACh receptors) efficacy than 5-Br-cy, or even cy (e.g. at hα4β2 nACh receptors). It seems paradoxical that affinity and efficacy should have different structural requirements. According to thermodynamic and ligand–protein interaction principles (Weber, 1992), the molecular forces that control affinity are the same as those that influence the effect of the ligand on the conformation of the receptor. However, differences between the structural requirements for affinity and efficacy are often observed (e.g. imidazole agonists of α-adrenergic receptors: Ruffolo and Waddell, 1983) and probably reflect the interaction of ligands with the ensemble of receptor conformations. These interactions are complex, leading to changes in the receptor conformation ensemble, which in turn may result in ligands of similar affinity but different efficacy or vice versa (Freire, 2000).

Bromination at C(5) of cy and *N*-Me-cy reduces binding and functional potency, the only exception being 5-Br-*N*-Me-cy at hα7 nACh receptors where the halogenated isostere is more potent than the parent compound. This effect is more marked at hα4β2 receptors where 5-Br-cy cannot elicit any currents even at concentrations as high as 1–3 mM (Houlihan et al., 2001b). Iodination at the same position causes a similar effect, albeit less pronounced, on hα7 and hα4β4 nACh receptors, but at hα4β2 there is a significant increase in relative efficacy. The factors that control the effects of the C(5) substituent on affinity and efficacy are clearly different from those that operate at C(3). The lack or detrimental effects of C(5) halogenation upon affinity and efficacy may reflect the fact that this position is not as close as C(3) to the putative hydrogen bond accepting oxygen of cy, which may buffer the effects of the halogen atoms upon hydrogen bonding. It is also possible, as suggested above, that the hypothetical hydrophobic pocket that accommodates halogen atoms present at C(3) is a small region that does not include the area that C(5) faces in the receptor binding site. Thus, the interaction of the C(5) substituent with the receptor binding site should be different from that of the C(3) substituent, leading to different patterns of effects on affinity and efficacy.

Cy and its most potent halogenated isosteres (3-Br-cy and 3-I-Cy) yielded biphasic agonist concentration–response curves at both hα4β2 and hα4β4 nACh receptors. This finding is in accord with previous reports showing biphasic agonist concentration–response curve at hα4β4 (Houlihan et al., 2001a) and hα4β2 (Buisson et al., 2000; Covernton and Connolly, 2000; Houlihan et al., 2001a; Zwart and Vijverberg, 1998) nACh receptors. Biphasic agonist concentration–response curves might arise for a variety of reasons such as assembly of α4 and β2 or β4 subunits into receptors with at least two different stoichiometric arrangements (Zwart and Vijverberg, 1998). Biphasic concentration–response curves could also arise by the existence of stable and distinct conformations of a single population of receptors. Recently, we have shown that the agonist concentration–response curves of hα4β2 nACh receptors can be transformed into a monophasic concentration–response curves in the presence of low extracellular Ca^{2+} or by inhibitors of PKC-dependent phosphorylation (Houlihan et al., 2001a), which is consistent with the idea that biphasic agonist concentration–response curves could result from the presence of two distinct conformations of a receptor stabilized, for example, by phosphorylation.

Interestingly, 5- or 3,5-diBr-cy and *N*-Me-cy, which display low or modest affinity relative to cy, 3-Br-cy or 3-I-cy at hα4β2 or hα4β4 nACh receptors, yielded monophasic concentration–response curves. Plots comparing radioligand competition $\log K_i$ values to functional $\log EC_{50}$ values are better fit by linear regression when using data for high-affinity rather than low-affinity components for those agents inducing biphasic functional concentration–response curves. In previous studies we have shown that application of mid to high agonist concentrations, such as those required to achieve saturation of agonists DRCs, results in other effects such as competitive inhibition, desensitization and/or ion channel block of nACh receptors (Houlihan et al., 2001a,b), which may distort EC_{50} values. Thus, correlation values between K_i and low-affinity EC_{50} may not directly reflect the relationship between ligand binding and ligand-induced function.

It is clear from our studies that the effects of cy, *N*-Me-cy and their halogenated isosteres on nACh receptors are influenced by receptor subtype, which provides further evidence that the topology of the binding site of the nACh receptor is determined by the type of α and β subunit present (Chavez-Noriega et al., 1997; Luetje and Patrick, 1991; Parker et al., 1998; this study). The β subunit plays a crucial role in defining cy efficacy and affinity; cy and its halogenated isosteres consistently display higher affinity and efficacy at hα4β4 nACh receptors. However, the type of α subunit is also important for affinity and efficacy. For example, cy, *N*-Me-cy and their derivatives, like other nACh receptor agonists, are more potent at hα4β2 than at hα7 nACh receptors. Indeed Curtis et al. (2000) have recently reported that the second transmembrane domain of the α4 subunit influences the affinity and efficacy of cy.

In summary, we show here that halogenation at C(3) of the pyridone ring of cy or *N*-Me-cy significantly increases the binding and functional potency of the parent compounds on hα7, and to a lesser extent on hα4β2 and hα4β4 nACh receptors, while halogenation at C(5) or both C(3) and C(5) causes the opposite effect. We discuss here that the electronic effects and the size of the halogen substituent may play crucial roles in determining the affinity and efficacy of the halogenated cy derivatives. We also show that ligands with high affinity discriminate between two distinct receptor populations on both hα4β2 and hα4β4 nACh receptors while those with modest or low affinity only reveal one receptor population.

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