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Activity of cytisine and its brominated isosteres on recombinant human α7, α4β2 and α4β4 nicotinic acetylcholine receptors

Lee M. Houlihan,* Yvonne Slater,* Doris L. Guerra,† Jian-Hong Peng,‡ Yen-Ping Kuo,‡ Ronald J. Lukas,‡ Bruce K. Cassels† and Isabel Bermudez*

**School of Biological and Molecular Sciences, Oxford Brookes University, Oxford, UK*

†Millennium Institute for Advanced Studies in Cell Biology and Biotechnology and Department of Chemistry, Faculty of Sciences, University of Chile, Santiago, Chile

‡Division of Neurology, Barrow Neurological Institute, Phoenix, Arizona, USA

Abstract

Effects of cytisine (cy), 3-bromocytisine (3-Br-cy), 5-bromocytisine (5-Br-cy) and 3,5-dibromocytisine (3,5-diBr-cy) on human (h) α7-, α4β2- and α4β4 nicotinic acetylcholine (nACh) receptors, expressed in *Xenopus* oocytes and cell lines, have been investigated. Cy and its bromo-isosteres fully inhibited binding of both [α-^{125}I]bungarotoxin ([α-^{125}I]BgTx) to hα7- and [^{3}H]cy to hα4β2- or hα4β4-nACh receptors. 3-Br-cy was the most potent inhibitor of both [α-^{125}I]BgTx and [^{3}H]cy binding. Cy was less potent than 3-Br-cy, but 5-Br-cy and 3,5-diBr-cy were the least potent inhibitors. Cy and 3-Br-cy were potent full agonists at hα7-nACh receptors but behaved as partial agonists at hα4β2- and hα4β4-nACh receptors. 5-Br-cy and 3,5-diBr-cy had low potency and were partial agonists at hα7- and hα4β4-nACh receptors, but they elicited no responses on hα4β2-nACh receptors. Cy and 3-Br-cy produced dual dose–response curves (DRC) at both hα4β2- and hα4β4-nACh receptors, but ACh produced dual DRC only at hα4β2-nACh receptors. Low concentrations of cy, 3-Br-cy and 5-Br-cy enhanced ACh responses of oocytes expressing hα4β2-nACh receptors, but at high concentrations they inhibited the responses. In contrast, 3,5-diBr-cy only inhibited, in a competitive manner, ACh responses of hα4β2-nACh receptors. It is concluded that bromination of the pyridone ring of cy produces marked changes in effects of cy that are manifest as nACh receptor subtype-specific differences in binding affinities and in functional potencies and efficacies.

Keywords: cytisine, neuronal nicotinic acetylcholine receptor, two-site receptor occupation model, *Xenopus* oocyte.

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Nicotinic acetylcholine (nACh) receptors in the nervous system are assembled from subunits that are distinct, though homologous to, those that compose muscle nACh receptors (Galzi and Changeux 1995; Corringer *et al*. 2000). To date, nine different α subunits (α2 to α10) and three β subunits (β2 to β4) have been isolated from avian, rodent or human neuronal tissue, providing for a potentially large number of nACh receptor subtypes (Galzi and Changeux 1995; Gotti *et al*. 1997; Lukas *et al*. 1999). However, as for the $GABA_A$ receptor, only a small number of naturally occurring nACh receptor combinations and stoichiometries have been identified to date. An abundant brain nACh receptor is proposed to assemble from two copies of the α4 subunit plus three copies of the β2 subunit (to form α4β2 nACh receptors; Cooper *et al*. 1991). However, other stoichiometries have been proposed (Zwart and Vijverberg 1998), and there is evidence for inclusion of α5 subunits in addition to α4 and β2 subunits in one naturally expressed nACh receptor subtype (Conroy and Berg 1998). High expression of β4 subunits in primate brain (Quik *et al*. 2000) suggests that α4β4-nACh receptors may be more abundant in primates than in rodents. Although pairwise combinations of α3 with β2 or β4 subunits can assemble to form functional nACh receptors

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Address correspondence and reprint requests to Dr I Bermudez, School of Biological and Molecular Sciences, Oxford Brookes University, Gipsy Lane, Oxford OX3 0BP, UK.

E-mail: P0054922@brookes.ac.uk

Abbreviations used: ACh, acetylcholine; DRC, dose–response curve; cy, cytisine; 3-Br-cy, 3-bromocytisine; 5-Br-cy, 5-bromocytisine; 3,5-diBr-cy, 3,5-dibromocytisine; nACh, nicotinic acetylcholine; α-BgTx, α-bungarotoxin.

in heterologous expression systems, so too can more complex combinations with three (α3β4α5; Gerzanich *et al*. 1998) or four (α3β2β4α5; Wang *et al*. 1996) different subunits. α7, α8 and α9 subunits differ from others in being able to form functional, heterologously expressed homomeric nACh receptors in oocytes or cell lines (Gotti *et al*. 1997). Homomeric forms of α7-nACh receptor appear to exist naturally, although the α7 subunit also may form heteromers in native neuronal tissue (Yu and Role 1998).

Different nACh receptor subtypes have unique localizations and functions, and the diverse subunits of neuronal nACh receptors provide bases for structural and functional heterogeneity that is manifest as distinctive biophysical and pharmacological properties of individual receptor subtypes. For example, α4β2-nACh receptors are involved in nociception (Marubio *et al*. 1999), glutamate release in the hippocampus is mediated through α7-containing nACh receptors (Radcliffe and Dani 1998), whilst GABA release is modulated by both α7 (Frazier *et al*. 1998) and β2-containing nACh receptors (Léna 1997). The α and β subunits of nACh heteromeric receptors determine features such as conductance states and kinetics of desensitization (Papke and Heinemann 1994; Fenster *et al*. 1997). Subunit makeup also influences Ca^{2+} permeability and agonist and/or antagonist pharmacology. Homomeric α7-nACh receptors have the highest Ca^{2+} permeability of the nACh receptors and are blocked by α-bungarotoxin (α-BgTx). In contrast, heteromeric receptors have much lower Ca^{2+} permeabilities and are insensitive to α-BgTx (Gotti *et al*. 1997).

Cytisine (cy) is a natural alkaloid that occurs in a large number of plants of the Leguminosae family, and is well known as the main toxic principle of the common garden *Laburnum*. Both radioligand binding (Pabreza *et al*. 1991; Hall *et al*. 1993; Monteggia *et al*. 1995; Parker *et al*. 1998) and functional (Rapier *et al*. 1990; Luetje and Patrick 1991; Papke and Heinemann 1994) studies have shown that cy has one of the highest affinities of all drugs examined for α4β2-nACh receptors. Effects of cy on nACh receptors are markedly sensitive to receptor subunit composition. Cy binds with nM affinity to nACh receptors containing β2 or β4 subunits (Parker *et al*. 1998), but its efficacy is much lower at receptors containing β2 subunits instead of β4 subunits (Luetje and Patrick 1991; Papke and Heinemann 1994; Chavez-Noriega *et al*. 1997). It behaves as a full agonist at homomeric α7-nACh receptors, but its potency at α7-nACh is lower than that at heteromeric nACh receptors (Peng *et al*. 1994; Chavez-Noriega *et al*. 1997).

Given the diversity of nACh receptors and the differential potency and efficacy of cy, it is surprising that only a few attempts have been made to modify the cy skeleton. Such studies have been mostly restricted to substitutions of the basic nitrogen atom (Barlow and McLeod 1969; Boido and Sparatore 1999). More recently, *N*-protected and -deprotected pyridone ring-substituted derivatives of cy have been prepared, but neither their affinities nor their functional effects at nACh receptors have been characterized (Marrière *et al*. 2000). As an aid to explore structural and functional diversity of brain nACh receptors, and toward development of additional, selective pharmacological tools for potential therapeutic agents targeting nACh receptor subtypes, we have synthesized three bromo-isosteres of cy, 3-bromocytisine (3-Br-cy), 5-bromocytisine (5-Br-cy) and 3,5-dibromocytisine (3,5-diBr-cy) (Fig. 1). We show here that each of these compounds acts competitively and with distinct agonist efficacies and potencies at human (h) recombinant α7-, α4β2- and α4β4-nACh receptors. We also show that the changes in the efficacy of cy brought about by bromination cause interesting changes in the effects of cy on functional responses of hα4β2-nACh receptors.

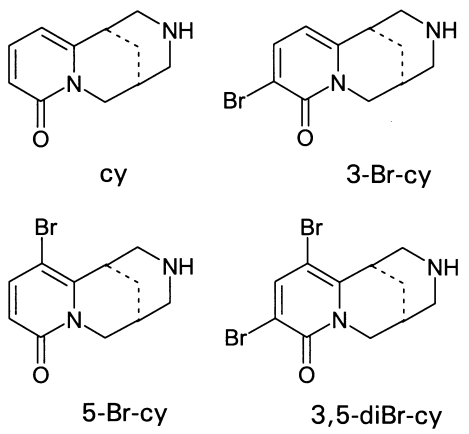

Fig. 1 Structure of cy and its bromo-isosteres.

Materials and methods

Materials

Xenopus laevis were purchased from Blades Biological (Kent, UK). All chemicals, with the exception of cy and its brominated isosteres, were purchased from Sigma (St Louis, MO, USA). [^{3}H]Cytisine ([^{3}H]cy; 30–32 Ci/mmol) and [α-^{125}I]BgTx (134 Ci/mmol) were from NEN (Boston, MA, USA). Solutions of ACh and cy were prepared freshly in Ba^{2+}-Ringer solution (in mM: 115 NaCl, 2.5 KCl, 10 HEPES, 2.4 $BaCl_2$, pH 7.2), while the bromo-isosteres of cy were made up as stock solutions in Ba^{2+}-Ringer and frozen at −20°C. Individual aliquots were thawed and diluted in Ba^{2+}-Ringer solution at the desired concentration.

Chemistry

Cy was purified from *Sophora secundiflora* seeds using standard methodology. 3,5-DiBr-cy was prepared as described by Luputiu and Moll (1971) and 3-Br-cy and 5-Br-cy were obtained by treating cy with slightly more than one molar equivalent of bromine in acetic

Table 1 Comparison of binding affinity for cy and brominated isosteres at hα7-, hα4β2- and hα4β4-nACh receptors

Drug	IC_{50}	K_I	nHill
hα7-nACh receptors (K_d 1 ± 0.15 nM)			
3-Br-cy	31.60 ± 1.5 nM	16.0 ± 0.8 nM	1.01 ± 0.05
Cy	16.70 ± 1.4 μM	8.36 ± 0.7 μM	0.98 ± 0.08
3,5-DiBr-cy	26.97 ± 1.7 μM	13.50 ± 0.9 μM	1.09 ± 0.07
5-Br-cy	31.80 ± 1.2 μM	10.10 ± 0.6 μM	0.99 ± 0.04
hα4β2-nACh receptors (K_d 0.43 ± 0.082 nM)			
3-Br-cy	0.30 ± 0.01 nM	0.082 ± 0.003 nM	1.0 ± 0.05
Cy	3.74 ± 0.22 nM	1.07 ± 0.060 nM	1.01 ± 0.06
3,5-DiBr-cy	1.50 ± 0.04 μM	0.42 ± 0.004 μM	1.1 ± 0.05
5-Br-cy	5.4 ± 0.06 μM	1.54 ± 0.009 μM	0.98 ± 0.05
hα4β4-nACh receptors (K_d 0.10 ± 0.02 nM)			
3-Br-cy	0.28 ± 0.01 nM	0.026 ± 0.001 nM	1.01 ± 0.04
Cy	1.05 ± 0.09 nM	0.096 ± 0.008 nM	0.97 ± 0.08
3,5-DiBr-cy	0.25 ± 0.02 μM	23.10 ± 1.8 nM	0.98 ± 0.07
5-Br-cy	0.75 ± 0.03 μM	68.50 ± 3.0 nM	1.0 ± 0.4

Data represent the means ± SEM values of 4–5 experiments. In competition studies using hα7, hα4β2- or hα4β4-nACh receptors the radiolabelled ligand concentration was 1 nM. K_d is the equilibrium dissociation constant.

acid. Bromo-isosteres were separated by column chromatography on silica gel, crystallized to homogeneity and characterized by ^1H and ^{13}C NMR and high resolution mass spectrometry.

Preparation of RNA transcripts and *Xenopus* oocyte injection

The hα7, hα4, hβ2 and hβ4 nACh receptor subunit cDNAs were provided by Professor Jon Lindstrom (University of Pennsylvania, PA, USA). *In vitro* transcripts were prepared using MegaScript T7 (hβ4) or SP6 (hα7, hα4, hβ2) capped RNA transcription kits (Ambion, Inc., Austin, TX). *Xenopus laevis* oocytes were prepared and injected with hα7 or combinations of hα4 + hβ2 or hβ4 (at a 1 : 1 ratio) nACh receptor subunit cRNAs as previously described (Houlihan *et al*. 2000). Injected oocytes were incubated at 20°C for up to a week in Barth's solution containing (in mM): 88 NaCl, 1 KCl, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 2.5 $NaHCO_3$, 0.5 theophylline, 10 HEPES; pH 7.2, supplemented with 5% heat-inactivated horse serum, 0.1 mg/mL gentamicin sulphate, 0.01 mg/mL streptomycin sulphate and 0.01 mg/mL penicillin-G.

Electrophysiological recordings

Recordings were made 3–5 days following injection of cRNAs. Oocytes were placed in a 100-μL bath that was gravity perfused continuously at 4 mL/min with Ba^{2+}-Ringer solution. This solution ($CaCl_2$ in conventional Ringer solution was replaced by $BaCl_2$) was used to record from oocytes in order to minimize the activation of Ca^{2+}-gated chloride conductance, which is endogenous to the *Xenopus* oocyte and may be activated by Ca^{2+} entry through nACh receptors (see for example Sands *et al*. 1993). We found no differences in the EC_{50}s or IC_{50}s determined in the presence of Ba^{2+}- or Ca^{2+}-containing Ringers, which confirms previous reports that nominally Ca^{2+}-free external solution does not affect the pharmacology of nACh receptors expressed in *Xenopus* oocytes (see for example, Cachelin and Rust 1994; Chavez-Noriega *et al*. 1997; Houlihan *et al*. 2000). Oocytes were voltage-clamped at between −60 and −80 mV, depending on nACh receptor expression levels. Whole-cell currents were measured as described before (Houlihan *et al*. 2000). Drugs were applied by gravity perfusion using a manually activated valve. Agonists were applied for a period sufficient (approximately 10–15 s) to obtain a stable plateau response (at low concentrations) or the beginning of a sag after a peak (at higher concentrations). At least 3 min wash time was allowed between each drug application to allow clearance of the drug and to prevent receptor desensitization. Dose-response curves (DRC) for agonists were constructed by normalizing to the maximal response of the agonist and used to generate EC_{50} and nHill estimates. 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 3b and c). 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. 3a). To construct antagonist or potentiator DRCs in studies of hα4β2-nACh receptors, the responses elicited by the coapplication of ACh and increasing concentrations of drugs were normalized to the responses evoked by ACh alone. Constant responses to ACh were obtained before the coapplication of ACh and drug. In these studies, hα4β2-nACh receptors were not preincubated with the test drugs prior to the coapplication procedure to avoid cross-desensitization of the receptors that might have arisen from the agonist effects of these compounds.

Cell lines

The [α-^{125}I]BgTx binding was assayed utilizing the SH-SY5Y-hα7 neuroblastoma clonal cell line which over-expresses hα7 nACh

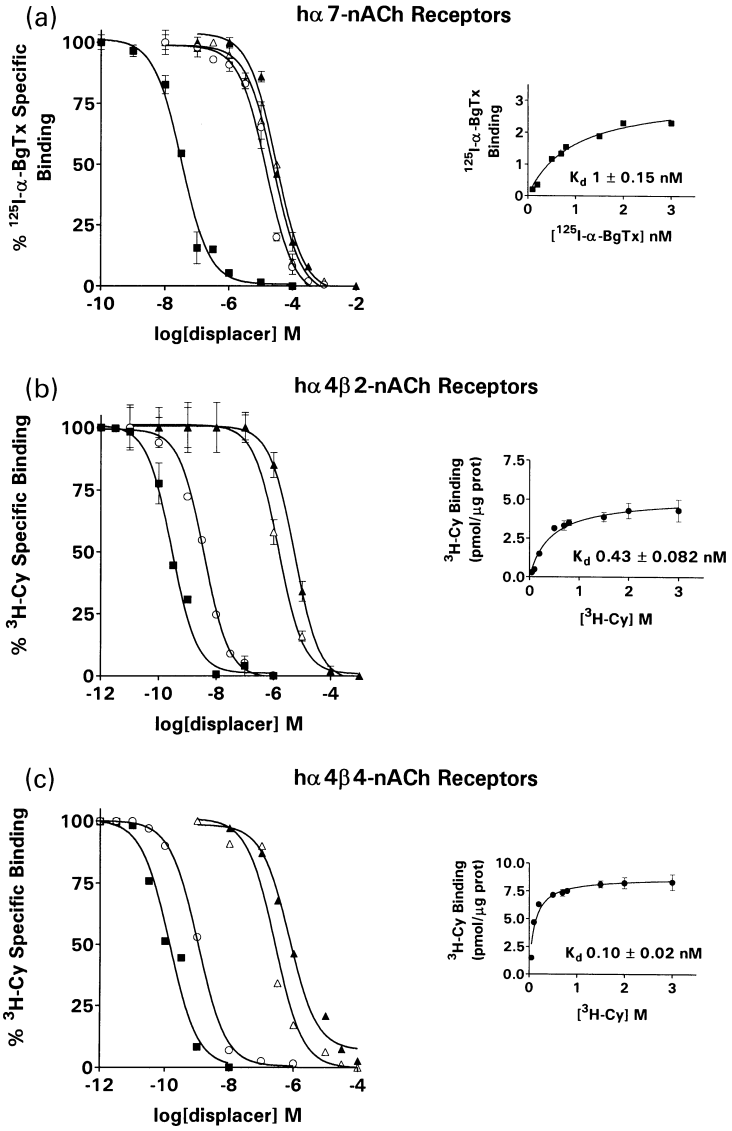

Fig. 2 Competition for [α-^{125}I]BgTx and [^{3}H]cys binding sites in hα7- (a) hα4β2- (b) or α4β4- (c) nACh receptors by unlabelled cy and its brominated isosteres. Homogenates were incubated with 8–10 concentrations of drugs before addition of 1 nM [α-^{125}I]BgTx or [^{3}H]cy. Curves are representative of three to five determinations for each drug. Where no error bars are seen, they are smaller than the symbols. Symbols: cy, ○; 3-Br-cy, ■; 5-Br-cy, ▲; 3,5-diBr-cy, △.

receptors (Houlihan *et al*. 2000). To generate the SH-SY5Y-hα7 cell line (Lukas and Peng, unpublished work), wild-type SH-SY5Y cells were transfected with hα7 subunits (kindly provided by Dr Sherry Leonard) subcloned into the pCEP4 vector. [^{3}H]cy binding studies were carried out on membrane homogenates prepared from the human clonal cell lines SH-EP1-pcDNA-hα4β2 (SH-EP1-hα4β2) and SH-EP1-pCEP4/hygro/α4-pcDNA/zeo/β4-hα4β4 (SH-EP1-hα4β4), which express hα4β2- and hα4β4-nACh receptors, respectively (Peng *et al*. 1999a; Eaton *et al*. 2000). To generate the SH-EP1-hα4β2 cell line (Peng *et al*. 1999a), native nACh receptor-null SH-EP1 cells (Lukas *et al*. 1993) were transfected with hα4 and hβ2 subunits (kindly provided by Dr Ortrud Steinlein) subcloned into pcDNA3.1-zeo and pcDNA3.1-hygro vectors, respectively, using conventional techniques (Puchacz *et al*. 1994; Peng *et al*. 1999b). To generate the SH-EP1-hα4β4 cell line (Eaton *et al*. 2000), wild-type SH-EP1 cells were transfected with hα4 and hβ4 subunits (kindly provided by Drs Ortrud Steinlein and Jon Lindstrom, respectively) subcloned into pCEP4 and pcDNA3.1-zeo vectors, respectively. Cells were maintained as low passage number (1–26 from our frozen stocks) cultures, to ensure stable expression of phenotype, in serum-supplemented medium (Lukas *et al*. 1993) augmented with zeocin (0.5 mg/mL, Invitrogen, Carlsbad, CA, USA) and hygromycin (0.4 mg/mL, Calbiochem, San Diego, CA, USA) to select for transfectants and passaged once weekly by splitting just-confluent cultures 1/10–1/20 to maintain cells in proliferative growth.

Ligand binding assays

For binding assays, confluent SH-SY5Y-hα7, SH-EP1-hα4β2 or SH-EP1-hα4β4 cells were rinsed with ice-cold phosphate-buffered saline, mechanically disaggregated and homogenized using a Polytron homogenizer for 10 s. The homogenates were centrifuged at 40 000 *g* at 4°C for 20 min, and the pellets resuspended in ice-cold binding saline to give a final protein concentration in the assay tubes of approximately 30–50 μg. The binding saline used in

[α-^{125}I]BgTx studies consisted of (in mM): 140 NaCl, 1 EDTA and 50 Tris-HCl at pH 7.4, whilst for [^{3}H]cy binding the saline contained (in mM) 120 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 50 Tris, pH 7.0.

The [α-^{125}I]BgTx binding was carried out as described previously (Houlihan *et al*. 2000). The [^{3}H]cy binding was carried out as described by Pabreza *et al*. (1991) with some modifications. Briefly, incubations were carried out in a final volume of 250 μL in 1 nM [^{3}H]cy and cell membrane homogenate for 75 min at 4°C in the presence or absence of unlabelled drugs. The same solution and total cell membrane protein were used in saturation binding studies, but the concentration of [^{3}H]cy varied from 0.05 to 4 nM. Non-specific binding was defined using 10 μM nicotine. Incubations were terminated by vacuum filtration over GF/C glass fibre filters presoaked in 0.1% polyethyleneimine, and filters were washed twice with 3 mL of ice-cold binding saline. Bound radioactivity was measured by liquid scintillation counting.

Data analysis

Inward currents were recorded on a flat bed chart recorder for later analysis. DRC data for agonists and antagonists were fitted by non-linear regression (Prism 3.01, GraphPad, USA) to the equations:

(a) $I = I_{max}/[1 + (EC_{50}/x)^{nHill}]$, or

(b) $I = I_{max}/[1 + (x/IC_{50})^{nHill}]$,

wherein I_{max} = maximal normalized current response (in the absence of antagonist for inhibitory currents), x = agonist or antagonist concentration, EC_{50} = concentration of agonist eliciting a half-maximal response, IC_{50} = antagonist concentration eliciting half-maximal inhibition, and nHill = Hill coefficient. Biphasic agonist data were fitted to the sum of two empirical Hill equations comparable to those used by Covernton and Connolly (2000) and Buisson and Bertrand (2001). A comparison was always made between the fitting of the mean agonist DRC with either one or two components (assuming either one or two distinct sites). The

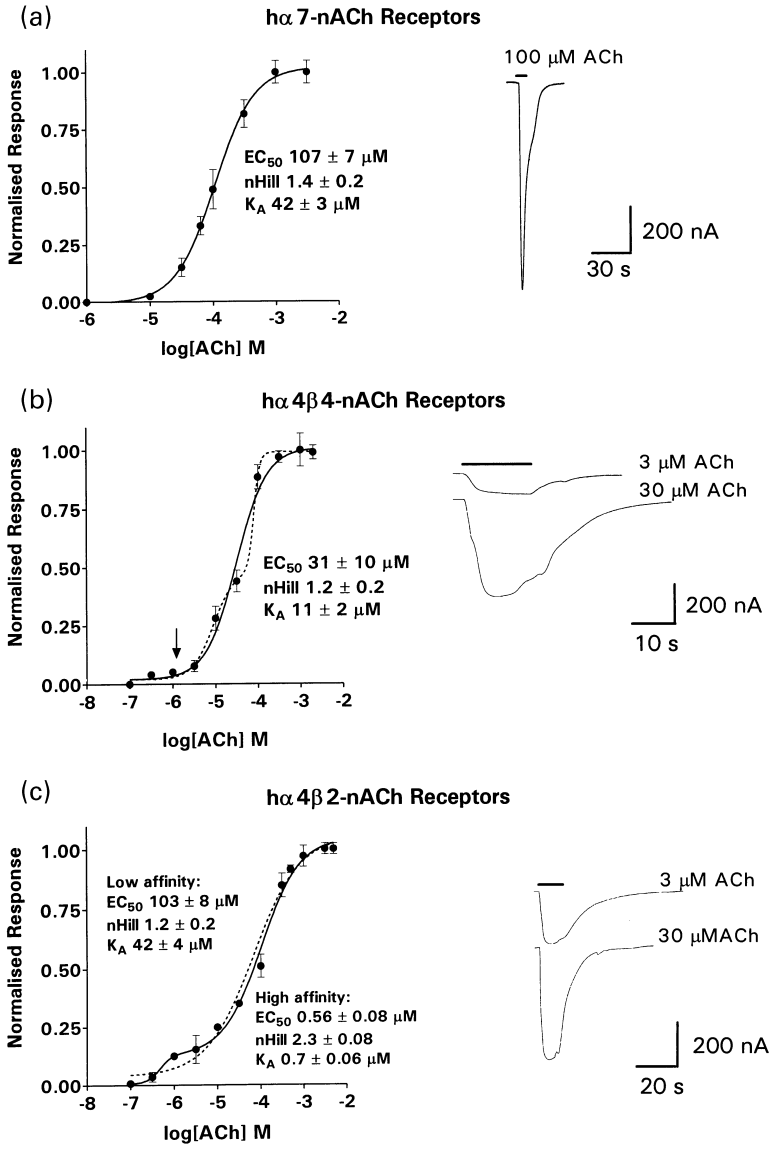

Fig. 3 Functional DRC for ACh with hα7- (a), hα4β4- (b) or α4β2- (c) nACh receptors. The ACh responses were normalized to the maximal ACh response on each receptor type. Each point is the average of 3–7 experiments carried out at −60 mV. The DRC data for the effects of ACh on hα7- (a) and hα4β4-nACh receptors (b) were best fitted to a single Hill equation ($p < 0.05$); dashed lines correspond to the curve obtained by fitting the data to the sum of two Hill equations. The arrow in (b) shows data points at low ACh concentration, which were not adequately fitted with a single Hill equation but whose amplitudes were too small to be resolved as a distinct high affinity receptor population. In contrast, the ACh DRC data for hα4β2-nACh receptors (c) were best fitted using the sum of two Hill equations as described in Materials and methods. Dashed line in (c) represents the fitting of the same data to a single Hill equation. Fitting of the DRC data shown in (a), (b) and (c) to the equilibrium two-site receptor occupation equation described in Materials and methods yielded curves that were indistinguishable from those obtained by fitting the data to one or two Hill equations. Traces in (a), (b) and (c) and represent typical ACh responses of *Xenopus* oocytes expressing hα7, hα4β4 or hα4β2 nACh receptors, respectively.

magnitude of the responses to the agonist concentrations greater than 2–3 mM decreased in a concentration dependent manner due to receptor desensitization and/or agonist-induced open channel block and were excluded from the analysis of the data.

The apparent functional affinity of agonists for nACh receptors was estimated from the DRC data by fitting the data to the equilibrium two-site receptor occupation equation:

$$\text{(c) } I = I_{max}\{c_A/(1 + c_A)\}^2,$$

where c_A represents the concentration of ACh divided by its K_A value (x/K_A). Data consisting of two components were fitted using the sum of two equations in the form:

$$\text{(d) } I = I_{max1}\{c_{A1}/(1 + c_{A1})\}^2 + I_{max2}\{c_{A2}/(1 + c_{A2})\}^2,$$

where c_{A1} and c_{A2} represent $[x/K_{A1}]$ and $[x/K_{A2}]$.

The effects of cy, 3-Br-cy and 5-Br-cy on the ACh responses of hα4β2 nACh receptors expressed in *Xenopus* oocytes produced bell shaped DRC curves that consisted of a potentiation phase followed by an inhibitory phase (see for example Figs 7, 8 and 9). The bell-shaped data were analysed as follows. Potentiation DRC were fitted to equation (a) for concentrations up to and including those concentrations of cy, 3-Br-cy or 5-Br-cy that produced maximal potentiation. Inhibition DRC were fitted to equation (b) for concentrations of cy, 3-Br-cy or 5-Br-cy at or above those concentrations that achieved maximal potentiation. This method has been previously used to account for the dual effects of zinc on rat neuronal nACh receptors (Hsiao *et al*. 2001).

The binding parameters (K_d and B_{max}) of [^{3}H]cy binding were determined from saturation binding isotherm data using the equation $Y = B_{max} \times X/K_d + X$, wherein B_{max} = maximal binding, K_d = apparent equilibrium dissociation binding constant, X = concentration of ligand and Y = binding. The K_i value of the test compounds was determined using the equation of Cheng and Prusoff, $K_i = EC_{50}/1 + [x]/K_d$.

Results are presented as mean ± standard error of the mean of at least four separate experiments from at least two different batches of oocytes. Where appropriate, one-way ANOVA or Student's *t*-test for paired or unpaired data were used, and values of $p \leq 0.05$ were regarded as significant. *F*-tests were carried out using the Prism software to determine the best fit to the data (e.g. one or two independent sites).

Results

Effects of cy and its brominated isosteres on [α-^{125}I]BgTx and [^{3}H]cy binding

We first examined the effects of cy and its brominated isosteres on the binding of [^{3}H]cy to hα4β2- and hα4β4-nACh receptors in membrane fractions from SH-EP1-hα4β4 or SH-EP1-hα4β2 cells, respectively, and on [α-^{125}I]BgTx binding to hα7-nACh receptors in membrane fractions from SH-SY5Y-hα7 cells (Table 1; Fig. 2). The K_d for [α-^{125}I]BgTx binding to hα7-nACh receptors and [^{3}H]cy binding to hα4β4- and hα4β2-nACh receptors were comparable to previously published data (Hall *et al*. 1993; Monteggia *et al*. 1995; Houlihan *et al*. 2000). Specific binding of both [α-^{125}I]BgTx to hα7-nACh receptors and [^{3}H]cy to hα4β2- or hα4β4-nACh receptors was fully displaced by cy and its brominated isosteres in a dose-dependent manner. 3-Br-cy was the most potent inhibitor of both [α-^{125}I]BgTx and [^{3}H]cy binding with K_i values ranging from 0.026 nM (hα4β4-nACh receptor) to 0.082 nM (hα4β2-nACh receptor) and 16 nM (hα7-nACh receptor). Unlabelled cy was also a potent inhibitor of [α-^{125}I]BgTx and [^{3}H]cy binding with K_i values in the low (hα4β4-nACh receptors) or moderate (hα4β2-nACh receptors) nanomolar range or in the moderate (hα7-nACh receptor) micromolar range. 5-Br-cy and 3,5-diBr-cy were the least potent inhibitors of [α-^{125}I]BgTx and [^{3}H]cy binding. The nHill coefficient value estimated from the DRCs was close to one for all compounds (Table 1). The rank order of potency for blockade of [^{3}H]cy binding to hα4β2- and hα4β4-nACh receptors was 3-Br-cy > cy > 3,5-diBr-cy > 5-Br-cy and for blockade of [α-^{125}I]BgTx binding to

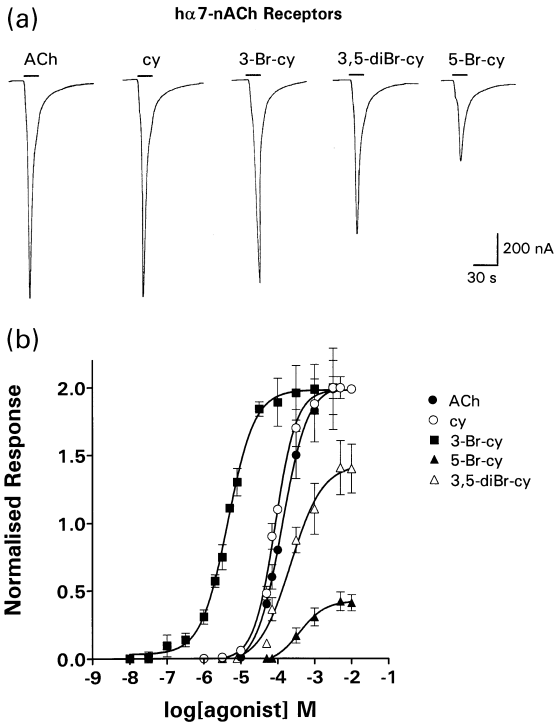

Fig. 4 Functional agonist effects of cy and its brominated isosteres on hα7-nACh receptors. (a) Representative traces showing the currents elicited by application of 100 μM ACh and 1 mM test compounds in oocytes expressing hα7-nACh receptors and voltage-clamped at −60 mV. (b) DRC for the agonist effects of the test compounds on hα7-nACh receptors (for comparison the ACh DRC is also shown). The data were normalized to the responses elicited by 100 μM ACh (approximately EC_{50} of the ACh response at hα7-nACh receptors) and then fitted to a single Hill equation. The data was not adequately fitted to a two-component Hill equation (not shown). Data points in B represent the mean ± SEM of 5 −10 experiments. Where no error bars are seen, they are smaller than the symbols.

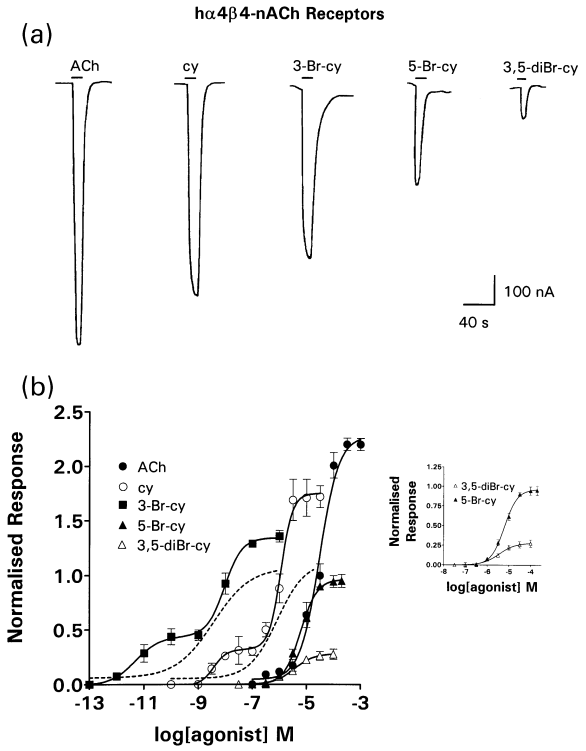

Fig. 5 Functional agonist effects of cy and its brominated isosteres on hα4β4-nACh receptors. (a) Representative traces showing the current responses to E_{max} ACh and test compounds in oocytes expressing hα4β4-nACh receptors and voltage-clamped at −60 mV (b) DRC curves for ACh and the test compounds on hα4β4 -nACh receptors. DRC data for cy and the bromo-isosteres were normalized to 30 μM ACh (approximately, the ACh EC_{30} in hα4β4-nACh receptors). Data points for cy and 3-Br-cy were best fitted to the sum of two Hill equations ($p < 0.05$); dashed lines shows the fitting of the same data to a single Hill equation. Data points for ACh, 5-Br-cy and 3,5-diBr-cy could only be fitted to a single Hill equation. The insert shows clearly that the DRC data of 5-Br-cy nor 3,5-diBr-cy produced monophasic DRC. Data points represent the mean ± SEM of 3–7 experiments. Where no error bars are seen, they are smaller than the symbols.

hα7-nACh receptors was 3-Br-cy >> cy ~ 3,5-diBr-cy ~ 5-Br-cy.

ACh DRC

Application of ACh onto oocytes expressing the hα7-, hα4β2- or hα4β4-nACh receptors induced dose-dependent inward currents. The DRC data for ACh at hα7-nACh receptors were best fitted with a single Hill equation ($p < 0.001$); the estimated EC_{50} and nHill coefficient were 107 ± 7 μM and 1.4 ± 0.2, respectively (Fig. 3a). The apparent functional affinity for ACh (K_A) estimated by fitting the DRC data shown in Fig. 3(a) to the equilibrium two-site receptor occupation equation was 42 ± 3 μM. The DRC data for ACh on the hα4β4-nACh receptor were best fitted with only one single component ($p < 0.05$; Fig. 3b).

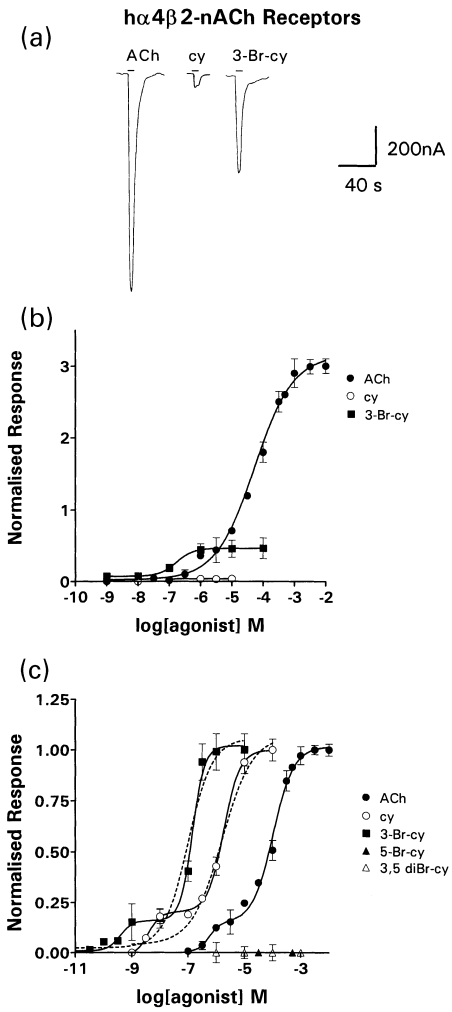

Fig. 6 Functional agonist effects of cy and its brominated isosteres on hα4β2-nACh receptors. (a) Representative traces showing the current responses to 30 μM ACh and 100 μM cy or 3-Br-cy in oocytes expressing hα4β2-nACh receptors and voltage-clamped at −60 mV. Note that 5-Br-cy and 3,5-diBr-cy did not evoke ion currents, even at 1 mM (b) Relative efficacy of cy and 3-Br-cy at hα4β2-nACh receptors. The relative efficacy was determined by normalizing the current response in each oocyte to 30 μM ACh (ACh EC_{100} in the high-affinity hα4β2-nACh receptor population and about EC_{20-25} of the ACh response of the low-affinity receptor population). (c) DRC for cy and 3-Br-cy on hα4β2-nACh receptors normalized to their respective maximal responses. As shown, both 3-Br-cy and cy activated the high- and low-affinity hα4β2-nACh receptor populations, which resulted in biphasic DRC. Data points were best fitted to the sum of two Hill equations; dashed lines represent the curves obtained by fitting the data to a single Hill equation. For comparison, the DRC for ACh is included. Data points for (b) and (c) represent the mean ± SEM of 3 −7 experiments. Where no error bars are seen, they are smaller than the symbols.

However, an additional high-affinity component representing less than 5–6% of the total receptor population might have been present at low (0.1–3 μM) ACh concentrations (indicated by arrow in Fig. 3b). The estimated EC_{50} value

Table 2 Functional potency and relative efficacy of cy and its brominated isosteres on recombinant hα7-, hα4β2- and hα4β4-nACh receptors

Drug	EC_{50}	nHill	I_{max}	K_A
hα7-nACh receptors				
Cy	83 ± 6 μM	1.1 ± 0.09	1.97 ± 0.2	66.71 ± 3 μM
3-Br-cy	1.3 ± 0.2 μM	0.8 ± 0.06	1.94 ± 0.10	1.8 ± 0.2 μM
5-Br-cy	197 ± 10 μM	1.3 ± 0.10	0.45 ± 0.03	207 ± 12 μM
3,5-diBr-cy	87 ± 12 μM	1 ± 0.10	1.43 ± 0.12	88 ± 7 μM
hα4β4-nACh receptors				
Cy				
High-affinity	3.6 ± 0.2 nM	2.1 ± 0.1	0.4 ± 0.02	1.3 ± 0.08 nM
Low-affinity	1.1 ± 0.09 μM	1.98 ± 0.1	1.4 ± 0.08	0.37 ± 0.03 μM
3-Br-cy				
High-affinity	4.9 ± 0.4 pM	0.97 ± 0.08	0.4 ± 0.04	1.6 ± 0.2 pM
Low-affinity	9.1 ± 0.7 nM	1.3 ± 0.1	0.9 ± 0.07	6 ± 0.5 nM
5-Br-cy	6.1 ± 0.2 μM	1.30 ± 0.4	0.97 ± 0.03	2.4 ± 0.08 μM
3,5-DiBr-cy	3.5 ± 0.5 μM	1.2 ± 0.2	0.28 ± 0.02	1.6 ± 0.2 μM
hα4β2-nACh receptors				
Cy				
High-affinity	4.50 ± 0.3 nM	1.98 ± 0.13	0.22 ± 8	0.25 ± 0.02 nM
Low-affinity	2.13 ± 0.2 μM	1.53 ± 0.09	0.78 ± 9	0.71 ± 0.08 μM
3-Br-cy				
High-affinity	0.44 ± 0.09 nM	1.96 ± 0.08	0.3 ± 0.05	0.07 ± 0.001 nM
Low-affinity	0.16 ± 0.01 μM	1.98 ± 0.07	0.7 ± 0.09	0.08 ± 0.006 μM

Relative efficacy (I_{max}) was determined by normalizing the responses of the agonists to 30 μM ACh (hα4β4-nACh receptors) or 100 μM ACh (hα7-nACh receptors) responses. I_{max} values for the hα4β2-nACh receptors were estimated from DRC data normalized to the maximal responses evoked by cy or 3-Br-cy. Data represent the means ± SEM values of 3–7 experiments.

and nHill coefficient were 31 ± 10 μM and 1.2 ± 0.2, respectively; the K_A estimated by fitting the data to the equilibrium two-site receptor occupation equation was 11 ± 2 μM. The DRC for ACh on the hα4β2-nACh receptor in comparison to that on the hα4β4- or hα7-nACh receptors revealed a distinct plateau between 1 μM and 10 μM ACh, which was followed by a further increase in slope (Fig. 3c). Fitting of the curve to a single component was poor at low concentrations (dashed lines in Fig. 3c). However, a better fit ($p < 0.05$) was obtained by using a two-component approach ($p < 0.05$; continuous curve in Fig. 3c). The EC_{50} and nHill estimated from the two-component curves were: EC_{50} 0.56 ± 0.08 μM; nHill 2.3 ± 0.08 (high affinity component); EC_{50} 103 ± 8 μM; nHill 1.2 ± 0.2 (low affinity component). The DRC data shown in Fig. 3(c) were also fitted using the sum of two independent equilibrium two-site receptor occupation equations, which yielded K_As of 0.70 ± 0.06 μM for the high affinity component and 42 ± 4 μM for the low affinity component. The ratios of the high- and low-affinity components of the DRC for ACh were 18 ± 3% and 82 ± 7%, respectively.

Agonist effects of cy and its brominated isosteres

Marked receptor subtype differences were observed in the potency and relative efficacy displayed by cy and its bromo-isosteres at hα7-, hα4β2- and hα4β4-nACh receptors (Figs 4–6; parameters estimated from DRC are summarized in Table 2). Cy and its three bromo-isosteres evoked inward currents in oocytes expressing hα7- or the hα4β4-nACh receptors, whilst only cy and 3-Br-cy elicited inward currents in oocytes expressing hα4β2-nACh receptors. The DRC data for the effects of cy, 3-Br-cy, 5-Br-cy and 3,5-diBr-cy on hα7-nACh receptors were best fitted to a single Hill equation (Fig. 4; $p < 0.005$). The most potent agonist at the hα7-nACh receptor was 3-Br-cy with an EC_{50} in the low micromolar range, which was about 65-fold more potent than that of cy or 3,5-diBr-cy. 5-Br-cy was the least potent agonist with an EC_{50} in the high micromolar range. The nHill coefficients estimated from these curves were all close to unity. Both cy and 3-Br-cy were full agonists of hα7-nACh receptors, but 5-Br-cy and 3,5-diBr-cy behaved as partial agonists with relative efficacies that were approximately 75% (3,5-diBr-cy) or 25% (5-Br-cy) of that of cy.

The agonist effects of cy and 3-Br-cy on hα4β4-nACh receptors yielded biphasic DRCs (Fig. 5b), which were best

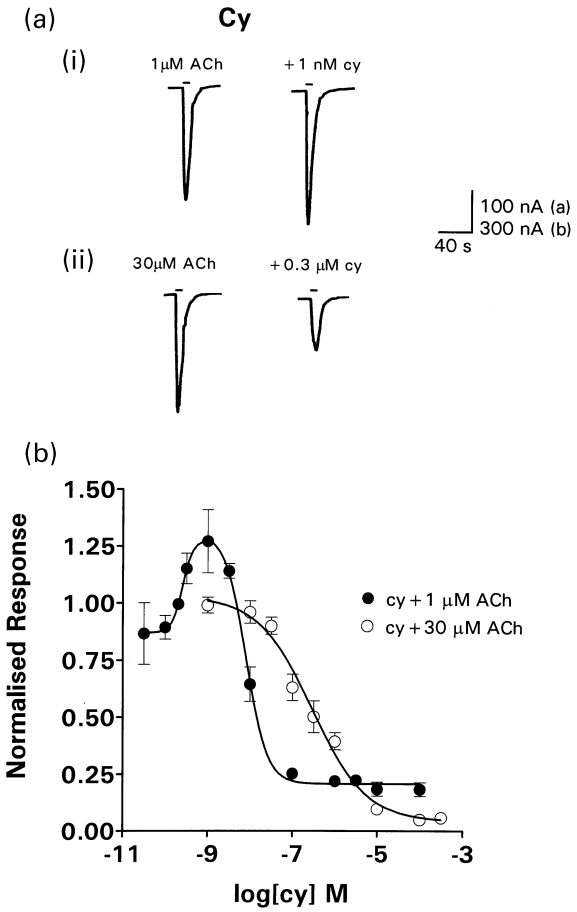

Fig. 7 Effects of cy on the ACh responses of hα4β2-nACh receptors. (a) Representative traces of the effects of cys on currents elicited by either 1 μM (i) or 30 μM (ii) ACh recorded from oocytes expressing hα4β2-nACh receptors and voltage-clamped at −60 mV (b) DRC for the effects of cy on currents elicited by either 30 μM or 1 μM ACh. Control responses elicited by ACh alone were used to normalise the responses obtained in the presence of cy. The DRC data were fitted as indicated in Materials and methods. In the presence of 30 μM ACh, the effect of cy was only inhibitory. Each point is the mean ± SEM of 3–6 independent experiments. Where no error bars are seen, they are smaller than the symbols.

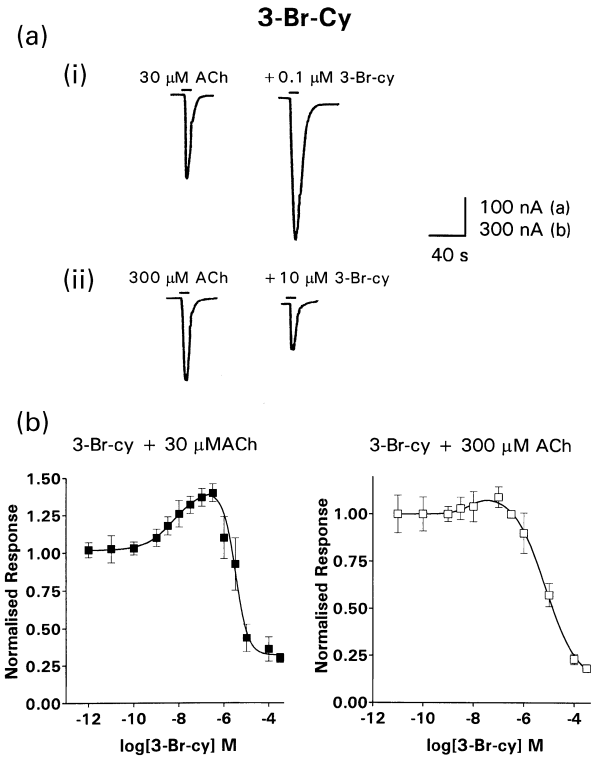

Fig. 8 Effects of 3-Br-cy on the ACh responses of hα4β2-nACh receptors. (a) Representative traces obtained from oocytes expressing hα4β2-nACh receptors and clamped at −60 mV. The traces show that at low ACh and 3-Br-cy concentrations 3-Br-cy potentiated the ACh responses, whilst at high ACh or 3-Br-cy the effect of 3-Br-cy was inhibitory. (b) DRC for the effects of 3-Br-cy on cationic currents evoked by 30 μM. A maximal potentiation of 40% was achieved with 0.32 μM of 3-Br-cy. (c) DRC for the effects of 3-Br-cy on cationic currents evoked by 300 μM. A maximal potentiation of 10% was achieved with 32 μM 3-Br-cy. The biphasic data were fitted as indicated in Materials and methods. Data points represent the mean ± SEM of 5–7 experiments. Where no error bars are seen, they are smaller than the symbols.

fitted to the sum of two independent Hill equations ($p < 0.05$ and $p < 0.001$, respectively). To our knowledge, this is the first time that a two-component DRC for the agonist effects of cy on hα4β4-nACh receptors has been reported. However, a dual DRC has been observed previously for the agonist effects of cy on rat α4β2-nACh receptors expressed on *Xenopus* oocytes (Papke and Heinemann 1994). The EC_{50} of the high- and low-affinity components estimated from the dual DRC for 3-Br-cy were in the low nanomolar and moderate nanomolar range, respectively. Cy was less potent than 3-Br-cy; the estimated EC_{50} values for the high- and low-affinity components were in the moderate nanomolar (3.6 nM) and low micromolar (1.1 μM) range, respectively. 3-Br-cy was less efficacious than cy on hα4β4-nACh receptors, a rank order that is in contrast to the one observed on hα7- and hα4β2-nACh receptors (see Figs 4 and 6, Table 2). The ratios of the high- and low-affinity components unmasked by cy and 3-Br-cy were not significantly different: cy, high-affinity component = 22 ± 7%; low-affinity component = 76 ± 9%; 3-Br-cy: high-affinity component = 30 ± 5%; low-affinity component = 70 ± 9%), which suggests that cy and 3-Br-cy unmasked the same DRC components on hα4β4-nACh receptors. In contrast to cy and 3-Br-cy, the agonist effects of 5-Br-cy and 3,5-diBr-cy on hα4β4-nACh receptors were monophasic, yielding DRC data that were best fitted using a single component approach ($p < 0.05$) (Fig. 5b and insert). These results indicate that neither 5-Br-cy nor 3,5-diBr-cy can reveal the high- and low-affinity components of the

agonist DRC of cy and 3-Br-cy on hα4β4-nACh receptors. 5-Br-cy was less efficacious than cy or 3-Br-cy at hα4β4-nACh receptors, but 3,5-diBr-cy was the least efficacious of the compounds tested at hα4β4-nACh receptors activating currents that were only about 20% of those activated by EC_{30} ACh concentrations.

Hα4β2-nACh receptors were not activated by 5-Br-cy or 3,5-diBr-cy, even at concentrations as high as 1 mM (Fig. 6). In contrast, cy and 3-Br-cy activated currents on oocytes expressing hα4β2-nACh receptors, albeit with low efficacy (Fig. 6b, Table 2), which is in agreement with previous studies of the effects of cy on hα4β2-nACh receptors (Chavez-Noriega *et al*. 1997). Because the low efficacy of cy and 3-Br-cy made it difficult to analyse reliably the effects of these agonists on hα4β2-nACh receptors, the responses evoked by cy or 3-Br-cy were normalized to the maximal response evoked by them. Figure 6(c) shows that when the responses of cy or 3-Br-cy were normalized to their respective maximal responses, the resulting DRC were clearly biphasic. As for the hα4β4-nACh receptors, the DRC for cy and 3-Br-cy were best fitted using a two independent sites approach ($p < 0.001$; in Fig. 6c dashed lines represent the fitting of the data to a single Hill equation). The ratios of the high- and low affinity components of the DRC for ACh (18 ± 3% and 82 ± 7%), cy (22 ± 8% and 78 ± 9%) and 3-Br-cy (19 ± 6% and 81 ± 8%) on hα4β2-nACh receptors were not significantly different from each other.

The results so far have shown that the rank orders of functional potency for cy and its bromo-isosteres are qualitatively comparable on hα7- and hα4β4-nACh receptors: 3-Br-cy >> cy ≈ 3,5-diBr-cy > 5-Br-cy. In the case of hα4β2-nACh receptors the rank order of potency is 3-Br-cy > cy. The rank order of efficacy relative to that of ACh changes on each receptor type as follows. Hα7-nACh receptor: ACh = 3-Br-cy = cy > 3,5DiBr-cy > 5-Br-cy. Hα4β2-nACh receptor: ACh >> 3-Br-cy > cy. Hα4β4-nACh receptor: ACh > cy > 3-Br-cy > 5-Br-cy > 3,5-diBr-cy.

Effects of cy and brominated analogues on ACh currents

In addition to its partial agonist effects, cy has been reported to inhibit in a competitive manner the ACh responses of rat α4β2-nACh receptors (Papke and Heinemann 1994). The results so far show that cy and its brominated isosteres display low efficacy at hα4β2-nACh receptors, in comparison to their efficacy at hα7- or hα4β4-nACh receptors. Therefore, the inhibitory effects of cy and the bromo-isosteres on currents elicited by ACh were investigated on oocytes expressing hα4β2-nACh receptors and the resulting DRC data were normalized to the responses elicited by ACh alone. Figure 7(a and b) show that cy had a biphasic effect on the responses elicited by 1 μM ACh. Cy concentrations in the range of 0.3–3 nM enhanced the responses elicited by 1 μM ACh (Fig. 7b, traces Fig. 7ai). A maximal potentiation of 25 ± 4% was induced by 1 nM cy, with an EC_{50} for potentiation of 0.24 ± 0.03 nM. At higher cy concentrations, the degree of potentiation was diminished until at concentrations higher than 5 nM, cy produced only inhibition of ACh responses. Inhibition of ACh responses had an IC_{50} of 8 ± 0.8 nM. Inhibition of ACh currents was also observed at concentrations of cy lower than 0.3 nM (Fig. 7b). In order to determine whether potentiation by cy is competitive or non-competitive, the effect of cy was also examined on ion currents elicited by 30 μM ACh. The effects of cy on the responses evoked by 30 μM ACh were only inhibitory and yielded monophasic DRC (Fig. 7b and traces in Fig. 7aii). Inhibition of responses mediated by 30 μM ACh by cy had an IC_{50} of 0.3 ± 0.02 μM. These results then indicate that the potentiation of ACh responses by cy is competitive.

The effects of 3-Br-cy on 30 μM ACh responses were biphasic consisting of a potentiating effect at concentrations ranging from 1 nM to 2 μM (a maximum potentiation 40 ± 7.6% was achieved with 0.32 μM 3-Br-cy) followed by an inhibitory phase (Fig. 8b, traces in Fig. 8ai,ii). Potentiation of hα4β2 nACh receptors by 3-Br-cy had an EC_{50} of 3.8 ± 0.3 nM whilst inhibition had an IC_{50} of 3.4 ± 0.8 μM. The potentiating effect of 3-Br-cy was markedly decreased in the presence of 300 μM ACh (Fig. 8c). A maximal potentiation of 10 ± 1% was achieved with 32 nM 3-Br-cy, with an EC_{50} of 8 ± 0.9 nM. Concentrations of 3-Br-cy higher than 0.32 μM caused only inhibition of the responses evoked by 300 μM; inhibition had an IC_{50} of 7.3 ± 0.8 μM.

3,5-DiBr-cy, which did not elicit responses in oocytes expressing hα4β2-nACh receptors, even at concentrations as high as 1 mM, was a competitive inhibitor of ACh responses (Fig. 9a). 3,5-DiBr-cy produced concentration-dependent inhibition of 1 μM ACh with an IC_{50} of 0.44 ± 0.02 μM. Current responses elicited by 30 μM ACh were also fully inhibited by 3,5-diBr-cys (IC_{50} 16 ± 2 μM) and the DRC produced was shifted to the right in comparison to that obtained in the presence of 1 μM ACh. Both DRC were equally well fitted to a single Hill equation. Overall, these results indicate that 3,5-diBr-cy is a competitive inhibitor of hα4β2-nACh receptors.

5-Br-cy which, like 3,5-diBr-cy, did not induce activation of hα4β2-nACh receptors, potentiated the responses evoked by 30 μM ACh at concentrations ranging from 0.1 μM to 0.3 mM. A maximal potentiation of 116 ± 7% was induced by 100 μM 5-Br-cy, with an EC_{50} of 8.6 ± 0.7 μM (Fig. 9c, traces in Fig. 9b). Potentiation was reversed at concentrations higher than 0.1 mM and at concentrations higher than 1 mM the ACh responses were inhibited (IC_{50} 2.1 ± 0.5 mM). As shown in Fig. 9(c), the potentiating effect of 5-Br-cy was surmounted in the presence of 300 μM ACh. 5-Br-cy inhibited the currents elicited by 300 μM ACh

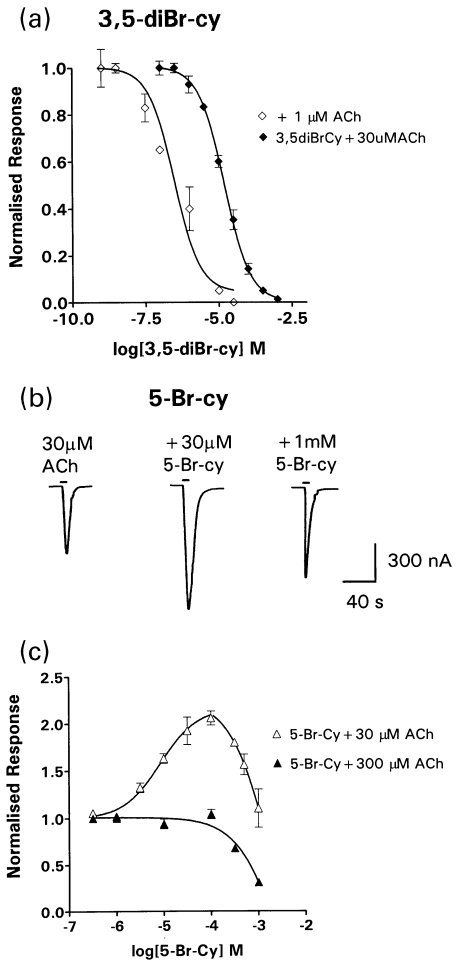

Fig. 9 Effects of ACh concentration on the action of 3,5-diBr-cy and 5-Br-cy on hα4β2-nACh receptors. (a) 3,5-diBr-cy inhibited ACh responses activated by either 30 μM or 1 μM ACh. The data were fitted to a single Hill equation. (b) 5-Br-cy both enhanced and inhibited ACh responses. The data were normalized to control responses evoked by ACh alone and the resulting DRC were bell shaped consisting of a potentiation phase followed by an inhibitory phase (c). The data were fitted as indicated in Materials and methods. At 300 μM ACh, the effects of 5-Br-cys were inhibitory only, producing monophasic DRC that were fitted well to a single Hill equation.

with an IC_{50} of 2.6 ± 0.4 mM, which is not significantly different from the IC_{50} value obtained with 30 μM ACh. The results then show that 5-Br-cy enhanced the ACh responses of hα4β2-nACh receptors in a competitive manner, but that inhibition was not affected by the concentration of ACh.

Discussion

The primary finding of this study is that bromination of C′3, C′5 or both of the pyridone ring of cy, alters both the potency and efficacy of its agonist effects. 3-Br-cy was the most potent agonist evaluated, increasing potency by about tenfold in hα4β2-, 40-fold in hα7- and more than 100-fold in hα4β4-nACh receptors. In contrast, 5-Br-cy was the least potent of the cy bromo-isosteres tested. However, bromination of C′5 of the cy pyridone ring did not affect potency to the same extent as bromination of C′3: 5-Br-cy was only around sixfold less potent than cy on the hα4β4-nACh receptor and twofold less potent on hα7-nACh receptors. 3,5-Br-cy was slightly more potent than 5-Br-cy, which suggests that the low potency of 5-Br-cy may reflect molecular mechanisms different from those influencing the potency of 3-Br-cy. In ligand binding assays for [α-^{125}I]BgTx or [^{3}H]cy binding sites the rank order of potency of cy and its bromo-isosteres was similar to that determined for the agonist effects. Comparison of EC_{50} values shows the former to be generally five- to 20-fold, which reflects the tendency of agonists to bind preferentially the high-affinity desensitized conformation, thus preventing the quantitative comparison of equilibrium binding data with functional estimates of agonist potency (Grady *et al.* 1992). Bromination of the cy skeleton did not change the ability of these compounds to fully displace [α-^{125}I]BgTx binding to hα7-nACh receptors or [^{3}H]cy binding to hα4β2- and hα4β4-nACh receptors or the unitary slope of the DRCs. Assuming that the biphasic agonist DRC of cy and 3-Br-cy on hα4β2- and hα4β4-nACh receptors results from the presence of two distinct populations of receptors, the results imply that desensitized states of the high- and low-affinity receptor populations of hα4β2- and hα4β4-nACh receptors have the same affinity for cy and the bromo-isosteres.

Cy and its bromo-isosteres displayed distinct efficacies at hα7-, hα4β2- and hα4β4-nACh receptors, which confirms the importance of the α and β subunits in determining the overall pharmacology of neuronal nACh receptors (Luetje and Patrick 1991; Figl *et al.* 1992). Thus, for example, 5-Br-cy and 3,5-diBr-cy did not evoke currents in hα4β2-nACh receptors, but activated currents on hα4β4- and hα7-nACh receptors. 3-Br-cy was more efficacious at the hα4β4-nACh receptor than at the hα4β2-nACh receptor, but behaved as a full agonist at the hα7 nAChR. 5-Br-cy was the least efficacious agonist at the hα7-nACh receptor, but at the hα4β4-nACh receptor was more efficacious than 3,5-diBr-cy. The bromo-isosteres of cy were more potent and more efficacious agonists at hα4β4-nACh receptors than at hα4β2-nACh receptors, mirroring the pattern of functional agonist potency and efficacy of cy on these nACh receptors (Luetje and Patrick 1991; Chavez-Noriega *et al.* 1997; Parker *et al.* 1998; this study).

How might bromination of C′3 of the pyridone ring of cy bring about an increase in potency? Bromination of cy would be expected to increase its lipophilicity, but knowledge of the nACh receptor pharmacophores is too scanty (Glennon and Dukat 1998) to speculate extensively regarding the effects of cy halogenation on its pharmacology. The

best known pharmacophore for agonists of nACh receptors comprises a cationic centre (a positive nitrogen) that binds the agonist site by coulombic forces and an electronegative centre (e.g. the carbonyl oxygen of ACh) which is presumed to engage in hydrogen bonding (Sheridan *et al*. 1986). The importance of the cationic centre has long been recognized as crucial for the potency of nicotinic agonists, but recent studies have shown that the hydrogen bonding centre also contributes to the potency and efficacy of nicotinic agonists. For example, as the demonstration that epibatidine, which contains a 2-chloro-5-aminoalkyl-substituted pyridine moiety, is a potent nicotinic agonist, a large number of 2(6)-substituted nicotine, azetidinylmethoxypyridine, and related substances have been synthesized and assayed at nACh receptor preparations, and the presence of halogen substituents at this position appears to generally increase the functional binding potency by several orders of magnitude (Holladay *et al*. 1998; Dukat *et al*. 1999). It may be then that halogenation of atoms adjacent to the hydrogen bonding centre of nicotinic agonists generally enhances potency. This is supported by the observation that in 3-Br-cy the Br atom is placed at a position that appears to be congruent with the position of the halogen in epibatidine and in the more potent substituted nicotine derivatives and azetidinyl-methoxypyridines. Interestingly, as shown by our studies and those of 5-substituted nicotine analogues (Rondahl 1977), halogenation of positions farther away from the hydrogen bonding centre produces a decrease in potency. The molecular basis of the effect of halogenation of atoms adjacent to the hydrogen bonding centre of nicotinic agonists is not apparent. Although halogenation of the rigid pyridone ring next to the carbonyl group might be expected to hinder hydrogen bonding by virtue of the bulk of the halogen atom, unlike the situation with flexible anatoxin analogues (Wonnacott *et al*. 1991), the electronic effect of bromine on the pyridone ring and more specifically on the hydrogen bond-accepting oxygen atom, might favour this interaction. On the other hand, it is conceivable that the neuronal nACh receptors used in this study may have a hydrophobic pocket near the hydrogen bond donor moiety, which might be able to accommodate a bromine atom, thus increasing affinity for the appropriately substituted compounds.

ACh, cy and 3-Br-cy produced biphasic agonist DRCs on hα4β2-nACh receptors consisting of high- and low-affinity components. In addition, cy and 3-Br-cy also produced agonist DRCs on hα4β4-nACh receptors. The results are therefore in agreement with previous reports showing that ACh produces biphasic DRC on human (Buisson and Bertrand 2001) and rat (Zwart and Vijverberg 1998; Buisson *et al*. 2000; Covernton and Connolly 2000) α4β2-nACh recombinant receptors. ACh, which is several orders of magnitude less potent than cy and 3-Br-cy on hα4β4-nACh receptors, did not differentiate the high- and low-affinity components of this receptor type. This finding provides further evidence that subunit composition determines the pharmacology of neuronal nACh receptors and suggests that potent agonists are more likely to produce biphasic DRC on α4β2- and α4β4-nACh receptors than agonists of modest or low potency. Thus, both 5-Br-cy and 3,5-diBr-cy, which were less potent than ACh on hα4β4-nACh receptors, produce monophasic DRC on hα4β4-nACh receptors. Interestingly, regardless of the preparation, the ratios of the high- and low-affinity components are similar (20–30% high-affinity component, 70–80% low-affinity component; Zwart and Vijverberg 1998; Buisson *et al*. 2000; Covernton and Connolly 2000; Buisson and Bertrand 2001; this study). The implication of this is that the ratios of the high- and low-affinity components do not occur in a random fashion, but are fixed by mechanisms as yet unknown.

How might dual DRC arise? One possibility, suggested by Zwart and Vijverberg (1998) is that other stoichiometries besides the 2α4 : 3β2 (Anand *et al*. 1991; Cooper *et al*. 1991) may be synthesized. Injection of different ratios of α4 and β2 rat cDNA subunits into *Xenopus* oocytes results in the synthesis of distinct neuronal nACh receptors each characterized by a specific agonist and antagonist profile (Zwart and Vijverger 1998), and presumably, a different stoichiometric arrangement of the α4 and β2 subunits. We have shown in this study that the two populations of the hα4β2- and hα4β4-nACh receptors express distinct sensitivities towards ACh, cy and its bromo-isosteres, which supports the idea that the observed biphasic DRC result from the presence of two different receptor populations. The observation that α7-nACh receptors, which are homomeric, produce agonist monophasic DRC (Chavez-Noriega *et al*. 1997; Covernton and Connolly 2000; this study) supports the idea that dual agonist DRC found in this study reflect the coexistence of two different hα4β2- or hα4β4-nACh receptor populations. An alternative possibility is that a receptor with a single stoichiometry may exist in two independent conformations. However, the existence of two independent and stable conformations of a single receptor stoichiometry would require the stabilization of those conformations by, for example, post-translational modifications of the proteins; to our knowledge there is no evidence of post-translational modifications to neuronal nACh receptors expressed in *Xenopus* oocytes. Another possibility is that some agonists may have differential affinities for the various allosteric conformations of the hα4β2- and hα4β4-nACh receptors and be able to stabilise some of these conformations to the extend of producing dual DRC. Such a possibility would be consistent with the idea that nACh receptors are allosteric proteins that may exist under several discrete and interconvertible conformational states (Monod *et al*. 1965).

The results of the functional and [α-^{125}I]BgTx and [^{3}H]cy binding studies results confirm the competitive nature of the

effects of cy on neuronal nACh receptors (Luetje and Patrick 1991; Papke and Heinemann 1994) and demonstrate that all three cy bromo-isosteres are also competitive ligands of neuronal nACh receptors. Changes in the functional efficacy of cy revealed functional effects on the ACh responses of hα4β2-nACh receptors, which ranged from pure competitive antagonism (3,5-diBr-cy) to competitive potentiation with (cy and 3-Br-cy) or without (5-Br-cy) agonism. We concluded that the potentiating effects of cy, 3-Br-cy and 5-Br-cy are competitive based on the surmountability of the effects by elevating the concentration of ACh. Competitive enhancement of the ACh responses of hα4β2-nACh receptors by cy, 3-Br-cy and 5-Br-cy was comparable to the effects of a diverse range of molecules, such as choline, atropine and tubocurarine, on the ACh responses of β2- or β4-containing neuronal nACh receptors (Cachelin and Rust 1994; Zwart and Vijverberg 1997, 2000). Competitive potentiation of α4β2- and α4β4-nACh receptors by the acetylcholinesterase inhibitors tacrine and physostigmine has also been reported (Zwart *et al*. 2000), although there is evidence that these inhibitors may be positive allosteric modulators of α4β2-nACh receptors (e.g. Pereira *et al*. 1994).

In summary, we show here that bromination of C′3 of the pyridone ring of cy significantly increases the binding and functional potency of cy on hα4β2-, hα4β4- and hα7-nACh receptors, whilst bromination of C′5, or C′3 and C′5 causes the opposite effect. Moreover, we show that the increased potency achieved by C′3-bromination allows the ligand to discriminate between two distinct receptor populations on both hα4β2-, and, for the first time, hα4β4-nACh receptors. The functional effects of cy and its bromo-isosteres are largely determined by their agonist efficacy at each nACh receptor. This, in turn, is determined by factors not investigated here, such as the interactions with the amino-acids that result in conformational changes leading to the opening of the ion channel.

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