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5-I A-85380 and TC-2559 differentially activate heterologously expressed α4β2 nicotinic receptors

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Abstract

The neuronal nicotinic acetylcholine receptor α4 and β2 subunits expressed in heterologous expression systems assemble into at least two distinct subunit stoichiometries of α4β2 receptor. The $(\alpha4)_2(\beta2)_3$ stoichiometry is about 100-fold more sensitive to acetylcholine than the $(\alpha4)_3(\beta2)_2$ stoichiometry. In order to investigate if agonists in general distinguish high- and low-affinity α4β2 nicotinic acetylcholine receptors, we have expressed human α4 and β2 nicotinic acetylcholine receptor subunits in two different expression systems. The relative amounts of α4β2 nicotinic acetylcholine receptors with high- and low-affinity for acetylcholine were manipulated by (a) injecting the subunit cDNAs at different α:β ratios into *Xenopus* oocytes and (b) by culturing HEK-293 cells stably expressing α4β2 nicotinic acetylcholine receptors overnight at different temperatures. The sensitivities of the α4β2 nicotinic acetylcholine receptors to the agonists acetylcholine, 5-I A-85380, and TC-2559 were investigated using the voltage-clamp technique on *Xenopus* oocytes and using a fluorescent imaging plate reader to measure calcium responses from HEK-293 cells. Like acetylcholine, 5-I A-85380 produced biphasic concentration–response curves and the high-affinity component became larger when the cells were manipulated to produce a greater proportion of $(\alpha4)_2(\beta2)_3$ nicotinic acetylcholine receptors. Interestingly, under all circumstances, TC-2559 produced monophasic concentration–response curves. In oocytes injected with α4 and β2 subunits in the 1:1 ratio the maximum effect of TC-2559 was 28% of that of acetylcholine. The EC_{50} for TC-2559 was not changed when oocytes were manipulated to express exclusively $(\alpha4)_2(\beta2)_3$ nicotinic acetylcholine receptors, however, the maximum effect of TC-2559 was dramatically enhanced. These results suggest that TC-2559 is a selective agonist of the $(\alpha4)_2(\beta2)_3$ nicotinic acetylcholine receptor stoichiometry.

Keywords: α4β2 nicotinic acetylcholine receptor; *Xenopus* oocyte; HEK-293 cell; Voltage clamp; Heterologous expression

1. Introduction

Neuronal nicotinic acetylcholine receptors are ligand-gated ion channels, which are found throughout the central and peripheral nervous system. Molecular cloning studies have provided evidence for the existence of at least 9 different types of neuronal nicotinic acetylcholine receptor α subunits (α2–α10) and three different types of neuronal nicotinic acetylcholine receptor β subunits (β2–β4). Some of these subunits form homo-pentameric receptors when expressed in heterologous expression systems (α7, α8 and α9), while other subunits assemble into hetero-pentameric structures with various combinations of α and β subunits. Different subunit combinations yield functional nicotinic acetylcholine receptors that differ considerably in their functional and pharmacological properties (Luetje and Patrick, 1991; Papke, 1993; Chavez-Noriega et al., 1997). The predominant subtype of nicotinic acetylcholine receptor in the central nervous system consists of α4 and β2 subunits (Flores et al., 1992; Picciotto et al., 2001). These α4β2 nicotinic acetylcholine receptors are possible targets for drugs to treat pain, nicotine addiction, and diseases like Alzheimer's and Parkinson's (Holladay et al., 1997; Lloyd and Williams, 2000). The exact subunit composition of α4β2 nicotinic acetylcholine receptors in the brain is still largely unknown.

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When expressed in *Xenopus* oocytes, α4 and β2 subunits have long been thought to assemble into a pentameric structure with $(\alpha4)_2(\beta2)_3$ stoichiometry, and with the subunits arranged around the ion channel pore in the order: αβαββ (Anand et al., 1991; Cooper et al., 1991). Later, it has been found that combinations of α4 and β2 subunits assemble into multiple subtypes of α4β2 nicotinic acetylcholine receptor (Zwart and Vijverberg, 1998; Khiroug et al., 2004; Lopez-Hernandez et al., 2004; Karadsheh et al., 2004) and direct evidence has been obtained for the existence of at least two different stoichiometries of α4β2 nicotinic acetylcholine receptor. Besides $(\alpha4)_2(\beta2)_3$, α4 and β2 subunits can also assemble with an $(\alpha4)_3(\beta2)_2$ stoichiometry (Nelson et al., 2003; Zhou et al., 2003). The two different α4β2 nicotinic acetylcholine receptors differ about a hundred-fold in their sensitivity to the neurotransmitter acetylcholine: the $(\alpha4)_3(\beta2)_2$ stoichiometry has a low sensitivity to acetylcholine ($EC_{50} \sim 100$ μM), whereas the $(\alpha4)_2(\beta2)_3$ stoichiometry is much more sensitive ($EC_{50} \sim 1$ μM). Upon co-expression of α4 and β2 subunits in heterologous expression systems, a mixture of the two stoichiometries of α4β2 nicotinic acetylcholine receptors is formed, and as a result concentration–response curves for acetylcholine consist of two components. This has been observed for α4 and β2 subunits from different species, such as human (Buisson and Bertrand, 2001; Houlihan et al., 2001; Zuo et al., 2003; Zhou et al., 2003; Nelson et al., 2003), rat (Zwart and Vijverberg, 1998; Covernton and Connolly, 2000; Khiroug et al., 2004) and mouse (Karadsheh et al., 2004). Similar to the concentration–response curve of the endogenous neurotransmitter acetylcholine, the nicotinic acetylcholine receptor agonists nicotine (Buisson and Bertrand, 2001; Nelson et al., 2003), cytisine, and cytisine-derivatives (Houlihan et al., 2001) also have been shown to produce biphasic agonist concentration–response curves at α4β2 nicotinic acetylcholine receptors. It has been reported that native mouse thalamic α4β2 nicotinic acetylcholine receptors also have biphasic agonist concentration–response characteristics (Marks et al., 1999; Butt et al., 2002). Both response components require the β2 subunit and most likely the α4 subunit (Zoli et al., 1998; Marks et al., 1999), suggesting that native α4 and β2 nicotinic acetylcholine receptor subunits also assemble into multiple subunit stoichiometries. However, it cannot be ruled out that native α4β2 nicotinic acetylcholine receptors contain additional subunits.

In this study, we addressed the question of whether α4β2 nicotinic acetylcholine receptor agonists in general distinguish between α4β2 nicotinic acetylcholine receptor subunit stoichiometries with high- and low-sensitivity or whether there could be differences between various agonists from different chemical classes. We have investigated this by measuring the concentration–response relationships for three α4β2 nicotinic acetylcholine receptor agonists in oocytes expressing α4β2 nicotinic acetylcholine receptors with high and low sensitivities to acetylcholine using the voltage clamp technique. Since the proportion of α4β2 receptors with high sensitivity to acetylcholine increases also when stably transfected HEK-293 cells are cultured at a lower temperature (Nelson et al., 2003), we further investigated the properties of the same agonists on α4β2 nicotinic acetylcholine receptors expressed in HEK-293 cells cultured at either 29 °C or 37 °C. Part of this work has appeared in abstract form (Zwart et al., 2005).

2. Materials and methods

2.1. Oocyte expression and two-electrode voltage clamp recording

Stage V and VI *Xenopus* oocytes were prepared as previously described (Zwart et al., 2002). The human α4 and β2 nicotinic acetylcholine receptor subunits, ligated into the pcDNA3.1 plasmid vector, were dissolved in distilled water at approximately equal concentrations of 102±12 and 106±9 μg/ml (mean±standard deviation of triplicate spectrophotometric determinations), respectively. Mixtures of these solutions at 1:5 and 1:1 α:β ratios were injected into the nuclei of oocytes in a volume of 18.4 nl/oocyte, using a variable volume automatic oocyte microinjector (Drummond Broomall, PA, USA). The total amount of cDNA injected per oocyte was kept constant (2 ng). After injection the oocytes were incubated at 18 °C in a 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 $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM Hepes, and 50 mg/l neomycin (pH 7.6 with NaOH; osmolarity 235 mOsm). Experiments were performed on oocytes after 3–5 days of incubation.

Oocytes were placed in a recording chamber (internal diameter 3 mm), which was continuously perfused with a saline solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, 10 mM Hepes, pH 7.3 with NaOH, 235 mOsm) at a rate of ~10 ml/min. Dilutions of drugs in external saline were prepared immediately before the experiments and applied by switching between control and drug-containing saline using an 8-channel valve control system (BPS-8, ALA Scientific Inc., Westbury, NY, USA). Agonist applications were alternated by 5 min of superfusion with agonist-free saline to allow the receptors to recover from desensitization.

Oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–2.5 MΩ) and voltage clamped using a modified Geneclamp 500B amplifier (Axon Instruments, Union City, CA, USA). The external saline was clamped at ground potential by means of a virtual ground circuit using an Ag/AgCl reference electrode and a Pt/Ir current passing electrode. The membrane potential was held at −60 mV. The current needed to keep the oocyte membrane at the holding potential was measured. Membrane currents were low-pass filtered (4-pole low pass Bessel filter, −3 dB at 100 Hz), digitized (300 Hz), and stored on disk for off-line computer analysis. Data are expressed as mean±standard deviation. All experiments were performed at room temperature.

For agonist concentration–response curves, ion current amplitudes were measured and normalized to the amplitude of control responses induced by the near-maximum effective concentration of 1 mM acetylcholine. Control responses were evoked alternately, in order to adjust for small variations in

response amplitude over time. Concentration–response curves were fitted to the data and mean±standard deviation of estimated parameters were calculated. Agonist data were fitted according to the equations: $i/i_{max}=E_{max}/[1+(EC_{50}/[X])^{nH}]$ and $i/i_{max}=E_{maxa}/1+(EC_{50a}/[X])+E_{maxb}/1+(EC_{50b}/[X])$, for one- and two-component activation curves, respectively. In these equations, [X] is the concentration of agonist, *nH* is the Hill coefficient, and i/i_{max} is the normalized current amplitude. Curve fitting was performed using Prism 3.01 (GraphPad Software, San Diego, CA, USA). The *F*-test was applied to evaluate whether a two-component concentration–response model fitted the data better than a single-component concentration–response model. When the *F*-test resulted in $P<0.05$ it was concluded that the two-component model fitted the data significantly better.

2.2. FLIPR assay

HEK-293 cells stably expressing α4β2 nicotinic acetylcholine receptors were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 50 mg/ml geneticin. For Fluorescent Imaging Plate Reader (FLIPR; Molecular Devices, Winnersh, UK) experiments, HEK-293 cells stably expressing α4β2 nicotinic acetylcholine receptors were plated in black-walled, transparent bottomed 96-well plates (Poly-D-lysine coated, Marathon Laboratories), at a density of 0.5×10^6 cells/ml, and incubated overnight at 29 °C or 37 °C in a humidified atmosphere containing 5% CO_2.

For experiments, growth medium was removed from the 96 wells and replaced with Hepes-buffered saline solution (HBSS) containing 10 μM Fluo-3-AM, 0.05% pluronic F-127, with the aid of a multidrop pipettor (Lab Systems, Helsinki, Finland). The cells were loaded with the dye for 1 h at room temperature, the buffer removed, and replaced with fresh HBSS without Fluo-3. The plates were then transferred to the FLIPR for experiments. The dye was excited by light of 488 nM wavelength generated by an Argon laser, while the emitted fluorescence was passed through a 510- to 570-nm filter, before being detected by a cooled CCD camera (Princeton Instruments, Trenton, NJ, USA). Drugs were prepared in a 96-well plate using a Biomek 2000 (Beckman Instruments, Fullerton, CA, USA). Parameters for drug addition were programmed on the computer and delivery was automated through a 96-tip pipettor. More details about FLIPR assays can be found in Schroeder and Neagle (1996). The peak amplitudes of the calcium responses to each agonist application were normalized to the peak amplitude of control, 1 μM epibatidine-induced, calcium responses. In this assay epibatidine rather than acetylcholine, was used as a control agonist, because the HEK-293 cell line expressing α4β2 nicotinic acetylcholine receptors also contains endogenous muscarinic receptors, which would be activated by acetylcholine, but not by epibatidine. Data files were saved to a Dell Optiplex GX110 computer and stored for offline analysis using the FLIPR system software and Origin 6.1 (OriginLab, Northampton, MA, USA). The FLIPR data were fitted to the same equations and using the same software as described for the oocyte experiments.

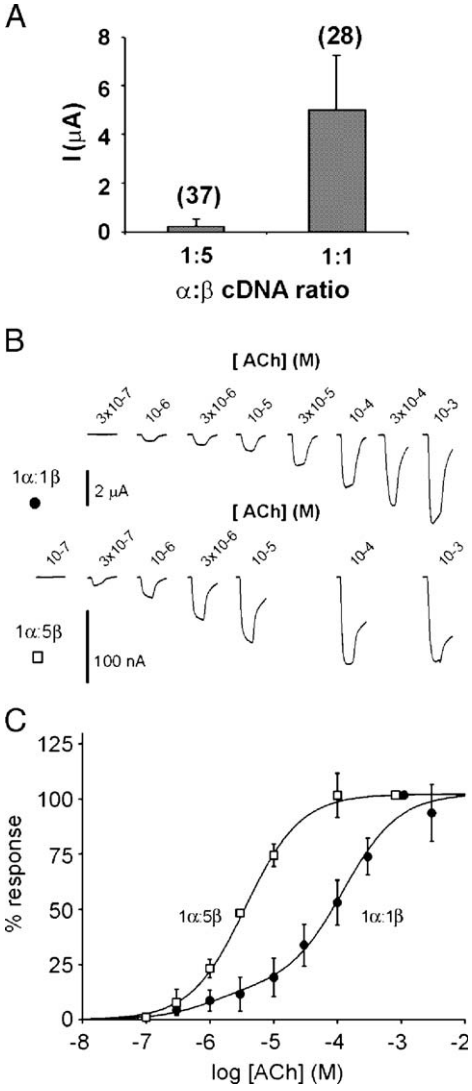

Fig. 1. Effect of α4:β2 subunit ratio on ion currents induced by acetylcholine. (A) 1-mM acetylcholine-induced peak inward current amplitudes in oocytes expressing human α4β2 nicotinic acetylcholine receptors are significantly reduced at the 1:5 α4:β2 subunit ratio as compared to 1:1 α4:β2 ratio (*t*-test; $P<0.001$). Error bars represent standard deviation values of the peak amplitudes from the number of oocytes indicated between brackets. (B) Inward currents evoked by 10-s periods of superfusion with external saline containing acetylcholine at the concentrations indicated for oocytes expressing nicotinic acetylcholine receptors after 1:1 α4:β2 injection (●) and 1:5 α:β injection (□). Note the difference in vertical scale bar between responses obtained at the 1:1 α:β subunit ratio and the responses obtained at the 1:5 α:β subunit ratio. (C) Concentration–response curves for acetylcholine measured from oocytes injected with α4 and β2 subunits in the 1:1 (●; $n=7$) and 1:5 (?; $n=3$) subunit ratios. Response amplitudes were normalized to the response amplitude induced by 1 mM acetylcholine in the same oocyte. The data obtained at the 1:1 ratio were best fitted by a two-component concentration–response curve with Hill slopes set to 1 and data obtained at the 1:5 ratio could be fitted by a single component concentration–response curve. The estimated parameters of the fitted curves are presented in Tables 1 and 2.

Table 1
Effects of agonists on human α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes injected with cDNAs encoding α and β subunits in a 1:1 ratio

	EC_{50}(A) (μM)	E_{max}(A) (%)	*nH*(A)	EC_{50}(B) (μM)	E_{max}(B) (%)	*nH*(B)
Acetylcholine (n=7)	1.1 (0.6–2.7)	15±4	1.0 (fixed)	124 (74–162)	87±5	1.0 (fixed)
5-I-A-85380 (n=3)	0.010 (0.007–0.015)	14±4	1.0 (fixed)	18 (15–20)	89±6	1.0 (fixed)
TC-2559 (n=13)	1.2 (0.5–2.9)	28±2	0.9±0.1			

E_{max} and *nH* values represent the mean±S.D., EC_{50} values represent the mean and the 95% confidence intervals are indicated between brackets.

2.3. Materials

The human cDNAs encoding the α4 and β2 nicotinic acetylcholine receptor subunits and the cell line stably transfected with α4β2 nicotinic acetylcholine receptors were obtained from Merck Research Laboratories (La Jolla, CA, USA). (±)-Epibatidine and 5-iodo-3-(2(*S*)-azetidinylmethoxy) pyridine (5-I A-85380; Mukhin et al., 2000) were purchased from Tocris (Avonmouth, UK). Acetylcholine chloride was purchased from Sigma (Poole, UK). [(*E*)-*N*-Methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine (TC-2559; Bencherif et al., 2000) was synthesized at Eli Lilly and Company (Windlesham, UK). The purity of TC-2559 was >98%. Stock solutions of 1 M acetylcholine chloride and 10 mM TC-2559 were prepared in distilled water. All other drugs were prepared as 10 mM stock solutions in dimethylsulfoxide. Dilutions of agonists in recording saline were prepared immediately before the experiments. Fluo-3-AM and pluronic F-127 were purchased from Molecular Probes (Leiden, The Netherlands). All cell culture reagents were from GIBCO-BRL (Parsley, UK), except fetal calf serum, which was from Invitrogen (Carlsbad, CA, USA).

3. Results

3.1. Effects of α4:β2 ratio on acetylcholine-induced ion current amplitude

Application of 1 mM acetylcholine to voltage-clamped oocytes expressing human α4β2 nicotinic acetylcholine receptors after injection of α4 and β2 subunit cDNAs in the α:β ratios of 1:1 and 1:5 resulted in nicotinic acetylcholine receptor-mediated inward currents in approximately 80% and 50% of the cells, respectively. An oocyte was considered to be responsive when application of 1 mM acetylcholine resulted in a deflection of the baseline current of more than 10 nA. The effect of varying the subunit ratio on response amplitude was investigated in oocytes obtained from a single batch, because expression levels can vary considerably between different batches. The largest inward currents were observed in oocytes injected with α4 and β2 subunit cDNAs in the 1:1 ratio (4.9 ±2.2 μA, n=28; Fig. 1A). At the 1:5 α:β ratio the amplitudes of inward currents evoked by 1 mM acetylcholine were significantly smaller (310±207 nA; n=37; t-test, P<0.001; Fig. 1A). The results show that, after injection of the α4 and β2 subunit cDNAs in the 1:5 α:β ratio, while maintaining the total cDNA injected approximately constant, acetylcholine-induced inward current amplitudes are reduced, indicating that the response amplitude is limited by the availability of α subunits.

3.2. Effects of α4:β2 ratio on the concentration–response curve for acetylcholine

Voltage clamped oocytes injected with α4 and β2 subunits in the 1:1 and 1:5 α:β ratios were superfused with various concentrations of acetylcholine. In both cases this resulted in concentration-dependent inward currents (Fig. 1B). The peak amplitudes of these currents were measured, normalized to the amplitude of the current induced by 1 mM acetylcholine and the normalized data plotted against the acetylcholine concentration. The data obtained from oocytes that were injected with α4 and β2 subunits in the 1:1 α:β ratio were best fitted by the sum of two concentration–response curves with Hill slopes set to 1. The data obtained from oocytes that were injected with α4 and β2 subunits in the 1:5 α:β ratio were adequately fitted with a single component concentration–response curve. The estimated parameters of the fitted curves (Fig. 1C) are summarized in Tables 1 and 2.

3.3. Effects of α4:β2 ratio on the concentration–response curves for 5-I A-85380 and TC-2559

The chemical structures of 5-I A-85380 and TC-2559 have been published in Mukhin et al. (2000) and Bencherif et al. (2000), respectively. Voltage clamped oocytes injected with α4 and β2 subunits in the 1:1 and 1:5 α:β ratios were superfused with various concentrations of 5-I A-85380, and TC-2559. The peaks of the ion current amplitudes were normalized to the peak amplitude of 1 mM acetylcholine-induced ion current from the same oocyte. 5-I A-85380 produced a clear two-component concentration–response relationship in oocytes that were injected with α4 and β2 subunits in the 1:1 ratio. Like acetylcholine, 5-I A-85380 activated only the high-affinity component in oocytes injected with the α4 and β2 subunits in the 1:5 ratio, and the data were best fitted by a monophasic concentration–response curve (Fig. 2). TC-2559, however, produced monophasic concentration–response curves regardless the ratio in which the α4 and β2 nicotinic acetylcholine

Table 2
Effects of agonists on human α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes injected with cDNAs encoding α and β subunits in a 1:5 ratio

	EC_{50} (μM)	E_{max} (%)	*nH*
Acetylcholine (n=3)	3.5 (2.8–4.2)	102±2	1.0±0.1
5-I A-85380 (n=3)	0.0076 (0.004–0.014)	209±16	0.9±0.2
TC-2559 (n=7)	1.9 (1.2–3.0)	357±24	0.9±0.1

E_{max} and *nH* values represent the mean±S.D., EC_{50} values represent the mean and the 95% confidence intervals are indicated between brackets.

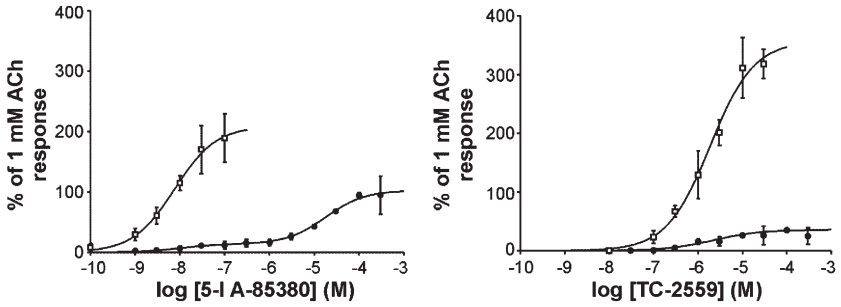

Fig. 2. Concentration–response curves of 5-I A85380 and TC-2559 obtained from oocytes injected with α4 and β2 subunits in the 1:1 (●) and 1:5 (□) ratios. Response amplitudes were normalized to the response amplitude induced by 1 mM acetylcholine in the same oocyte. The data obtained with 5-I A85380 on the 1:1 α4:β2 subunit ratio were best fitted by a two-component concentration–response curve, whereas the data obtained with 5-I A85380 on the 1:5 α4:β2 could be fitted by a single component concentration–response curve. The data obtained with TC-2559 could be fitted by single component concentration–response curves, regardless the α:β subunit ratio injected into the oocyte. The values of the estimated parameters of the fitted curves are presented in Tables 1 and 2.

receptor subunits were injected into the oocytes. The α4:β2 subunit ratio did not have a significant effect on the EC_{50} for TC-2559. However, the maximum effect of TC-2559 in oocytes injected with α4 and β2 subunits in the 1:1 ratio was only 28% as compared to the maximum effect of acetylcholine, and the maximum effect of TC-2559 was considerably larger (357%) than the maximum effect of acetylcholine when oocytes were injected with α4 and β2 subunits in the 1:5 ratio. The estimated parameters of the fitted curves (Fig. 2) are summarized in Tables 1 and 2.

3.4. Effects of agonists on human α4β2 nicotinic acetylcholine receptors stably expressed in HEK-293 cells after overnight incubation at 37 °C or 29 °C

The relative proportion of α4β2 nicotinic acetylcholine receptors with high sensitivity to activation by acetylcholine can be upregulated by incubating transfected HEK-293 cells overnight at 29 °C instead of 37 °C (Nelson et al., 2003). We have therefore evaluated the effects of the same nicotinic acetylcholine receptor agonists that we investigated in the oocytes, on intracellular calcium levels in Fluo-3 loaded HEK-293 cells stably expressing human α4β2 nicotinic acetylcholine receptor, which were incubated 24 h prior to the experiments at either 37 °C or 29 °C. Because HEK-293 cells express endogenous muscarinic acetylcholine receptors 100 nM of the muscarinic receptor antagonist atropine was added to the buffer solution in order to block possible contributions of the muscarinic receptors to the acetylcholine-induced calcium responses. Peak amplitudes of the calcium responses to each agonist application were normalized to the peak amplitude of 1 μM epibatidine-induced calcium response. The concentration–response data obtained with acetylcholine and 5-I A-85380 appeared biphasic and were best fitted by two-component concentration–response curves. TC-2559 produced concentration–response data that could be adequately fitted by a single component concentration–response curve. The estimated parameters of the fitted curves (Fig. 3) are summarized in Table 3.

4. Discussion

The concentration–response curves obtained with different α4β2 nicotinic acetylcholine receptor agonists demonstrate that not every α4β2 nicotinic acetylcholine receptor agonist distinguishes between two different α4β2 nicotinic acetylcholine receptor subunit stoichiometries. The results from both the oocyte and FLIPR experiments show that 5-I A-85380 mimicked acetylcholine in generating two-component concentration–response curves. These observations are interesting,

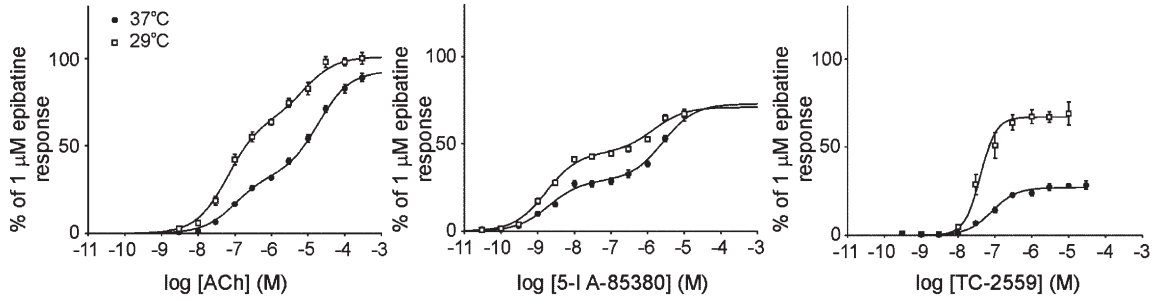

Fig. 3. Concentration–response curves of the nicotinic acetylcholine receptor agonists acetylcholine, 5-I A-85380, and TC-2559 obtained from HEK-293 cells stably transfected with α4 and β2 subunits that were cultured overnight at 29 °C (□) and 37 °C (●). Acetylcholine responses were measured in the presence of 100 nM atropine, in order to block muscarinic acetylcholine receptors which are endogenous to HEK-293 cells. Peak amplitudes of responses were normalized to the peak response amplitude induced of 1 μM epibatidine. The data obtained with acetylcholine and 5-I A-85380 were clearly biphasic and have been fitted by two-component concentration–response curves. The data obtained with TC-2559 were adequately fitted by single component concentration–response curves. The values of the estimated parameters of the fitted curves are presented in Table 3.

Table 3
Effects of agonists on human α4β2 nicotinic acetylcholine receptors stably expressed in HEK-293 cells after overnight incubation of cells at 37 °C and 29 °C

	EC_{50}(A) (nM)	E_{max}(A) (%)	*nH*(A)	EC_{50}(B) (μM)	E_{max}(B) (%)	*nH*(B)
37 °C						
Acetylcholine (*n*=14)	98 (90–107)	33±5	1 (fixed)	18.1 (16.3–20.1)	60±6	1 (fixed)
5-I-A-85380 (*n*=16)	2.0 (1.4–3.0)	29±3	1 (fixed)	2.6 (0.4–18.6)	44±5	1 (fixed)
TC-2559 (*n*=4)	93 (72–120)	28±1	1.1±0.1			
29 °C						
Acetylcholine (*n*=14)	62 (40–96)	65±6	1 (fixed)	10.0 (6.3–15.9)	36±4	1 (fixed)
5-I-A-85380 (*n*=16)	1.7 (1.5–2.0)	45±7	1 (fixed)	1.4 (0.9–2.1)	23±6	1 (fixed)
TC-2559 (*n*=4)	42 (35–49)	68±1	1.4±0.1			

E_{max} and *nH* values represent the mean±S.E.M., EC_{50} values represent the mean and the 95% confidence intervals are indicated between brackets.

because other α4β2 nicotinic acetylcholine receptor agonists, such as nicotine, cytisine and cytisine derivatives produced biphasic concentration–response curves (Buisson and Bertrand, 2001; Houlihan et al., 2001; Nelson et al., 2003), indicating that these agonists distinguish the two α4β2 nicotinic acetylcholine receptor stoichiometries in a manner similar to acetylcholine and 5-I-A-85380. On the other hand, TC-2559 produced single-component concentration–response curves only, suggesting that this agonist is either selective for a particular α4β2 nicotinic acetylcholine receptor stoichiometry, or, alternatively, that both α4β2 nicotinic acetylcholine receptor stoichiometries have very similar sensitivities for TC-2559.

The concentration–response curves obtained with the α4β2 nicotinic acetylcholine receptor agonist TC-2559 are intriguing. In oocytes expressing mainly α4β2 nicotinic acetylcholine receptors with a low-affinity for acetylcholine and a minor fraction of α4β2 with a high-affinity for acetylcholine (1:1 α:β ratio), TC-2559 produced a monophasic concentration–response with an efficacy value of only 28% as compared to acetylcholine. A surprising result was obtained when a homogeneous population of α4β2 nicotinic acetylcholine receptors was expressed that show only high-affinity for acetylcholine. In these oocytes TC-2559 appeared to be a super-efficacious agonist with an efficacy that is about 3.5 times larger than the efficacy of acetylcholine. Under these conditions, TC-2559 still produced a monophasic concentration–response curve, with an EC_{50} value similar to the EC_{50} value obtained from oocytes expressing both subtypes of α4β2 nicotinic acetylcholine receptors. The combination of a large increase in efficacy and no shift in EC_{50} when the α:β subunit ratio is changed from 1:1 to 1:5 is similar to the effects of 5-I A-85380 on α4β2 nicotinic acetylcholine receptors with high-affinity for acetylcholine. Also the efficacy of 5-I A-85380 is largely increased when the α:β subunit ratio is changed from 1:1 to 1:5. This suggests that TC-2559 is selective for α4β2 nicotinic acetylcholine receptors with high-affinity for acetylcholine and that TC-2559 does not activate the alternative α4β2 nicotinic acetylcholine receptor stoichiometry. On the other hand, the resemblance between the effects of TC-2559 and 5-I A-85380 on the receptors with high affinity for acetylcholine suggests that it is unlikely that TC-2559 activates both α4β2 nicotinic acetylcholine receptor stoichiometries with similar potency.

The results with TC-2559 obtained in the FLIPR experiments confirm the phenomena observed with TC-2559 in oocytes, indicating that this effect is not unique to the oocyte expression system. In HEK-293 cells cultured at 37 °C TC-2559 produced a monophasic concentration–response curve. In HEK-293 cells in which the proportion of α4β2 nicotinic acetylcholine receptors with high-affinity for acetylcholine was increased by incubating the cells at the lower temperature of 29 °C, the efficacy of TC-2559 to activate α4β2 nicotinic acetylcholine receptors increased dramatically, with only minor changes in EC_{50} values. The present results obtained by expression of human α4 and β2 nicotinic acetylcholine receptor subunits at different α:β subunit ratios resemble the previously published data on the corresponding rat α4 and β2 subunits (Zwart and Vijverberg, 1998). Upon expression of the rat α4 and β2 nicotinic acetylcholine receptor subunits at the 1α:1β ratio, a monophasic concentration–response curve for acetylcholine was obtained with EC_{50} of 66 ìM and a slope factor of 1.1. When the rat α4 and β2 subunits were expressed at the 1:9 α:β ratio, a biphasic curve was obtained, with a large proportion (66%) of high-affinity receptors for acetylcholine with an EC_{50} of 1.8 ìM and a slope factor of 1. The remainder of the channels had a low affinity for acetylcholine. The data obtained with the human α4 and β2 subunits, however, differ quantitatively from the rat counterparts, because the concentration–response curve for acetylcholine is biphasic even after 1:1 α:β injection. A small component (15%) shows high sensitivity to acetylcholine and a major component (85%) shows low sensitivity to acetylcholine. When the human α4 and β2 subunits were injected at the 1:5 subunit ratio, a homogeneous population of α4β2 nicotinic acetylcholine receptors is expressed with high sensitivity to acetylcholine. This comparison indicates that the proportion of high- and low-affinity α4β2 nicotinic acetylcholine receptors differs between the previous study on rat α4β2 nicotinic acetylcholine receptors and the present study on human α4β2 nicotinic acetylcholine receptors. This might be caused by differences in expression vectors in which the subunits were ligated, and the presence or absence of untranslated regions, which might influence the transcription efficiency and thereby might have an effect on the subunit ratio.

The fact that some agonists recognize two different subpopulations of α4β2 nicotinic acetylcholine receptors, whereas others appear to be selective for one particular subpopulation of α4β2 nicotinic acetylcholine receptor raises

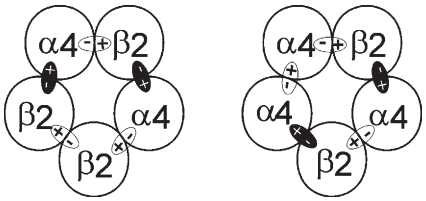

Fig. 4. The two subunit stoichiometries of α4β2 nicotinic acetylcholine receptors. In this cartoon + represents the principle component, and − represents the complimentary component of the agonist binding site. The binding sites indicated in black represent the "classical" agonist binding sites, which are located at the interfaces between α- and β-subunits. Note that both subunit assemblies do not differ in the nature and number of the "classical" agonist binding sites, both contain two identical "classical" binding sites. Binding sites indicated in white represent possible agonist binding sites. Besides having two α/β interfaces in common, both subunit assemblies also have two β/α interfaces in common. The main difference between the two subunit assemblies is that the $(\alpha4)_2(\beta2)_3$ stoichiometry contains a β/β interface and the $(\alpha4)_3(\beta2)_2$ stoichiometry contains a α/α interface.

questions about the nature of the agonist binding sites on α4β2 receptors. Classically, two identical binding sites per α4β2 nicotinic acetylcholine receptor are thought to be located at the interface of the α- and β-subunit (reviewed in Bertrand and Changeux, 1995). The α subunit contributes the principal component and the β subunit contributes the complementary component to the agonist binding site. Comparison of the high-affinity $(\alpha4)_2(\beta2)_3$ stoichiometry and the low-affinity $(\alpha4)_3(\beta2)_2$ stoichiometry shows that both types of α4β2 nicotinic acetylcholine receptor are the same in this respect (see Fig. 4). Both stoichiometries possess two identical "classical" binding sites, and therefore no pharmacological differences between these two receptor assemblies are expected. How, then, can we explain the significantly different pharmacology of the two stoichiometries here described? One possibility is that the subunit which does not contribute to a "classical" agonist binding site influences allosterically the affinity of the agonist binding sites. If this is the case, a β2 subunit in this position brings the "classical" binding sites of the receptors in a conformation with high affinity for acetylcholine, while an α4 subunit in this position brings the receptor in a conformation in which the binding sites have low-affinity for acetylcholine. It is conceivable that both conformations of the "classical" binding sites are sufficiently different to allow some agonists to be selective for a particular receptor subtype. An alternative explanation would be that other subunit interfaces than the "classical" α/β interfaces form binding sites for agonists on α4β2 nicotinic acetylcholine receptors. The main difference between the two subunit assemblies is that the $(\alpha4)_2(\beta2)_3$ stoichiometry contains a β/β interface and the $(\alpha4)_3(\beta2)_2$ stoichiometry contains an α/α interface (Fig. 4). Both α and β subunits contain most of the loops that contribute to the principal and complementary components of the agonist binding pocket. For example, the β2 subunit not only contains the complementary component of the agonist binding site, but it also contains loops A and B of the principal component (Corringer et al., 1995). If subunit interfaces other than α/β interfaces contribute to agonist binding sites on different α4β2 nicotinic acetylcholine receptors, then it would be easier to explain the existence of selective agonists for particular subtypes of α4β2 stoichiometries.

Testing the various agonists here described on α4β2 nicotinic acetylcholine receptors with a fixed subunit stoichiometry, e.g. using the concatenated subunit approach (Zhou et al., 2003; Minier and Sigel, 2004; Groot-Kormelink et al., 2006), might help in further understanding this selective pharmacology. Ultimately, we would need to understand which in vitro stoichiometries are actually present in native cells and in different tissues, an issue that is further complicated by the likely co-assembly of other subunits, such as α5, α6, and/or β3 (Champtiaux et al., 2003; Salminen et al., 2004) together with α4 and β2. A better understanding of the selectivity of α4β2 nicotinic acetylcholine receptor agonists for different subtypes of α4β2-containing receptors could drive a more rational approach to the development of efficacious and safer drugs acting on nicotinic α4β2 nicotinic acetylcholine receptors.

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