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Cholinergic drugs potentiate human nicotinic α4β2 acetylcholine receptors by a competitive mechanism

Chantal J.G.M. Smulders[a,1,2], Ruud Zwart[a,1,3], Isabel Bermudez[b], Regina G.D.M. van Kleef[a], Paul J. Groot-Kormelink[c,4], Henk P.M. Vijverberg[a,*]

[a] *Institute for Risk Assessment Sciences, Utrecht University, P.O. Box 80176, NL-3508 TD Utrecht, The Netherlands*
[b] *Department of Biological and Molecular Sciences, Oxford Brookes University, Gipsy Lane, Oxford OX3 OBP, United Kingdom*
[c] *Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium*

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Abstract

Effects of cholinergic drugs on human α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes have been investigated in electrophysiological and ligand binding experiments. Atropine, scopolamine, physostigmine, and tacrine combine potentiation of ion current induced by low concentrations of acetylcholine with inhibition of ion current evoked by high concentrations of acetylcholine. Rivastigmine, galanthamine, and dichlorvos cause only inhibition of ion current evoked by low concentrations of acetylcholine. Binding experiments show that the potentiating cholinergic drugs atropine, scopolamine, and physostigmine are competitive ligands of human α4β2 nicotinic acetylcholine receptors. Conversely, the inhibitory cholinergic drugs galanthamine and rivastigmine are non-competitive. The non-competitive drugs are not allosteric, since they do not affect the saturation curve of the radioligand [^{3}H]cytisine. Effects of potentiating cholinergic drugs on nicotinic acetylcholine receptors are consistent with and predicted by a model comprising competitive drug effects at two equivalent agonist recognition sites on the nicotinic acetylcholine receptor combined with non-competitive ion channel block.

Keywords: Human nicotinic acetylcholine receptor; Galanthamine; Scopolamine; Tacrine; *Xenopus laevis* oocyte; Two-microelectrode voltage clamp

1. Introduction

The potential role of neuronal nicotinic acetylcholine receptors in various neurological disorders has prompted attention for the development and use of nicotinic drugs as therapeutic agents (for reviews, see Gotti et al., 1997; Lloyd and Williams, 2000). It appears that α4β2 receptors, which constitute the predominant subtype of nicotinic acetylcholine receptor in the central nerve system (Flores et al., 1992), are involved in behavioral functions as nicotine dependence, avoidance learning, and antinociception (Cordero-Erausquin et al., 2000). Nicotinic α4 receptor subunits are selectively reduced in number in Alzheimer brains (Warpman and Nordberg, 1995; Martin-Ruiz et al., 1999). In strategies to reinforce nicotinic neurotransmission, the action of endogenous acetylcholine is enhanced by administration of inhibitors of acetylcholinesterase, the acetylcholine-degrading enzyme (Giacobini, 1998).

Research into function and pharmacology of human α4β2 nicotinic acetylcholine receptors has been fostered by the availability of systems for the selective expression of specific subtypes of nicotinic acetylcholine receptors, such as *Xenopus laevis* oocytes (Gopalakrishnan et al., 1996; Chavez-Noriega et al., 1997), human embryonic kidney

* Corresponding author. Tel.: +31 30 253 5397; fax: +31 30 253 5077.
E-mail address: h.vijverberg@iras.uu.nl (H.P.M. Vijverberg).
[1] These authors contributed equally to this paper.
[2] Present address: Shell Health Services-Shell International BV, P.O. Box 162, 2501 AN The Hague, The Netherlands.
[3] Present address: Eli Lilly and Company, Lilly Research Centre, Sunninghill Road, Windlesham GU20 6PH, United Kingdom.
[4] Present address: Department of Pharmacology, University College London, London WC1E 6BT, United Kingdom.

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(HEK) cells (Gopalakrishnan et al., 1996; Buisson et al., 1996; Chavez-Noriega et al., 2000), and, more recently, human epithelial SH-EP1 cells (Eaton et al., 2003). Agonists of the nicotinic acetylcholine receptor interact with the agonist recognition sites of these receptors to induce conformational changes in the membrane protein, which lead to ion channel opening and receptor desensitization. Like muscle nicotinic acetylcholine receptors, heteromeric neuronal nicotinic acetylcholine receptors are supposed to contain two agonist binding sites located at the α/β subunit interfaces (for review, see Karlin, 2002). Historically, cholinergic pharmacology distinguishes nicotinic drugs, e.g., the nonselective nicotinic receptor antagonist D-tubocurarine, muscarinic drugs, e.g., the nonselective muscarinic receptor antagonist atropine, and acetylcholinesterase inhibitors, e.g., physostigmine. However, nicotinic and muscarinic drugs and acetylcholinesterase inhibitors show mixed effects and cross-react with different members of the distinct families of cholinergic receptors. For example, D-tubocurarine is a mixed competitive and non-competitive nicotinic receptor antagonist and, under the specific condition that low concentrations of D-tubocurarine are combined with low concentrations of acetylcholine, also acts as a coagonist to potentiate acetylcholine responses (Cachelin and Rust, 1994). The muscarinic receptor antagonist atropine causes mixed coagonist and antagonist effects on rat neuronal nicotinic receptors (Zwart and Vijverberg, 1997). Effects resembling those of D-tubocurarine and atropine on rat nicotinic acetylcholine receptors have been described for several acetylcholinesterase inhibitors, e.g., for galanthamine (Schrattenholz et al., 1996; Samochocki et al., 2003), neostigmine (Nagata et al., 1997), and physostigmine (Zwart et al., 2000). Despite the similar coagonist and antagonist effects of these diverse cholinergic drugs on nicotinic acetylcholine receptors, the mechanism of potentiation has not been agreed upon yet, not in the least because of the large variability in potentiation and inhibition reported and in the nicotinic acetylcholine receptor subtypes involved.

Although some drugs, e.g., galanthamine and physostigmine, have been suggested to interact with an allosteric site located in the vicinity of the agonist recognition site on the extracellular domain of the nicotinic receptor α subunit (Schrattenholz et al., 1996; Costa et al., 2003), the ligand binding properties and the specificity of such an allosteric site remain to be established. Conversely, D-tubocurarine as well as galanthamine, physostigmine and tacrine, i.e., drugs with similar potentiating and inhibitory action on nicotinic acetylcholine receptors, appear to be competitive ligands at the agonist recognition sites of the receptor (Svensson and Nordberg, 1997; Zwart et al., 2000). In addition, inhibition by high concentrations of cholinergic receptor agonists and antagonists is generally recognized to be non-competitive and is caused by ion channel block (Colquhoun et al., 1979; Shaw et al., 1985; Zwart and Vijverberg, 1997). Here, it is anticipated that the specific features of potentiating and inhibitory effects on nicotinic acetylcholine receptors can be accounted for by competitive and non-competitive mechanisms common to a wide class of cholinergic drugs. In order to test this hypothesis, we have expressed human α4β2 nicotinic acetylcholine receptors in *Xenopus* oocytes and studied the functional effects and receptor binding properties of various cholinergic drugs. The results demonstrate that competitive and non-competitive, but not allosteric interactions account for the effects of the cholinergic drugs tested on the human α4β2 nicotinic acetylcholine receptors.

2. Materials and methods

2.1. Animals

All experiments were conducted with the approval of the Utrecht University Ethical Committee, and in accordance with Dutch law.

Adult female specimen of *X. laevis* frogs were obtained from AmRep (Breda, The Netherlands) and kept in standard aquaria (0.5×0.4×1 m; 1–15 per aquarium) with a normal 12 h light/dark cycle (lights on at 7:00 a.m. and lights off at 7:00 p.m.). The water in the aquaria (copper-free, pH 7, 1.25 mmol CaO/l, 24 °C) was continuously refreshed at a rate of ~1000 l/day. The animals were fed earthworms three times a week (Hagens, Nijkerkerveen, The Netherlands).

2.2. Receptor expression and receptor binding

X. laevis were anaesthetized by submersion in 0.1% MS-222 and ovarian lobes were surgically removed. Oocytes were defolliculated manually after treatment with 1.5 mg/ml collagenase type I in Ca^{2+}-free Barth's solution for 1.5 h at room temperature. Plasmids coding for the human α4 and β2 subunits of neuronal nicotinic acetylcholine receptors, dissolved in distilled water at a 1:1 molar ratio, were co-injected into the nuclei of stage V and stage VI oocytes within 8 h after harvesting, using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA, USA). Approximately 0.2 ng of each plasmid containing α4 or β2 cDNA was injected in a total injection volume of 18.4 nl/oocyte. After injection oocytes were incubated at 19 °C in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 50 μg/ml neomycin (pH 7.6 with NaOH). Experiments were performed on oocytes after 3–6 days of incubation (Zwart and Vijverberg, 1997).

For ligand binding experiments, *X. laevis* oocytes (100–150 oocytes per assay) expressing human α4β2 nicotinic acetylcholine receptors 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 for [^{3}H]cytisine (Perkin Elmer Life Sciences, Boston, MA, USA) contained (in mM) 120 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 50 Tris, pH 7.0. [^{3}H]cytisine binding was carried out as described previously (Houlihan et al., 2001). Non-specific binding was measured in the presence of 10 μM nicotine (Sigma, St. Louis, MO, USA). The saturation binding of [^{3}H]cytisine was measured in the absence and presence of 10 μM atropine, 10 μM physostigmine, 30 μM scopolamine, 300 μM rivastigmine, and 300 μM galanthamine. Competition experiments were carried out at a [^{3}H]cytisine concentration of 1 nM.

2.3. Electrophysiology and data acquisition

Oocytes were voltage clamped using two microelectrodes (0.5–2.5 MΩ) filled with 3 M KCl and a custom-built voltage clamp amplifier with high-voltage output stage according to the methods described by Stühmer (1998). The external saline was clamped at ground potential by means of a virtual ground circuit employing an Ag/AgCl reference electrode and a Pt/Pt-black current-passing electrode. Membrane current was measured with a current-to-voltage converter incorporated in the virtual ground circuit. The membrane potential was held at −40 mV. All experiments were performed at room temperature (22–24 °C).

Oocytes were placed in a silicon tube with an inner diameter of 3 mm, which was continuously perfused with saline solution (115 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.2 with NaOH). The superfusion rate was approximately 20 ml/min, resulting in a fluid velocity around the oocyte >47 mm/s. Aliquots of freshly thawed, concentrated stock solutions of acetylcholine chloride, atropine sulfate, scopolamine hydrobromide, physostigmine hemisulfate salt, tacrine hydrobromide, galanthamine hydrochloride, and rivastigmine hydrogen tartrate in water and metrifonate and dichlorvos in DMSO were added to the saline immediately before the experiments. Drugs were applied by switching the superfusate between control and drug-containing saline using a servomotor-operated valve. Drugs were either applied for 20 s during the 40 s acetylcholine application or coapplied with acetylcholine after 2 min wash-in. It was verified that the mode of application did not affect the drug effects quantitatively or qualitatively. Agonist applications were alternated by 5 min of superfusion with agonist-free saline to allow the receptors to recover from desensitization. Membrane currents were low-pass filtered (8-pole Bessel; −3 dB at 0.3 kHz), digitized (12 bits; 1024 samples/record), and stored on disk for computer analysis.

2.4. Chemicals

Acetylcholine chloride, physostigmine hemisulfate salt, scopolamine hydrobromide, dichlorvos (DDVP), metrifonate (trichlorfon), 3-aminobenzoic acid ethyl ester (MS-222), collagenase type I, dimethylsulfoxide (DMSO; ACS reagent), NaCl, and neomycin solution (10 mg neomycin/ml in 0.9% NaCl) were obtained from Sigma (St. Louis, MO, USA). $CaCl_2$ (1 M solution), $MgCl_2$ (1 M solution), $MgSO_4$, $NaHCO_3$, and NaOH were purchased from BDH Laboratory Supplies (Poole, England). $Ca(NO_3)_2$, HEPES, and KCl were from Merck (Darmstadt, Germany). Atropine sulfate was from Fluka (Buchs, Switzerland); galanthamine hydrochloride from Tocris Cookson (Bristol, UK); tacrine hydrochloride from RBI (Natick, MA, USA). Rivastigmine hydrogen tartrate ((*S*)-*N*-ethyl-*N*-methyl-3-[1-(dimethylamino)ethyl]-phenyl carbamate hydrogen-(2*R*,3*R*)-tartrate; SDZ ENA 713 hta) was kindly provided by Novartis Pharma AG Research (Basel, Switzerland). Stock solutions of acetylcholine chloride, atropine sulfate, scopolamine hydrobromide, physostigmine hemisulfate salt, tacrine hydrobromide, galanthamine hydrochloride, and rivastigmine hydrogen tartrate were prepared in distilled water and stock solutions of metrifonate and dichlorvos in DMSO.

The cDNAs encoding the human nicotinic acetylcholine receptor subunits ligated into the pcDNA3 plasmid vector (Groot-Kormelink and Luyten, 1997) were a kind gift from Janssen Pharmaceutica N.V. (Beerse, Belgium).

2.5. Data analysis, modeling, and statistics

Amplitudes of ion currents were measured and normalized to the amplitude of acetylcholine-induced control responses (100%) to adjust for differences in receptor expression levels among oocytes and for small variations in response amplitude over time. The percentage of inhibition or potentiation of the acetylcholine-induced ion current by the drugs was calculated from the quotient of the amplitude of the response after 20 s coapplication of the drug and that of the control response at the same time point. Concentration–effect curves were fitted to the data obtained in separate experiments and mean±S.D. of estimated parameters were calculated for *n* oocytes. Standard activation and inhibition curves were fitted according to the Hill equation. The apparent affinity of acetylcholine to activate α4β2 nicotinic acetylcholine receptors was estimated by fitting an equilibrium model for two-site receptor occupation to the agonist concentration–effect data according to the equation:

$$i/i_{\max} = \{cA/(1+cA)\}^2 \quad (1)$$

The hypothetical two-site model (Fig. 1), applied to the equilibrium effects of acetylcholine and a drug exerting competitive and non-competitive effects, was originally developed for D-tubocurarine (Cachelin and Rust, 1994) and was expanded for atropine (Zwart and Vijverberg, 1997) to:

$$i_{A,B}/i_{A,0} = \{1+2*f*cB/cA\}*\{(1+cA)/(1+cA+cB)\}^2 *\{1/(1+[B]/K_{block})\} \quad (2)$$

In Eqs. (1) and (2), cA and cB are the concentrations of acetylcholine (A) and of the drug (B) divided by their K_d

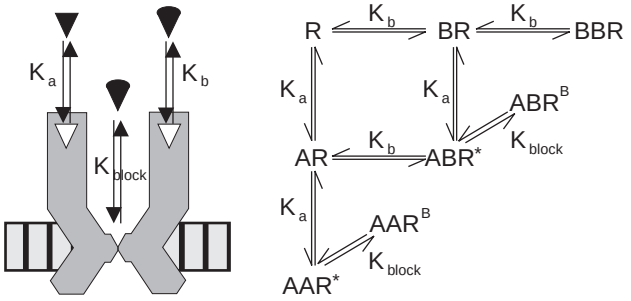

Fig. 1. Two-site model for the interaction of cholinergic drugs with the nicotinic acetylcholine receptor. The model assumes that the response is proportional to the degree of simultaneous occupation of two identical ligand binding sites (open triangles) by acetylcholine (closed triangle; affinity K_a). The occupation of ligand binding sites by other cholinergic drugs (cone shape; affinity K_b) may contribute to the agonist effect of acetylcholine (coagonists) or block the effect of acetylcholine (antagonists). In addition, the cholinergic drugs may show affinity (K_{block}) for ion channel block. When acetylcholine is not used at very high concentrations, ion channel block by acetylcholine can be neglected. The two-site model includes six different states of receptor occupation in the presence of two drugs with affinity for the ligand binding site and two additional receptor states in case of open ion channel block as indicated by the scheme.

values, which are denoted K_a and K_b, respectively. In Eq. (2), $i_{A,B}/i_{A,0}$ represents the current amplitude in the presence of acetylcholine and the drug relative to the control amplitude in the presence of acetylcholine alone and the term $2*f*cB/cA$ represents the contribution of receptors occupied simultaneously by one acetylcholine and one drug molecule to the response (see Fig. 1). The factor f accounts for the difference in response evoked by two acetylcholine molecules (f=1) and by the combination of one acetylcholine and one drug molecule occupying the two binding sites on the nicotinic acetylcholine receptor. Ion channel block by the drug, resulting in additional inhibition with an apparent affinity of K_{block}, is also accounted for in Eq. (2). The basic assumptions of the model are: (1) The response amplitude is proportional to agonist site occupation. (2) Each acetylcholine receptor contains two identical agonist binding sites. (3) Both agonist binding sites need to be occupied to produce a response (the contribution of receptors with no or one site occupied to the response is negligible). Curve fitting was performed using GraphPad Prism software (Version 3.00 for Windows; GraphPad Software, San Diego, USA) or Sigmaplot for Windows (Version 5.0; SPSS, Richmond, USA). Data are expressed as mean±S.D. of n oocytes. The statistical significance of concentration-dependent effects of drugs and acetylcholine was assessed by analysis of variance (ANOVA) with a post hoc Bonferroni t-test where applicable.

Radioligand displacement data were fitted by the Hill equation:

$$i/i_{max} = 1/(1 + [X/IC_{50}]^n) \quad (3)$$

where n is the Hill slope and IC_{50} is the concentration of unlabelled ligand that causes 50% inhibition of specific radioligand binding. The binding parameters (K_d and B_{max}) of [^{3}H]cytisine were determined from saturation binding data using the equation:

$$Y = B_{max}/(1 + K_d/X) \quad (4)$$

wherein B_{max}=maximal binding, K_d=apparent equilibrium dissociation binding constant, X=concentration of ligand, and Y=binding. K_d and B_{max} values are expressed as arithmetic mean±S.E.M. Results are derived from at least three independent experiments carried out using membrane homogenates prepared from different batches of *Xenopus* oocytes. Where appropriate the Student's t-test for unpaired data was used and values of $P<0.05$ were regarded as significant.

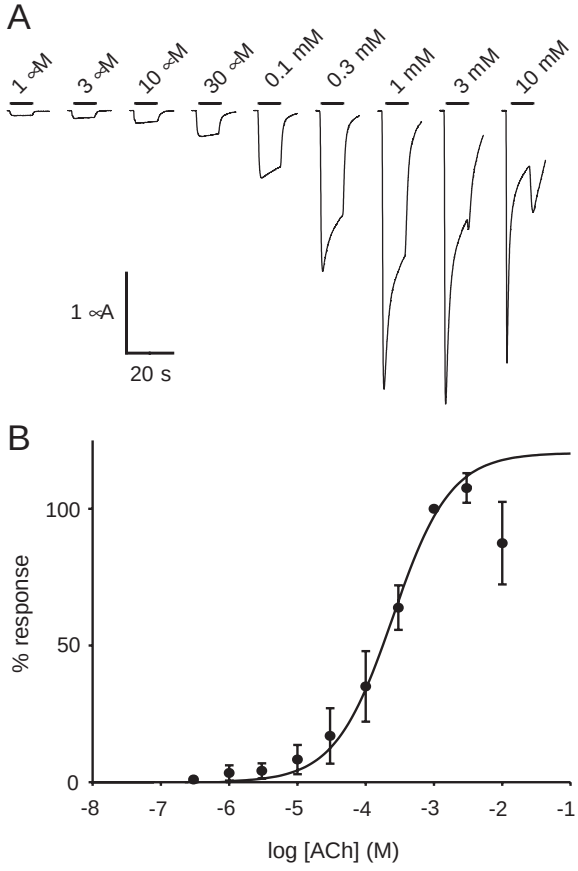

Fig. 2. Agonist sensitivity of human α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. (A) The amplitude of inward currents evoked by superfusion of the oocytes with external solution containing acetylcholine, as indicated by the bars on top of the current traces, depends on acetylcholine concentration. Ion channel block by acetylcholine (>1 mM) is indicated by the strong acceleration of the decay of inward current and by the rebound tail current on removal of acetylcholine. (B) Concentration–effect curve of acetylcholine. Data are mean±S.D. of 5 oocytes. The fitted line is a Hill curve with the mean values of the parameters obtained from the five separate experiments (EC_{50}=248 μM; Hill slope=1.03; E_{max}=121%). The data obtained at 10 mM acetylcholine were not included in curve fitting.

3. Results

3.1. Agonist effects

Superfusion of voltage clamped oocytes expressing human α4β2 nicotinic acetylcholine receptors with external solution containing the agonist acetylcholine resulted in ion currents with amplitudes that depended on acetylcholine concentration. The inward currents decayed slowly, except at acetylcholine concentrations >1 mM, which caused an accelerated decay and a rebound tail current on removal of the agonist. This is indicative of ion channel block at high concentrations of acetylcholine (Fig. 2A). Peak amplitudes of the acetylcholine-induced ion currents were measured, normalized to the response obtained with 1 mM acetylcholine, and plotted against acetylcholine concentration in each experiment for fitting a concentration–effect curve according to the Hill equation. The datapoint obtained at the highest concentration of 10 mM acetylcholine, which caused substantial block (see Fig. 2A), was omitted from the curve fit. The mean values for EC_{50}, E_{max}, and Hill slope of the fitted concentration–effect curves are 248±88 μM acetylcholine, 121±10%, and 1.03±0.31 (n=5), respectively. The calculated mean concentration–effect curve is drawn in Fig. 2B. In subsequent experiments, in which combined effects of acetylcholine and other cholinergic drugs were investigated, the concentration of acetylcholine did not exceed 1 mM to avoid ion channel block by acetylcholine.

Additional experiments were performed to investigate whether the various cholinergic drugs used in this study are able to activate the human nicotinic acetylcholine receptors. Oocytes expressing human α4β2 nicotinic acetylcholine receptors, which responded to superfusion with 1 mM acetylcholine with a large inward current, were also superfused with 10, 100, and 1000 μM of the muscarinic receptor antagonists atropine and scopolamine. No ion currents were observed upon superfusion with these drugs, demonstrating that these drugs by themselves are not agonists of α4β2 nicotinic acetylcholine receptors. In similar experiments, the cholinesterase inhibitors physostigmine, tacrine, rivastigmine, galanthamine, metrifonate, and dichlorvos also did not evoke detectable ion currents (results not shown). It is concluded that, unlike acetylcholine, all other cholinergic drugs tested are not agonists of the human α4β2 nicotinic acetylcholine receptors.

3.2. Potentiation and inhibition of acetylcholine-induced ion current

The muscarinic receptor antagonists atropine and scopolamine and the acetylcholinesterase inhibitors physostigmine and tacrine at concentrations between 1 and 100 μM enhanced ion currents evoked by the low concentration of 1 μM acetylcholine in oocytes expressing human α4β2 nicotinic acetylcholine receptors (Fig. 3). At this low acetylcholine concentration, a relatively high proportion of the receptors will have only one of the two agonist recognition sites occupied by acetylcholine. The same low concentrations of these drugs inhibited ion currents evoked by the high concentration of 1 mM acetylcholine (Fig. 3). At this high acetylcholine concentration, the majority of the agonist recognition sites is occupied by agonist molecules, resulting in a high proportion of receptors occupied by two molecules of acetylcholine. High concentrations of the cholinergic drugs inhibited the ligand-gated ion currents irrespective of the concentration acetylcholine used to evoke the responses.

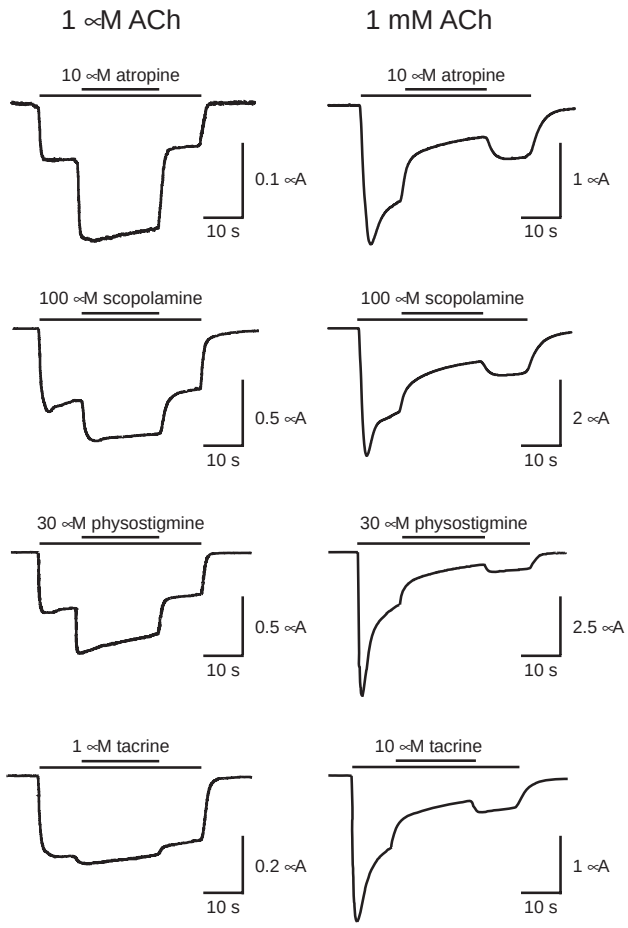

Fig. 3. Potentiation and inhibition of 1 μM and 1 mM acetylcholine-induced inward currents by atropine, scopolamine, physostigmine and tacrine. Drugs, at the concentrations indicated, were coapplied with acetylcholine for 20 s during the acetylcholine-induced ion current. For each of the drugs, the concentration chosen produced near maximum potentiation of the acetylcholine-induced ion current. Note that the concentration of tacrine at 1 mM acetylcholine is 10 times higher than that used to potentiate the 1 μM acetylcholine-induced ion current, since 1 μM tacrine caused little inhibition of the 1 mM acetylcholine-induced inward current.

The concentration–effect curves of atropine, scopolamine, physostigmine, and tacrine on 1 μM and on 1 mM acetylcholine-induced ion currents (Fig. 4) show that potentiation is observed only when low concentrations of the drugs are combined with low concentrations of acetylcholine. In addition, the left shift of the curves obtained at 1 mM acetylcholine indicates that the apparent

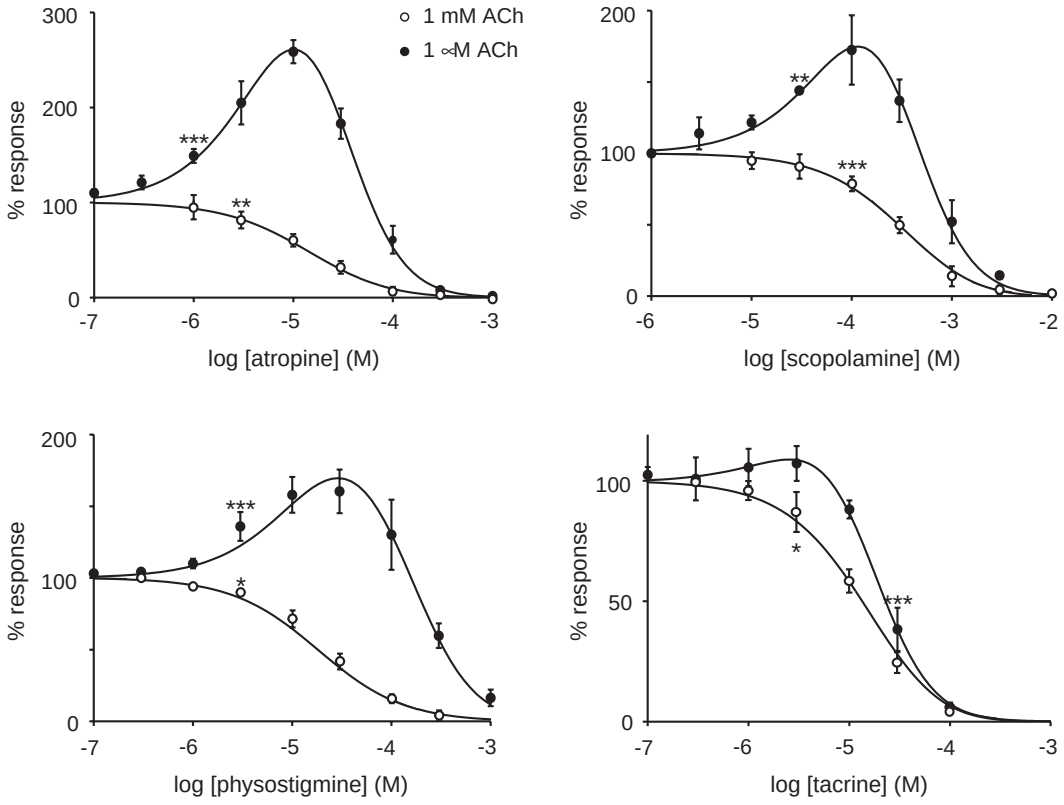

Fig. 4. Concentration dependence of the potentiating and inhibitory effects of atropine, scopolamine, physostigmine and tacrine on inward currents evoked by 1 μM acetylcholine and by 1 mM acetylcholine. Note that these drugs inhibit the 1 mM acetylcholine-induced ion current more potently than the 1 μM acetylcholine-induced ion current as indicated by the left shift of the concentration–effect curves at the higher agonist concentration. The lines drawn are the concentration–effect curves predicted by the two-site receptor occupation model, Eq. (2), fitted to the data obtained at high and low acetylcholine concentration simultaneously. All data are mean±S.D. of 3–4 oocytes. Asterisks indicate the lowest concentrations causing significant effects compared to control (P<0.05, 0.01, and 0.001 indicated by *, **, and ***, respectively). The potentiation by tacrine varied too much between oocytes to be significant.

potency of the inhibitory drugs is enhanced at the higher agonist concentration. Mean IC_{50} values and Hill slopes of inhibition curves fitted to the data obtained in replicate experiments with 1 mM acetylcholine are summarized in Table 1. A small potentiation by tacrine (Fig. 3) was observed in some oocytes. Overall, significant potentiation by tacrine could not be demonstrated (Fig. 4; n=3). Additional experiments, in which the acetylcholine concentration was lowered to 0.1 μM, also did not show significant potentiation by 3 μM tacrine (n=3). However, the inhibition curve of tacrine showed a significant shift of to the left when the agonist concentration was increased from 1 μM to 1 mM acetylcholine (two-way ANOVA $F_{1,5}$=41.28; P<0.001), similar to that of the potentiating drugs (Fig. 4).

The acetylcholinesterase inhibitors rivastigmine, dichlorvos, and galanthamine, applied at concentrations in the range of 0.1 μM–1 mM, inhibited ion currents evoked by 1 μM acetylcholine and by 1 mM acetylcholine (Fig. 5). Metrifonate hardly caused an effect when applied at concentrations in the range of 0.1 μM–0.1 mM. Coapplication of the solvent DMSO at concentrations up to 0.1% (v/v) with acetylcholine did not result in detectable effects. The

Table 1
Summary of the concentration-dependent effects of potentiating cholinergic drugs on human α4β2 nicotinic acetylcholine receptors

Compound	Hill inhibition curve[a], 1 mM acetylcholine		Two-site receptor model[b], 1 μM–1 mM acetylcholine			Receptor binding[c]
	IC_{50} (μM)	Hill slope	f	K_{block} (μM)	K_b (μM)	IC_{50} (μM)
Atropine	14.1±4.5	1.16±0.21	0.120	15	33	20±3
Scopolamine	278±56	1.27±0.37	0.050	399	308	298±24
Physostigmine	22.2±3.4	1.13±0.12	0.224	18	255	29±4
Tacrine	12.8±2.0	1.33±0.21	0.023	18	14	n.d.[d]

[a] Mean±S.D. of IC_{50} and Hill slope of 3–4 inhibition curves from responses induced by 1 mM acetylcholine in different oocytes.
[b] Parameter estimates from the equilibrium two-site model fitted to the data obtained at low and high concentrations of acetylcholine simultaneously.
[c] Mean±S.D. of IC_{50} of 3 displacement experiments performed in different batches of oocyte membranes.
[d] n.d.=not determined.

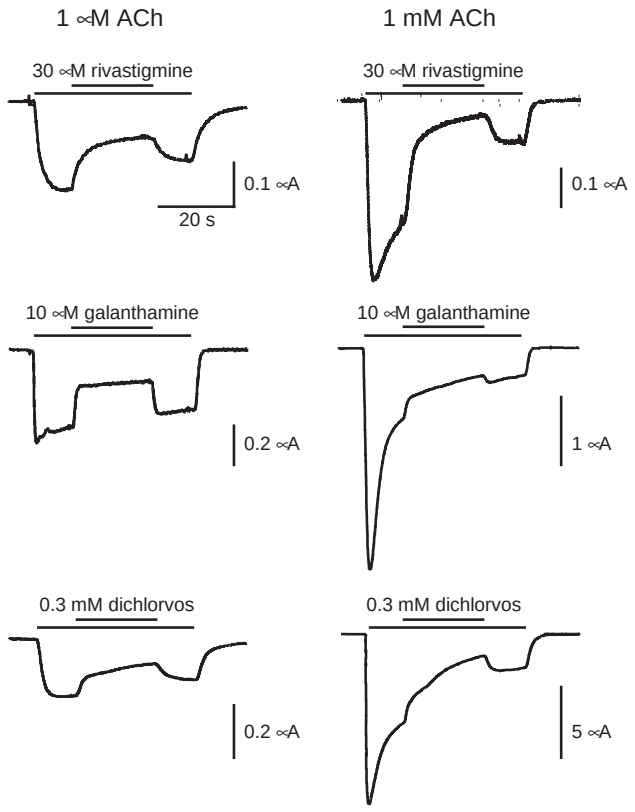

Fig. 5. Inhibition of acetylcholine-evoked inward currents in *Xenopus* oocytes expressing human α4β2 nicotinic acetylcholine receptors by rivastigmine, galanthamine, and dichlorvos. The traces show that 30 μM rivastigmine inhibits the 1 mM acetylcholine-induced ion current to a greater extent than the 1 μM acetylcholine-induced ion current, whereas the reverse is observed for inhibition of 1 μM and 1 mM acetylcholine-induced ion currents by 10 μM galanthamine. Dichlorvos inhibits 1 μM and 1 mM acetylcholine-induced ion currents to a similar extent. Each of the traces was obtained from a different oocyte.

concentration–effect curves of rivastigmine, dichlorvos, galanthamine, and metrifonate on 1 μM and on 1 mM acetylcholine-induced ion currents (Fig. 6) show the absence of potentiation at any of the combinations of a wide range of drug concentrations with a low and with a high concentration of acetylcholine. Mean IC_{50} values and Hill slopes of inhibition curves obtained from replicate experiments with 1 mM, 0.3 mM, and 1 μM acetylcholine are summarized in Table 2. Most notably, the results presented in Figs. 5 and 6 show that the effect of acetylcholine concentration on the position of the inhibition curves differs for the different drugs tested. Two-way ANOVA on the data, obtained from 3 to 4 replicate experiments on different oocytes for each drug and for each acetylcholine concentration, showed that an increased agonist concentration caused the inhibition curves to shift to the left for rivastigmine ($F_{1,6}$=25.96; P<0.001) and to the right for galanthamine ($F_{2,7}$=97.45; P<0.001). A significant shift was not observed for the inhibition curve of dichlorvos ($F_{1,9}$=1.36; P=0.25). In conventional pharmacology, the shift to the right of the inhibition curve of galanthamine and the absence of shift of the dichlorvos curve would be interpreted as competitive and non-competitive effects, respectively. However, the left shift observed with rivastigmine, as well as the left shift observed for the inhibitory effects of atropine, scopolamine, physostigmine, and tacrine (see Fig. 4), cannot possibly be explained on the basis of the same pharmacological conventions.

3.3. Potentiation and inhibition of cytisine- and nicotine-induced ion currents

The effects of potentiating and inhibitory drugs on ion currents evoked by the partial human α4β2 nicotinic acetylcholine receptor agonist cytisine and by the full human α4β2 nicotinic acetylcholine receptor agonist nicotine (Chavez-Noriega et al., 1997; Houlihan et al., 2001) were also investigated. The effects of the various drugs on ion currents evoked by these agonists were qualitatively similar to the effects on acetylcholine-evoked ion currents. Fig. 7 shows that 30 μM physostigmine potentiates the 0.3 μM nicotine-evoked ion current and inhibits the 10 μM nicotine-evoked ion current, whereas 10 μM galanthamine inhibits the ion current evoked by the high as well as by the low concentration of nicotine. The quantitative effects of these and other drugs on cytisine and nicotine-evoked ion currents (Table 3) show that potentiation and inhibition are systematically observed, irrespective of the type of agonist used.

3.4. Receptor binding

Ligand binding experiments have been performed to explicate the nature of the effects of cholinergic drugs on human α4β2 nicotinic acetylcholine receptors and to elucidate whether the cholinergic drugs affect the agonist affinity for the agonist binding site. The ability of the potentiating ligands atropine, physostigmine, and scopolamine and the inhibitory ligands galanthamine and rivastigmine to displace the radioligand [^{3}H]cytisine from the agonist recognition sites was determined on membranes of oocytes expressing human α4β2 nicotinic acetylcholine receptors (Fig. 8A). Neither galanthamine nor rivastigmine displaced [^{3}H]cytisine from its binding site. However, atropine, physostigmine, and scopolamine displace [^{3}H]cytisine with apparent affinities of 20±3 μM, 29±4 μM, and 298±24 μM, respectively. It was also investigated to which extent the drugs affected the saturation curve of [^{3}H]cytisine. Atropine and physostigmine at a concentration of 10 μM significantly (P<0.05, Student's t-test) decreased the apparent affinity of the radioligand for its binding site (Fig. 8B; Table 4). Similar, but smaller effects of 30 μM scopolamine were not statistically significant. None of the drugs tested caused a significant effect on B_{max}. Galanthamine and rivastigmine at a concentration of 300 μM showed no significant effect on the [^{3}H]cytisine saturation curve, indicating that these

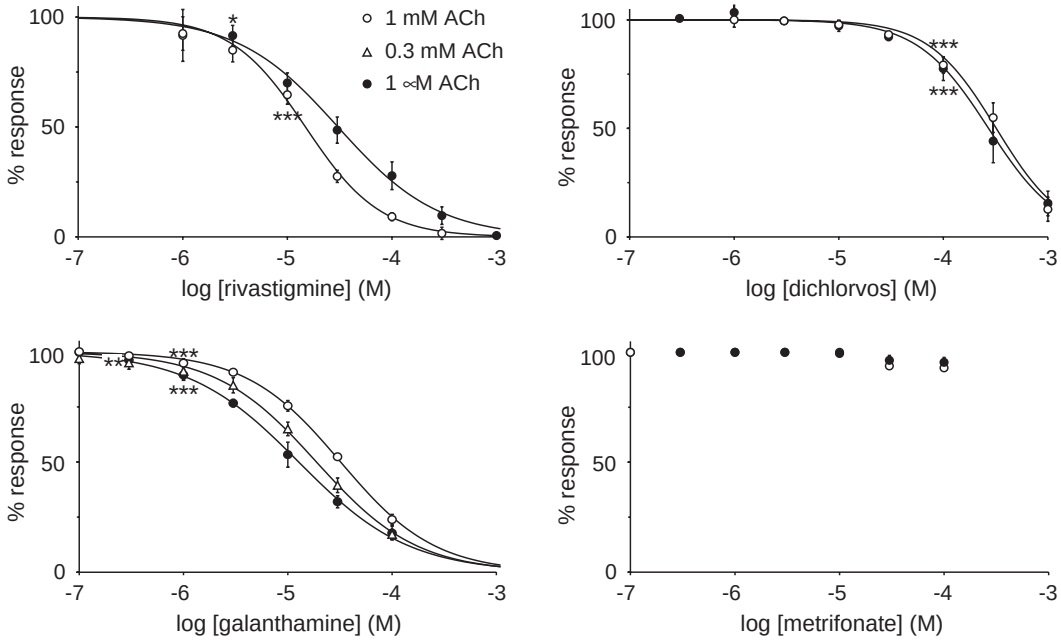

Fig. 6. Concentration-dependent inhibitory effects of rivastigmine, galanthamine, dichlorvos and metrifonate on inward currents evoked by low and high concentrations of acetylcholine. The inhibition curve of galanthamine shifts to the right at elevated acetylcholine concentration, whereas that of rivastigmine shifts to the left and that of dichlorvos appears to be independent of acetylcholine concentration. All data are mean±S.D. of 3–5 oocytes, except for metrifonate (n=2). Asterisks indicate the lowest concentrations causing significant effects compared to control (P<0.05, 0.01, and 0.001 indicated by *, **, and ***, respectively).

compounds do not affect the affinity of the radioligand for the agonist binding site (Fig. 8C).

3.5. A two-site receptor occupation model accounts for potentiating effects

The ligand binding experiments demonstrate that the potentiating cholinergic drugs, i.e., atropine, scopolamine, and physostigmine, are competitive ligands of human α4β2 nicotinic acetylcholine receptors. The functional effects of these potentiating, competitive ligands were modeled using the equilibrium two-site receptor occupation model, which combines competitive drug–receptor interactions with non-competitive channel block (Fig. 1). This model presumes that α4β2 nicotinic acetylcholine receptors contain two identical agonist binding sites, both of which need to be occupied to produce opening of ion channels. According to the model, potentiation by a cholinergic drug is due to the fact that, besides receptors with two acetylcholine molecules bound, receptors that have one acetylcholine molecule and one drug molecule bound also contribute to the response. For each drug, the model has been fitted to the concentration-dependent effects measured at low and high acetylcholine concentrations (Fig. 4). The estimated affinity of acetylcholine (K_a=87 μM), obtained from fitting an equilibrium two-site receptor occupation model to the concentration–effect data, was used as a constant in the model for evaluation of the drug effects. Estimates of the other model parameters, including the apparent affinity of the drugs for ion channel block, were obtained by fitting Eq. (2) to the data and are summarized in Table 1. The lines drawn in Fig. 4 are the fitted concentration–effect curves according to the two-site receptor occupation model. The close fits indicate that the model accounts for the effects of the potentiating cholinergic drugs on human α4β2 nicotinic acetylcholine receptors.

4. Discussion

Systematic investigation of the concentration-dependent effects of various cholinergic drugs on human α4β2

Table 2
Summary of the concentration-dependent effects of inhibitory cholinergic drugs on human α4β2 nicotinic acetylcholine receptors

Compound	Hill inhibition curve, 1 mM acetylcholine		Hill inhibition curve, 0.3 mM acetylcholine		Hill inhibition curve, 1 μM acetylcholine	
	IC_{50} (μM)	Hill slope	IC_{50} (μM)	Hill slope	IC_{50} (μM)	Hill slope
Rivastigmine	14.9±1.0	1.22±0.18			30.5±5.7	0.91±0.02
Galanthamine	32.1±2.2	0.96±0.04	18.8±2.9	0.90±0.11	12.7±2.5	0.82±0.05
Dichlorvos	321±71	1.35±0.03			271±73	1.28±0.14

Parameters estimated by fitting inhibition curves to data obtained at 1 mM, 0.3 mM, and 1 μM acetylcholine (mean±S.D., n=3–4).

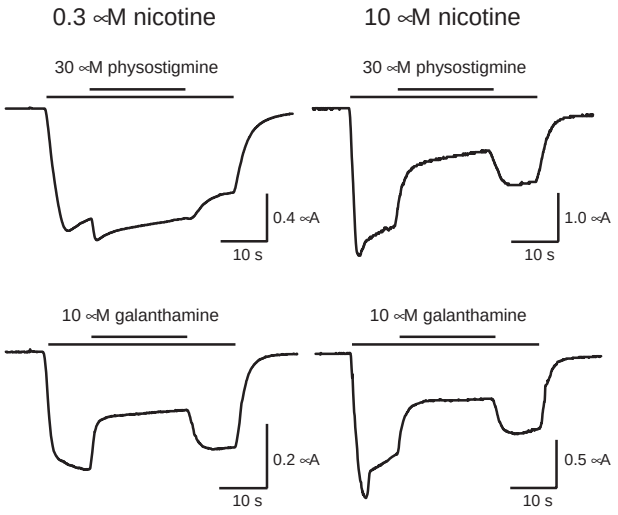

Fig. 7. Agonist-independent potentiation and inhibition of human α4β2 nicotinic acetylcholine receptors by physostigmine and galanthamine. Ion currents were evoked by 0.3 μM and 10 μM nicotine. The drugs were applied at the concentrations indicated for 20 s during the nicotine-evoked ion current. Note that nicotine-evoked inward current is potentiated and inhibited very much like acetylcholine-evoked inward current (see Figs. 3 and 5 and Table 3).

nicotinic acetylcholine receptors demonstrates that potentiation is consistently associated with responses evoked by low and not with responses evoked by high concentrations of acetylcholine, nicotine and cytisine (Figs. 3 and 7; Table 3). At high concentrations of acetylcholine the potentiating effects are surmounted. The surmountability indicates that the nature of the potentiating effects is competitive. Radioligand binding experiments confirm that the potentiating cholinergic drugs physostigmine, atropine, and scopolamine compete with the radioligand [^{3}H]cytisine for the agonist recognition sites of human α4β2 nicotinic acetylcholine receptors. Conversely, several of the cholinergic drugs are inhibitory only, both at low and at high agonist concentrations (Figs. 5 and 7; Table 3). The results of functional experiments are inconclusive with respect to the nature of the inhibitory effects. However, the inhibitory drugs galanthamine and rivastigmine are unable to displace [^{3}H]cytisine from the agonist binding site. Moreover, they fail to affect the saturation binding of [^{3}H]cytisine to human α4β2 nicotinic acetylcholine receptors. This demonstrates the non-competitive nature of the inhibitory effects of rivastigmine and galanthamine as well as the absence of allosteric interactions between the binding of these drugs and acetylcholine.

The EC_{50} of acetylcholine (248±88 μM) indicates that the receptors expressed have a low sensitivity to acetylcholine, consistent with previous observations on nicotinic ACh receptors expressed from rat α4 and β2 subunits at the same 1:1 ratio of α and β subunits (Zwart and Vijverberg, 1998). With the notion that the variation in EC_{50} values between oocytes is relatively large, the EC_{50} value of acetylcholine is not very different from a range of values reported in the literature for the "low affinity" α4β2 nicotinic acetylcholine receptors expressed in HEK293 cells (e.g., see Karadsheh et al., 2004). Previous results have shown that α4β2 receptors with high agonist affinity are apparent only after expression at an α:β subunit ratio much lower than the 1:1 ratio used in the present experiments (Zwart and Vijverberg, 1998). Moreover, similar potentiating and inhibitory effects of the drugs are observed, irrespective of the agonist used to evoke a cholinergic response (Table 3). Apart from the monophasic curves for ligand binding (Fig. 8) and receptor activation (Fig. 2), several other arguments indicate that a single population of receptors is involved in the effects observed.

4.1. Potentiating effects of cholinergic drugs

The binding experiments (Fig. 8) demonstrate that the potentiating drugs interact with an apparently single class of binding sites on α4β2 nicotinic acetylcholine receptors, consistent with the basic assumption of the model that the two agonist recognition sites of the nicotinic acetylcholine receptor are identical. Although it is not a prerequisite for the mechanism of potentiation that both agonist recognition sites are identical, the close correspondence between the apparent affinities of atropine and scopolamine for the agonist binding site and those estimated using the two-site model (Table 1) indicates that a single class of binding sites is sufficient to account for the potentiating effects. The apparent affinity of tacrine for neuronal chicken nicotinic

Table 3
Comparative effects of cholinergic drugs on acetylcholine-evoked ion currents with those on cytisine- and nicotine-evoked ion currents through human α4β2 nicotinic acetylcholine receptors

Compound	Acetylcholine		Cytisine		Nicotine	
	1 μM	1 mM	0.01 μM	10 μM	0.3 μM	10 μM
	% response		% response		% response	
Atropine, 30 μM	183±16	32±7	237±24	72±4	n.d.	n.d.
Physostigmine, 30 μM	160±15	42±6	201±10	76±3	128±5	52±10
Rivastigmine, 30 μM	49±6	28±3	88±4	77±1	n.d.	n.d.
Galanthamine, 10 μM	53±6	75±2	55±1	75±3	48±14	59±5

All values are response amplitudes normalized to the control response amplitude evoked by the same concentration of the same agonist (% response remaining in the presence of the drug; mean±S.D., n=3–5). For all drugs, the effects at high and low agonist concentrations differed significantly, except for the effect of galanthamine on nicotine-evoked ion currents. n.d.=not determined.

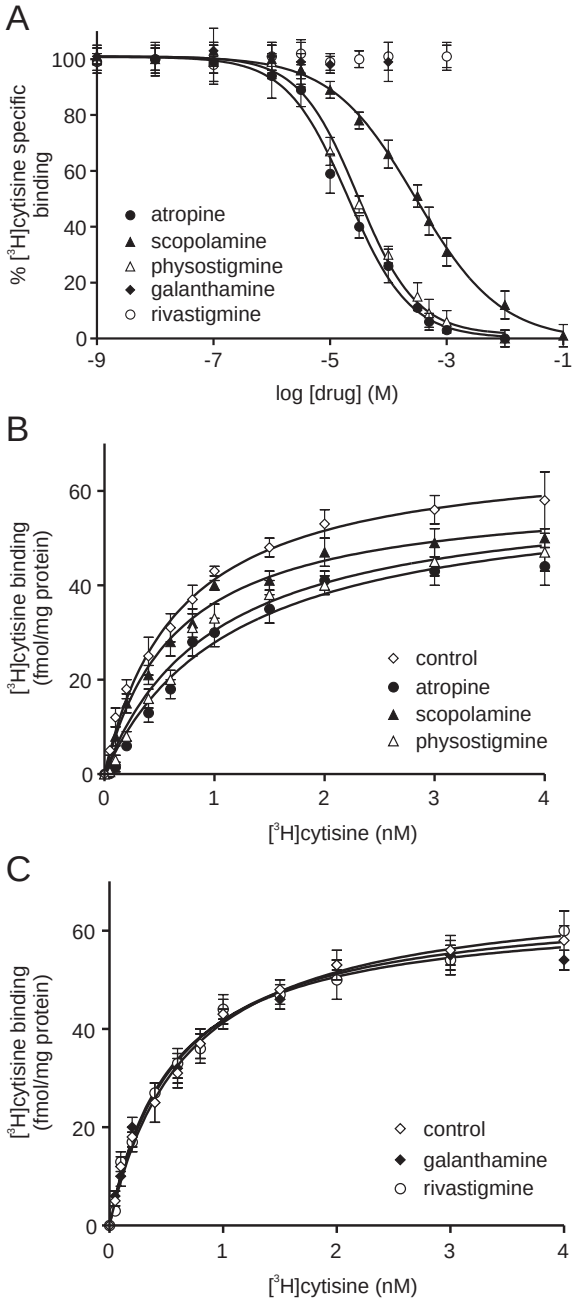

Fig. 8. Receptor binding experiments. (A) Atropine, scopolamine and physostigmine displace the radioligand [^{3}H]cytisine from its binding site. Galanthamine and rivastigmine showed no displacement of the radioligand. (B) Saturation of specific [^{3}H]cytisine binding to oocyte membranes expressing human α4β2 nicotinic acetylcholine receptors in the presence of 10 μM physostigmine, 10 μM atropine, or 30 μM scopolamine. (C) Saturation of specific [^{3}H]cytisine binding to oocyte membranes expressing human α4β2 nicotinic acetylcholine receptors in the presence of 300 μM galanthamine or 300 μM rivastigmine. Data are expressed as mean±S.E.M. for three experiments.

acetylcholine receptors expressed in M10 cells is 38 μM (Svensson and Nordberg, 1997) and for adult human muscle type nicotinic acetylcholine receptors expressed in HEK293 is 309 μM (Prince et al., 2002). With these values as reference, the present estimate of 14 μM for the apparent functional affinity of tacrine for human α4β2 nicotinic acetylcholine receptors does not appear unrealistic. Interestingly, the apparent affinity of physostigmine for the agonist binding site is higher than the affinity estimated by the model. One explanation might be that under the conditions of ligand binding experiments the nicotinic acetylcholine receptors are desensitized, whereas this is not the case in functional experiments. However, fitting only a potentiating, competitive effect to the bell-shaped curve in Fig. 4 yielded an estimate for the apparent affinity of physostigmine of 38 μM, which is close to the value obtained in binding experiments. Although this leads to a mismatch between the model and the inhibition curve of physostigmine obtained at 1 mM acetylcholine, the inhibitory effect may be more complex than accounted for by the two-site model (see below).

Potentiating effects of cholinergic drugs have been shown to vary with subunit composition of rat nicotinic acetylcholine receptors expressed in *Xenopus* oocytes (Zwart and Vijverberg, 1997; Zwart et al., 2000, Parker et al., 2003). A comparison of the present effects of physostigmine and tacrine on human α4β2 nicotinic acetylcholine receptors with those on rat α4β2 nicotinic acetylcholine receptors previously reported (Zwart et al., 2000) shows that the effects also vary with species. The presently found absence of a potentiating effect of galanthamine on human α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes and the previously reported potentiating effect on human α4β2 nicotinic acetylcholine receptors stably expressed in HEK293 cells (Samochocki et al., 2003) suggest that the effects may depend on the expression system as well. However, such a difference is not apparent from the effects of physostigmine, which potentiates responses mediated by rat α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes (Zwart et al., 2000) as well as in stably transfected HEK293 cells (Sabey et al., 1999) and in both systems more strongly at the low concentrations of acetylcholine. The differences between the effects of the drugs on human α4β2 nicotinic acetylcholine receptors and those reported for nicotinic acetylcholine

Table 4
Effects of cholinergic drugs on [^{3}H]cytisine saturation binding to human α4β2 nicotinic acetylcholine receptors in *Xenopus* oocyte membranes

Compound	K_d (nM)	B_{max} (fmol/mg protein)
Control	0.59±0.01	67±3
Atropine (10 μM)	1.20±0.20[a]	61±5
Scopolamine (30 μM)	0.65±0.10	60±6
Physostigmine (10 μM)	0.99±0.09[a]	60±6
Rivastigmine (300 μM)	0.60±0.02	63±8
Galanthamine (300 μM)	0.54±0.03	66±6

Values are mean (±S.E.M.) of K_d and B_{max} estimated from non-linear analysis of the saturation binding according to Eq. (4) in three independent experiments carried out in triplicate.

[a] $P<0.05$ compared to control.

receptors from other species stress the relevance of the use of human receptors for human drug research.

4.2. Inhibitory effects of cholinergic drugs

The drugs rivastigmine, dichlorvos, and galanthamine inhibit the human α4β2 nicotinic acetylcholine receptor-mediated acetylcholine responses (Figs. 5 and 6). The fact that these compounds inhibit the ion current without displacing the radioligand from the agonist binding site indicates that these compounds modulate nicotinic acetylcholine receptor function via a non-competitive mechanism. The lack of effect of galanthamine and rivastigmine on the saturation binding curve of [^{3}H]cytisine (Fig. 8C) indicates that these compounds do not affect the binding affinity of the radioligand for the agonist binding site. This implies that galanthamine and rivastigmine inhibit the acetylcholine-induced ion current in a non-allosteric way. Galanthamine also failed to displace [^{3}H]nicotine and [^{3}H]MLA from rat brain α4β2 and α7 nicotinic acetylcholine receptors, respectively, and [^{3}H]epibatidine from human α3* nicotinic acetylcholine receptors in SHSY-5Y cells. The saturation binding curves of these radioligands were also unaffected in the presence of 1 μM galanthamine (Dajas-Bailador et al., 2003). Despite the non-competitive binding, the inhibitory effects exerted by rivastigmine and galanthamine (Fig. 6) cannot simply be explained by non-competitive ion channel block. A more sophisticated mechanism is required to account for the anomalous concentration dependence of inhibition by galanthamine and rivastigmine. Various models for block of adult human muscle nicotinic acetylcholine receptors expressed in HEK293 cells by tacrine have been evaluated using single-channel data (Prince et al., 2002). The detailed kinetic analysis suggested that tacrine blocks the channel by binding to two sites in the open channel and to a site in the closed channel of the receptor. Closed channel block is equivalent to binding to a specific non-allosteric site, which would prevent ion channel opening. Although there is an obvious difference in binding affinity of tacrine for neuronal and muscle type nicotinic acetylcholine receptors (Svensson and Nordberg, 1997; Prince et al., 2002), it is conceivable that the effects of the same drug on distinct nicotinic acetylcholine receptor subtypes are qualitatively similar. The two-site model applied to the present results of tacrine on human α4β2 nicotinic acetylcholine receptors predicts similar affinities for channel block (18 μM) and for the agonist binding site (14 μM). This indicates that channel block contributes substantially to the inhibitory effect at all tacrine concentrations. For the inhibitory effects of physostigmine, a mechanism more complex than simple channel block appears to be required. This should warrant further studies using the two-site occupation model along with and complementary to the multiple-site channel block model. The latter may be applicable to inhibitory drugs, as well as to the inhibitory component of the effect of potentiating drugs.

In conclusion, the results demonstrate that distinct cholinergic drugs differentially modulate neuronal nicotinic acetylcholine receptor function. The differences in drug effects appear to be based on quantitative differences in the abilities of the drugs to bind to the agonist recognition sites of the nicotinic acetylcholine receptors and their abilities to block the ion channel. Distinguishing the various competitive and non-competitive effects may provide an important rationale for the further selection or development of specific drugs. However, it is yet unclear whether the therapeutic effects in various neurological disorders can be explained by one or several specific aspects of the action of cholinergic drugs on neuronal nicotinic acetylcholine receptors or whether they are due to a much wider spectrum of pharmacological actions.

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