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Impact of human D398N single nucleotide polymorphism on intracellular calcium response mediated by $\alpha 3\beta 4\alpha 5$ nicotinic acetylcholine receptors

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Abstract

The human *CHRNA5*D398N polymorphism (rs16969968) causes an aspartic acid to asparagine change in the nicotinic acetylcholine receptor (nAChR) $\alpha 5$ subunit gene. The N398 variant of *CHRNA5* is linked to increased risk for nicotine dependence. In this study, we explored the effect of the *CHRNA5*D398N polymorphism on the properties of human $\alpha 3\beta 4^*$ nicotinic acetylcholine receptors in human embryonic kidney (HEK) cells.

Addition of either D398 or N398 variant of $\alpha 5$ subunit in the $\alpha 3\beta 4^*$ receptor did not affect total [¹²⁵I]-epibatidine binding or surface expression of the receptor. However, addition of $\alpha 5^{D398}$ into $\alpha 3\beta 4^*$ receptor decreased the maximal response to agonist without significantly affecting EC₅₀ in aequorin intracellular calcium assay. $\alpha 3\beta 4\alpha 5^{N398}$ nAChRs showed further decreased maximal response. The differences in agonist efficacy between the receptor subtypes were found to be dependent upon the concentration of external calcium but independent of external sodium. Moreover, activation of $\alpha 3\beta 4\alpha 5$ nAChRs led to significantly greater intracellular calcium release from IP₃ stores relative to $\alpha 3\beta 4$ nAChRs although no effect of the $\alpha 5$ polymorphism was observed. Finally, inclusion of the $\alpha 5$ variant caused a small shift to the left in IC₅₀ for some of the antagonists tested, depending upon $\alpha 5$ variant but did not affect sensitivity of $\alpha 3\beta 4^*$ receptors to desensitization in response to incubation with nicotine.

In conclusion, addition of either variant of $\alpha 5$ into an $\alpha 3\beta 4\alpha 5$ receptor similarly affects receptor pharmacology and function. However, the N398 variant exhibits a reduced response to agonists when extracellular calcium is high and it may lead to distinct downstream cellular signaling.

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Authorship credit: AT carried out aequorin and binding assays, analyzed data, prepared figures and wrote the first version of manuscript, PH carried out aequorin assays, PL prepared the stably expressing cell lines, CE carried out the surface ELISA assays, JRL maintained the stably expressing cell lines and seeded cells in plates, GA designed and conducted the calcium permeability experiments and contributed to manuscript preparation, JAS designed the experiments, analyzed data and prepared the manuscript.

Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neuropharm.2012.07.022>.

Keywords

Nicotinic acetylcholine receptors; Polymorphism; rs16969968; Alpha5 subunit; *CHRNA5*; Intracellular calcium

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels of the cysteine loop superfamily, formed by five membrane-spanning subunits lining the central water-filled pore (for a recent review, see Barik and Wonnacott, 2009). When in open state, nAChRs are permeable to mono- and divalent cations, primarily sodium, Potassium and calcium ions. Mammalian neuronal nAChRs either consist of a combination of $\alpha 2$ - $\alpha 6$ and $\beta 2$ - $\beta 4$ subunits or are homomers of $\alpha 7$ or $\alpha 9$ subunits (Gotti et al., 2007). $\alpha 10$ forms heteromers with $\alpha 9$ subunit. At least two essential α subunits ($\alpha 2$, $\alpha 3$, $\alpha 4$ or $\alpha 6$) and two essential β subunits ($\beta 2$ or $\beta 4$) are needed to form functional heteromeric nAChRs and ACh binding sites. $\alpha 5$ and $\beta 3$ cannot form functional receptors without the essential subunits and they do not participate in forming the ACh binding sites. But they can be incorporated in the pentamer as accessory subunits (Wang et al., 1996; Ramirez-Latorre et al., 1996; Groot-Kormelink et al., 1998), which can have dramatic effects on the conductance and desensitization of the receptor (Wang et al., 1996; Ramirez-Latorre et al., 1996; Gerzanich et al., 1998; Broadbent et al., 2006). The accessory subunits may also be involved in forming binding sites for positive allosteric modulators (Kuryatov et al., 2008).

In the brain, the most abundant nAChR subtype is $\alpha 4\beta 2^*$ (the asterisk indicates that other subunits such as $\alpha 5$ can contribute to the receptor) but there are numerous other heteromeric nAChR subtypes. An example of the diversity of nAChRs are the five different subtypes – $\alpha 4\alpha 6\beta 2\beta 3$, $\alpha 6\beta 2\beta b3$, $\alpha 6\beta 2$, $\alpha 4\beta 2$, $\alpha 4\alpha 5\beta 2$ – that are expressed on dopaminergic nerve terminals (Zoli et al., 2002; for a review, see Grady et al., 2007). The $\alpha 3\beta 4^*$ subtype is abundant in autonomic and sensory ganglia and is often dubbed the ganglionic subtype (Conroy and Berg, 1995; Xu et al., 1999a, 1999b; Mao et al., 2006). $\alpha 3\beta 4^*$ receptors may contain $\alpha 5$, $\beta 2$ or $\beta 3$ as additional subunits (Conroy and Berg, 1995; Mao et al., 2006; Vernallis et al., 1993; Sheffield et al., 2000; Grady et al., 2009). In rodent superior cervical ganglia 25–30% of nAChRs are of $\alpha 3\beta 4\alpha 5$ subtype (Mao et al., 2006; David et al., 2010). The distribution of $\alpha 3\beta 4^*$ subtype is very limited in the brain, mainly confined to structures of habenulo-interpeduncular pathway – medial habenula, fasciculus retroflexus and interpeduncular nucleus (IPN) – (Sheffield et al., 2000; Grady et al., 2009; Duvoisin et al., 1989; Baddick and Marks, 2011) as well as the inferior colliculus (Baddick and Marks, 2011) and pineal gland (Marks et al., 1998; Hernandez et al., 2004). The habenular complex is activated by negative reward or the absence of anticipated positive reward (Matsumoto and Hikosaka, 2007; Ji and Shepard, 2007; Salas et al., 2010) and, consequently, its activity is apparently increased during nicotine withdrawal (De Biasi and Salas, 2008). $\alpha 3\beta 4^*$ nAChRs in the habenulo-interpeduncular pathway also appear to be involved in nicotine self-administration and aversion. Glick and colleagues recently reported that inhibition of $\alpha 3\beta 4^*$ nAChRs in the habenula blocks self-administration of nicotine in rats while inhibition of $\alpha 3\beta 4^*$ nAChRs in the IPN increases nicotine self-administration (Glick et al., 2011). In addition, Frahm et al. (2011) reported that overexpression of the $\beta 4$ subunit in mice increased aversion to nicotine and this aversion was eliminated when $\alpha 5$ was overexpressed in the medial habenula. Based on available data, $\alpha 3\beta 4^*$ nAChRs may contribute to several aspects related to nicotine intake including reinforcement, aversion and withdrawal.

In 2007, Bierut and colleagues reported that a D398N (Asp398Asn, rs16969968) polymorphism in the human *CHRNA5* gene is associated with nicotine dependence (Saccone et al., 2007; Bierut et al., 2007). Subsequent studies have convincingly replicated this association (Bierut et al., 2008; Chen et al., 2009, 2011; Saccone et al., 2009a, 2009b; Wanget al., 2009; Johnson et al., 2010; Sherva et al., 2010; Hong et al., 2010; Smith et al., 2011) and refined it by showing a significant link between D398N and smoking quantity (Stevens et al., 2008; Sarginson et al., 2011; Falcone et al., 2011) or “pleasurable buzz” received from smoking (Sherva et al., 2008). A recent meta-analysis has also confirmed a highly significant link between D398N polymorphism and smoking quantity (Saccone et al., 2010). Furthermore, the D398N polymorphism also appears to be associated with cocaine dependence (Sherva et al., 2010; Grucza et al., 2008), alcohol abuse or dependence (Chen et al., 2009), opioid dependence (Sherva et al., 2010; Erlich et al., 2010), lung cancer (Wang et al., 2009; Young et al., 2008; Falvella et al., 2009; Lips et al., 2010; Truong et al., 2010; Sakoda et al., 2011), upper aerodigestive tract cancers (Chen et al., 2011; Lips et al., 2010) as well as chronic obstructive pulmonary disease (Young et al., 2008). These clinically relevant findings have spurred research to characterize $\alpha 5N^*$ nAChRs.

Recent studies have demonstrated that the D398N polymorphism affects the function of $\alpha 4\beta 2\alpha 5$ nAChRs (Bierut et al., 2008; Kuryatov et al., 2011). $\alpha 4\beta 2\alpha 5$ nAChRs containing the variant of $\alpha 5$ associated with increased risk for nicotine dependence (Asn at position 398) exhibited diminished agonist-evoked intracellular calcium response, reduced calcium permeability as well as enhanced short-term desensitization compared to $\alpha 4\beta 2\alpha 5$ nAChRs possessing the major variant of $\alpha 5$. However, conflicting results have been obtained regarding the effect of the polymorphism on the function of $\alpha 3\beta 4\alpha 5$ nAChRs. One study found a similar effect of the D398N polymorphism on the function of $\alpha 3\beta 4\alpha 5$ nAChRs (Frahm et al., 2011). However, two other reports indicated that the variant forms of the $\alpha 5$ subunit do not affect the electrophysiological properties of $\alpha 3\beta 4\alpha 5$ nAChRs (Kuryatov et al., 2011; Li et al., 2011). The aim of the present study was to employ additional approaches to further evaluate whether the $\alpha 5$ D398N polymorphism can affect the function and pharmacology of $\alpha 3\beta 4\alpha 5$ nAChRs.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals—Coelenterazine was purchased from AnaSpec (San Jose, CA), amphotericin B, penicillin, streptomycin and G418 from Fisher Scientific (Waltham, MA), hygromycin B from Invitrogen (Carlsbad, CA), mecamlamine from Merck (Rahway, NJ), [125 I]-epibatidine (specific activity 2200 Ci/mmol) from Perkin Elmer (Boston, MA) and FuGene transfection reagent from Roche Diagnostics (Indianapolis, IN). Acetylcholine (ACh), atropine, CdCl₂, cytosine, dextrose, *d*-tubocurarine, HEPES, meglumine, nicotine hydrogen tartrate and Triton-X100 were from Sigma (St. Louis, MO). Bupropion, ryanodine, 2,2,6,6-tetramethylpiperidin-4-yl heptanoate (TMPH), varenicline and xestospongine C were purchased from Tocris Bioscience (Ellisville, MO).

2.1.2. Cell culture—The HEK293 cell line stably expressing the human $\alpha 3\beta 4$ nAChR was developed as previously described (Li et al., 2011). The $\alpha 3\beta 4\alpha 5D$ and $\alpha 3\beta 4\alpha 5N$ stable lines were generated by transfecting the stable $\alpha 3\beta 4$ line with constructs containing the $\alpha 5D$ or $\alpha 5N$ subunits in pCDNA3 with a hygromycin-selectable element. Cells were doubly selected with G418 (1000 μ g/ml) and hygromycin B (200 μ g/ml). Surviving populations were then immunoselected three times with monoclonal antibody 35 (Sigma-Aldrich, St. Louis MO), which binds to an epitope on both the $\alpha 3$ and $\alpha 5$ subunits (see Li et al., 2011). The human $\alpha 5$ subunit cDNA was obtained from J. Lindstrom (University of Pennsylvania), originally provided by F. Clementi (Università Degli Studi di Milano, Milano, Italy). The

lines were maintained in DMEM (Fisher Scientific) supplemented with 4.5 g/l of glucose, L-glutamine, 10% of fetal bovine serum, 15 mM of HEPES and 1% each of penicillin, streptomycin and amphotericin B, 400 µg/ml of G418 and, for α3β4α5D/N lines, 100 µg/ml of hygromycin B.

2.2. Elisa

To measure cell surface nAChRs in stably expressing HEK293 cell lines, an ELISA was carried out. Cells seeded in 12-well dishes were washed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.3). The cells were blocked with 4% (w/v) powdered milk in PBS and then incubated with an antibody against the α3 and α5 nAChR subunits (mAb35; Sigma-Aldrich, St. Louis MO). After three additional washes with milk-PBS, the cells were incubated with horseradish peroxidase conjugated goat anti-rat secondary antibody, washed again with milk-PBS, and finally incubated with the horseradish peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine. The absorbance of the supernatant was measured with a plate reader (iMark, Biorad, Hercules CA). The nonspecific background binding of antibodies to cells was determined using non-transfected cells. The ELISA signal was normalized to the amount of protein in parallel wells and ratios between the α3β4-expressing cell line and cell lines expressing α3β4α5D or α3β4α5N were calculated.

2.3. Nicotinic acetylcholine receptor binding

2.3.1. Sample preparation—For binding samples of stably expressing HEK293 cell lines, the cells were seeded in 6-well plates as described above. For binding studies in nicotine-treated cells, the cells were incubated in 10 µM nicotine for 24 h before preparing the membrane samples. Control cells received equal amount of HBSS. Four days after plating, the cells were rinsed once with HBSS, scraped from wells and homogenized using a motorized tissue grinder with a Teflon pestle in 50 µl of 0.1× buffer (14 mM NaCl, 0.15 mM KCl, 0.1 mM MgSO₄, 0.2 mM CaCl₂). After homogenization, sample volumes were increased to 1 ml with 0.1× buffer and pelleted by centrifugation (5 min at 20,000 g). The homogenization – centrifugation wash cycle was repeated twice. Membrane homogenates were first incubated on ice for 1 h and then incubated in 0.01 mg/ml DNase I (Sigma, St Louis, MO) at 37 °C for 15 min. Incubated homogenates were pelleted by centrifugation at 15,000 g and resuspended three times in 1 ml of 0.1 × buffer. Pellets were stored at –80 °C under 0.1× buffer until binding assay.

2.3.2. Binding assay—Membrane pellets were homogenized in distilled deionized water. Reaction volume was 30 µl, consisting of membrane sample, 1× buffer (140 mM NaCl, 1.5 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂) and 200 pM [¹²⁵I]-epibatidine. 300 µM cytosine was included in non-specific binding samples. Reaction mixes were allowed to incubate for 2 h at room temperature. After incubation, samples were vacuum filtered to collect the [¹²⁵I]-epibatidine binding into polyethyleneimine-soaked filter paper. Radioactivity was recorded with Packard Cobra Auto-Gamma counter (Meriden, CT).

Protein concentration of the samples was assessed by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Samples were prepared in 96-well plates and they were analyzed at 562 nm wavelength with Epoch microplate spectrophotometer (BioTek, Winooski, VT).

2.4. Aequorin intracellular calcium assay

Functional nAChR activities were assessed in HEK293 cells stably expressing human α3, β4 and α5D/N subtypes by measuring intracellular calcium changes using aequorin, a calcium sensing luminescent protein, as previously described (Bierut et al., 2008; Karadsheh

et al., 2004). Cells were seeded in six-well plates (1.5×10^6 cells per well) and 24 h later they were transfected with human codon optimized aequorin cDNA (Vernon and Printen, 2002) using FuGene transfection reagent (Roche Diagnostics, Indianapolis, IN) and Opti-MEM reduced serum media (Invitrogen, Carlsbad, CA) as recommended by manufacturers.

Approximately 40–48 h after transfection, culture medium was replaced with DMEM with 0.1% fetal bovine serum plus 2.5 μ l coelenterazine per well and the cells were incubated for three hours at room temperature and minimal light exposure. After loading, the cells were aspirated from the wells, transferred into 1.5 ml microcentrifuge tubes and pelleted by centrifugation (5 min at 800 g, 4 °C). Thereafter, supernatant was discarded and the cells were resuspended into fresh assay buffer [Hank's Balanced Salt Solution (HBSS, Cellgro, Mediatech, Manassas, VA) supplemented to 10 mM CaCl_2] then pelleted and resuspended again. After washes the cells were incubated for one hour at 4 °C in fresh assay buffer under minimal light exposure. The cells were then pelleted by centrifugation (5 min at 800 g, 4 °C), resuspended in 1 ml of fresh assay buffer and 50 μ l of cell suspension was aliquoted on opaque white 96-well plates. Luminescence (lux) was recorded in a Victor V³ plate reader with chemiluminescence detector (PerkinElmer, Waltham, MA). Recordings included three steps: 1) one second pre-read, 2) 20-s stimulation read at 0.2 s intervals after addition of 50 μ l of appropriate agonist solution, 3) five-second lysis read at 0.1 s intervals after addition of 100 μ l of lysis solution (0.1% Triton X-100 and 100 mM CaCl_2 in distilled deionized water).

2.4.1. Agonist experiments—For agonist experiments, chemiluminescence was recorded immediately after cells were applied to 96-well plate. Concentration ranges were 1 μ M–1 mM for ACh, 3 μ M–1 mM for nicotine and 300 nM–100 μ M for varenicline. For experiments with SAP-102, the cells in half of the wells were transfected with SAP-102 plasmid and the cells were stimulated with 300 nM–1 mM ACh. 200 nM atropine was included in the assay mix to inhibit muscarinic receptors whenever ACh was used as the agonist.

2.4.2. Antagonist experiments—For antagonist experiments, the cells were pre-incubated with 10 nM–100 μ M mecamylamine, 100 nM–100 μ M α -tubocurarine, 10 nM–100 μ M bupropion or 3 nM–100 μ M TMPH for 10 min before chemiluminescence was recorded. Cells were stimulated with 1 mM ACh (200 nM atropine included in the assay mix).

2.4.3. Desensitization experiments—For desensitization experiments, the cells were incubated with nicotine (100 nM–300 μ M) for 30 min before chemiluminescence was recorded. Stimulation was carried out with 1 mM ACh in the presence of 10 mM Ca^{2+} . For experiments measuring stimulation of nicotinic receptors, 200 nM atropine was included in the assay mix. For experiments measuring stimulation of muscarinic receptors, 10 μ M mecamylamine was included in the assay mix.

2.4.4. Source of agonist-induced increase in intracellular calcium—To determine the mechanism of the agonist-induced change in intracellular calcium, the cells were incubated with CdCl_2 (100 μ M) to block voltage-operated calcium channels, ryanodine (30 μ M) to inhibit ryanodine receptor dependent calcium release from endoplasmic reticulum, xestospongin C (10 μ M) to antagonize IP_3 receptor-mediated calcium release, or a combination of ryanodine and xestospongin C for 30 min before chemiluminescence was recorded. 1 mM ACh was used as agonist in these experiments (200 nM atropine included in the assay mix). Since ryanodine and xestospongin C were dissolved in ethanol, control cells were incubated in HBSS with equal amount of ethanol.

For experiments with varying calcium concentrations, the calcium concentrations in assay mix ranged from 0 to 10 mM. 1 mM ACh was used as agonist (200 nM atropine included in the assay mix).

For experiments in the absence of sodium, one of these three solutions was used as the assay mix: 1) normal HBSS with 10 mM CaCl₂, 2) sodium-free HBSS with 10 mM CaCl₂, supplemented with dextrose to maintain osmolarity, 3) sodium-free HBSS with 10 mM CaCl₂ supplemented with meglumine to maintain osmolarity. 1 mM ACh was used as agonist in these experiments (200 nM atropine included in the assay mix).

2.5. Determination of calcium permeability

The cells were grown overnight to two days after plating. Macroscopic currents were recorded using standard whole-cell voltage clamp. The pipette solution contained: 130 mM sodium gluconate, 2 mM MgCl₂, 10 mM BAPTA, 10 mM HEPES, pH 7.4. The bath solution contained: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4. In addition, the bath solution contained 2 mM CaCl₂ ("low Ca²⁺") or 20 mM CaCl₂ ("high Ca²⁺"). The agonist (ACh) was applied through the bath using an SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT).

Calcium permeability estimates were based on the shift in the reversal potential of channel currents upon changes in the concentration of extracellular calcium. The cells were exposed to 1 s pulses of 100 μM ACh followed by a 9 s washout in bath. The cells were clamped at -25 to +30 mV with 5 mV step size and one current trace was recorded at each voltage. The currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 2 kHz, and acquired with a Digidata 1320 Series interface at 10 kHz. Data analysis was carried out using the pClamp 9.0 software package (Molecular Devices, Union City, CA). The peak current response at each voltage was determined following subtraction of leak currents measured in the absence of ACh at the same range of voltage steps. For each cell, measurements were conducted in "low Ca²⁺" (i.e., 2 mM CaCl₂-containing) and "high Ca²⁺" (20 mM CaCl₂-containing) bath solutions to estimate the shift in the reversal potential upon changes in extracellular Ca²⁺. The reversal potentials were estimated using linear interpolation on the data points immediately below and above the reversal potential. The individual estimated reversal potentials ranged from 2.9 mV to 19.7 mV. The average shift in the reversal potentials was 4.9 ± 1.6 mV (mean ± SD; 6 cells) for α3β4 receptors, and 5.6 ± 2.0 mV (5 cells) and 5.6 ± 1.1 mV (5 cells) for α3β4a5D and α3β4a5N receptors, respectively.

The permeability ratio, P_{Ca}/P_{Na} was calculated according to Uteshev (2010) (Equation (1))

$$\frac{P_{Ca}}{P_{Na}} = \frac{1}{4} - \frac{\gamma_{Na}^* [Na^+]_{out} e^{\frac{F(V_{rev} - V_{rev}^*)}{RT}} - \gamma_{Na} [Na^+]_{out}}{\gamma_{Ca} [Ca^{2+}]_{out} \left(1 + e^{\frac{FV_{rev}}{RT}}\right)^{-1} - \gamma_{Ca}^* [Ca^{2+}]_{out}^* e^{\frac{F(V_{rev} - V_{rev}^*)}{RT}} \left(1 + e^{\frac{FV_{rev}^*}{RT}}\right)^{-1}} \quad (1)$$

where $[Na^+]_{out}$ and $[Ca^{2+}]_{out}$ stand for the concentrations of Na⁺ and Ca²⁺ in the extracellular medium, V_{rev} stands for reversal potential, γ_{Ca} and γ_{Na} are the activity coefficients for calcium and sodium ions, and F , R , and T are the Faraday constant, universal gas constant, and absolute temperature, respectively. Parameters marked with asterisks stand for those in the presence of 20 mM external Ca²⁺. Parameters without asterisks mark those in the presence of 2 mM external Ca²⁺. The values of the individual activity coefficients were $\gamma_{Ca} = 0.27$, $\gamma_{Ca}^* = 0.29$, $\gamma_{Na} = 0.75$, and $\gamma_{Na}^* = 0.74$.

2.6. Data analysis

The data are presented as means $\pm$ SEM. To control for differences in cell number per well and variation in coelenterazine loading, results of the aequorin assays were calculated as $L/L_{\max}$ ratios, where L is the maximal peak value for agonist-stimulated luminescence and $L_{\max}$ the maximal peak agonist-stimulated luminescence plus maximal peak luminescence resulting from cell lysis in the presence of high calcium concentration. To reduce between-day variation, results from each experiment day were normalized as follows. Generally, 0% was set to be 0% $L/L_{\max}$ response and 100% the average of maximal response to agonist values in the $\alpha 3\beta 4$ group. However, inhibition and desensitization data were normalized to the maximal response within each subtype. Further, data from the experiment exploring source of agonist-induced increase in intracellular calcium were normalized to the response of corresponding control cells within each subtype. For nAChR antagonist data, results were normalized within each receptor subtype. Further analysis and plotting of the data from aequorin assays was carried out using GraphPad Prism (GraphPad Software, San Diego, CA). Nonlinear regression for response on log agonist or antagonist concentration was used as the curve fitting model to calculate EC_{50} or half-maximal inhibitory concentration (IC_{50}) values as well as minimum and maximum responses.

One-way and two-way ANOVAs were carried out in SPSS (IBM SPSS Statistics, Version 19). Because $R_{\max}$ and EC_{50} data did not meet criteria for normal distribution, Kruskal–Wallis ANOVA was used to test these data. Bonferroni comparisons were used as the post hoc test for one-way and two-way ANOVAs. Dunn's test was used as the post hoc test for Kruskal–Wallis ANOVA. Furthermore, t -test was used to test two-group comparisons. Results where $p < 0.05$ were considered significant.

3. Results

3.1. Influence of the addition of the variant forms of the $\alpha 5$ subunit on the number and surface expression of nAChRs

Fig. 1 shows [^{125}I]-epibatidine binding in HEK293 cell lines stably expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$. No significant cell line differences were observed. ELISA data also indicated that the surface expression of nAChRs for the three cell types did not differ: the $\alpha 3\beta 4\alpha 5/\alpha 3\beta 4$ ratio was 0.81 ± 0.08 ($t = 0.08$, $p > 0.05$) for $\alpha 3\beta 4\alpha 5D$ and 1.03 ± 0.05 ($t = 0.63$, $p > 0.05$) for $\alpha 3\beta 4\alpha 5N$.

3.2. Effect of nAChR subtype on agonist-induced increase in intracellular calcium

Representative calcium traces after stimulation with 1 mM nicotine are shown in Fig. 2. Nicotine, ACh and varenicline-induced increases in intracellular calcium in HEK293 cell lines stably expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$, or $\alpha 3\beta 4\alpha 5N$ are shown in Fig. 3. The calculated $R_{\max}$ and EC_{50} values as well as statistics calculated for the data are shown in Table 1. Independent of the agonist, addition of the $\alpha 5$ subunit into $\alpha 3\beta 4$ receptor decreases the response to agonist at high agonist concentrations without affecting the EC_{50} estimates. This effect is even more pronounced in $\alpha 3\beta 4\alpha 5N$ than in $\alpha 3\beta 4\alpha 5D$ receptors. Interestingly, $\alpha 3\beta 4\alpha 5N$ receptors showed higher EC_{50} for nicotine than $\alpha 3\beta 4\alpha 5D$ receptors. The $\alpha 3\beta 4\alpha 5N$ nAChRs exhibited reduced response relative to both the $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5D$ nAChRs at the highest two or three agonist concentrations tested.

Conroy et al. (2003) reported that the scaffolding protein SAP102 (*Dlg3*) specifically associates with $\alpha 5$ containing nAChRs. Therefore, SAP102 was tested for its effects on the function of the nAChR subtypes in the HEK293 stable cell lines. Transfection of the stably expressing HEK293 cell lines with SAP102 cDNA did not affect the response to ACh (data not shown) [$F_{2, 18} = 18.801$, $p < 0.001$].

3.3. Effect of nAChR subtype on noncompetitive antagonist inhibition of agonist-induced increase in intracellular calcium

The noncompetitive nicotinic receptor antagonists mecamylamine, bupropion, *α*-tubocurarine and TMPH were examined for their ability to inhibit ACh-induced calcium response in HEK293 cell lines stably expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$ (Fig. 4 and Table 1). nAChR subtype affected IC_{50} values of mecamylamine, bupropion and TMPH but not *α*-tubocurarine. Both $\alpha 5$ -containing nAChR subtypes differed from $\alpha 3\beta 4$ nAChRs in IC_{50} for TMPH while only the $\alpha 3\beta 4\alpha 5N$ variant differed from $\alpha 3\beta 4$ nAChRs for mecamylamine and bupropion. However, the magnitude of IC_{50} shift was moderate: in $\alpha 3\beta 4\alpha 5N$ expressing cells the IC_{50} values of mecamylamine, bupropion and TMPH were shifted only 1.6–2.5fold to lower concentration. The $\alpha 3\beta 4\alpha 5D$ and $\alpha 3\beta 4\alpha 5N$ subtypes did not differ in sensitivity to inhibition by any of the tested antagonists.

3.4. Desensitization of agonist-induced calcium influx in $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ and $\alpha 3\beta 4\alpha 5N$ cell lines

Nicotine-induced desensitization of agonist-induced calcium response in HEK293 cell lines stably expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$ is shown in Fig. 5 and IC_{50} values as well as results of statistical tests are shown in Table 1. The IC_{50} values for nicotine-induced desensitization were not statistically significant between the cell lines. The decrease in agonist-evoked changes in intracellular calcium following exposure to nicotine was due to desensitization and not loss of available aequorin due to extended exposure to agonist (Appendix A, Table 1). Nicotine pretreatment did not affect the function of endogenous muscarinic receptors (data not shown).

We probed the effect of change in external calcium concentration on receptor desensitization using whole-cell electrophysiology. Cells expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$ receptors were exposed to a 40 s pulse of 100 μM ACh. Desensitization was quantified by calculating the ratio of the mean current response at the end of the 40 s drug application relative to the peak current. The data indicate that an increase in the concentration of external calcium significantly enhances the extent of desensitization for $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5D$ receptors but not for $\alpha 3\beta 4\alpha 5N$ receptors (Fig. 6).

3.5. Effect of nAChR subtype on the source of agonist-induced increase of intracellular calcium

Agonist-evoked increases in intracellular calcium could arise through several sources including direct entry through the nAChR channel, activation of voltage operated calcium channels, or release from internal stores regulated by ryanodine or IP_3 receptors. Each of these potential sources of calcium was assessed for the potential effect of the D398N polymorphism and/or their contribution to the agonist-evoked increase in intracellular calcium in the HEK293 cell lines expressing the $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ and $\alpha 3\beta 4\alpha 5N$ nAChR subtypes. Electrophysiological experiments showed that the P_{Ca}/P_{Na} ratio was 2.9 ± 0.4 for $\alpha 3\beta 4$ ($n = 6$), 3.7 ± 0.7 for $\alpha 3\beta 4\alpha 5D$ ($n = 5$) and 3.7 ± 0.3 for $\alpha 3\beta 4\alpha 5N$ ($n = 5$). ANOVA with Bonferroni correction indicated that the differences between $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5D/N$ are not significant ($p > 0.5$). These results indicate that calcium permeability was not significantly different between the nAChR subtypes. In addition, $CdCl_2$, an inhibitor of voltage sensitive Ca^{2+} channels, did not affect the response induced by 1 mM ACh in any of the three cell lines (Appendix B, Fig. 1A). However, blockade of ryanodine receptors by ryanodine decreased the agonist-induced response and the effect was similar in all cell lines (Appendix B, Fig. 1B). Inhibition of the IP_3 receptors by xestospongine C also decreased the agonist-induced response. However, the xestospongine C-induced inhibition of agonist-induced calcium response was significantly less in $\alpha 3\beta 4$ -expressing cells than in cells expressing $\alpha 3\beta 4\alpha 5D/N$ receptors (see Fig. 7 and Appendix B, Fig. 1C).

Combination of ryanodine and xestospongin C produced an inhibition of agonist-induced calcium response that was larger in $\alpha 3\beta 4\alpha 5D/N$ -expressing cell lines than in the cell line expressing $\alpha 3\beta 4$ (see Fig. 7 and Appendix B, Fig. 1D). In the $\alpha 3\beta 4\alpha 5D/N$ -expressing cell lines the inhibition was also more pronounced than with ryanodine or xestospongin C alone. For $\alpha 3\beta 4\alpha 5$ nAChRs, genotype of the $\alpha 5$ subunit did not appear to have an effect on any of these measures.

Previous studies have shown that increasing concentrations of extracellular calcium can potentiate the function of nAChRs (Mulle et al., 1992; Vernino et al., 1992). To assess whether the three nAChR subtypes are differentially responsive to the potentiation effect of external calcium and to determine the necessity of external sodium for agonist-elicited responses, nAChR function was assessed under conditions of altered extracellular calcium concentrations and in the presence or absence of extracellular sodium. Effects of varying extracellular calcium (Panel A) and sodium concentrations (Panel B) on ACh-induced calcium response in HEK293 cell lines stably expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$ are shown in Fig. 8. Cell lines responded differently to varying extracellular calcium concentration. Differences in the ACh-stimulated intracellular calcium response between the cell lines were clearly visible only at 10 mM. At lower concentrations the differences were not observed. In addition, the effect of the D398N polymorphism on the response of $\alpha 3\beta 4\alpha 5$ nAChRs to 1 mM ACh was found to be independent of extracellular sodium as the effect of the polymorphism was maintained when sodium was removed from the assay buffer (Fig. 8B).

4. Discussion

In this study we found that the maximal agonist-induced calcium response is smaller in HEK293 cells expressing $\alpha 3\beta 4\alpha 5D$ nAChRs than $\alpha 3\beta 4$ nAChRs. The response is further diminished in cells expressing the $\alpha 3\beta 4\alpha 5N$ nAChR variant suggesting that the D398N polymorphism in the $\alpha 5$ subunit affects the function of $\alpha 3\beta 4\alpha 5$ nAChRs. The same general result was observed with all three agonists tested, acetylcholine, nicotine and varenicline. The cell line differences in nAChR subtype function could not be attributed to differences in receptor pharmacology, Ca^{2+} permeability, receptor number or surface expression of the nAChR subtypes. Therefore, we assessed whether inclusion of the $\alpha 5$ subunit affects downstream calcium signaling and if so, whether the $\alpha 5D$ or $\alpha 5N$ subunits differentially affect the release of calcium from intracellular stores. Ryanodine, an agent that inhibits ryanodine receptor-mediated calcium release from endoplasmic reticulum, diminished agonist-induced calcium response equally in all three cell lines. However, xestospongin C, which blocks IP_3 receptor-mediated intracellular calcium release, induced a much more pronounced inhibition of agonist-induced calcium response in the $\alpha 3\beta 4\alpha 5D$ and $\alpha 3\beta 4\alpha 5N$ -expressing cell lines relative to the $\alpha 3\beta 4$ -expressing cell line. These data indicate that despite similar pharmacologies and calcium permeability, $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ nAChRs differ not only in maximal response to agonists but also lead to distinct downstream cellular signaling. Consequently, activation of $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ nAChRs may produce disparate effects on neuronal function. This finding is in accord with an earlier discovery that different nAChR subtypes are differently coupled to specific Ca^{2+} sources (Dajas-Bailador et al., 2002). Regardless, the sources of agonist-evoked increases in intracellular calcium were similar from $\alpha 3\beta 4\alpha 5$ nAChRs containing either variant of the $\alpha 5$ subunit. Thus, the observed effect of the D398N variant on agonist-evoked responses cannot be attributed to a differential effect on the two intracellular calcium stores.

The effect of the variant forms of the $\alpha 5$ subunit on receptor function is consistent with our previous finding that the N398 variant of the $\alpha 5$ subunit leads to a reduced maximal Ca^{2+} response to agonist in $\alpha 3\beta 4\alpha 5$ nAChRs (Bierut et al., 2008). Recently, Frahm et al. (2011)

reported that the N398 variant of $\alpha 5$ leads to reduced function of $\alpha 3\beta 4\alpha 5$ nAChRs in frog oocytes. Kuryatov et al. (2011) also confirmed the effect of the D398N polymorphism in heterologously expressed $\alpha 3\beta 4\alpha 5$ nAChRs and reported that the variant forms of the $\alpha 5$ subunit affected function by altering calcium permeability and short-term desensitization. In contrast, this group did not observe an effect of the polymorphism on either of these parameters in $\alpha 3\beta 4\alpha 5$ nAChRs. Li et al. (2011) also did not find an effect of the $\alpha 5$ D398N polymorphism on a number of biophysical or pharmacological properties of $\alpha 3\beta 4\alpha 5$ nAChRs. Although the results of Kuryatov et al. (2011) and Li et al. (2011) appear to be in conflict with the data reported here, we point out that at the external calcium concentrations used by Kuryatov et al. (2011) and Li et al. (2011) we also did not see an effect of the D398N polymorphism.

Prior studies have shown that high external calcium concentrations potentiate the function of nAChRs (Mulle et al., 1992; Amador and Dani, 1995; Booker et al., 1998; Liu and Berg, 1999; Zhang et al., 1999). The data presented in this report also demonstrate the potentiating effect of external calcium on $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ and $\alpha 3\beta 4\alpha 5N$ nAChRs. As mentioned previously, this potentiating effect of external calcium is necessary to detect an effect of the D398N polymorphism on the function of $\alpha 3\beta 4\alpha 5$ nAChRs. Although the mechanism by which high external calcium unmasks the functional effect of the polymorphism is not known, it is not likely due to nAChR subtype specific effects on sodium flux through the receptor since the effect of the polymorphism at high calcium concentrations is independent of external sodium. The variant forms of $\alpha 5$ also might differentially affect the ability of calcium to potentiate the function of $\alpha 3\beta 4\alpha 5$ nAChRs. Further studies are needed to determine the mechanism by which increased external calcium unveils the functional effect of the $\alpha 5$ D398N polymorphism in $\alpha 3\beta 4\alpha 5$ nAChRs.

The D398N polymorphism is located within the membrane-associated stretch (MA stretch) of large cytoplasmic loop of the $\alpha 5$ polypeptide between transmembrane domains three and four. In addition to having an impact on channel conductance, the large cytoplasmic loop interacts with chaperone proteins and other subunits, affecting stability of subunit complexes as well as assembly, clustering and trafficking of nAChRs (Williams et al., 1998; Jeanclos et al., 2001; Ortiz et al., 2005; Zhao et al., 2009). Within the large intracellular loop, the MA stretch is a key location determining channel conductance in $\alpha 4\beta 2^*$ (Peters et al., 2006), $\alpha 3\beta 4\alpha 5$ (Frahm et al., 2011) and 5-HT₃ receptors (Kelley et al., 2003; Peters et al., 2006). Adding an $\alpha 5$ subunit to a receptor complex does not seem to affect nAChR binding as seen in this study and in an earlier report of $\alpha 4\beta 2^*$ receptors (Bierut et al., 2008). This is in accordance with the fact that the accessory subunits $\alpha 5$ and $\beta 3$ do not contribute to the ACh binding site (Gotti et al., 2007). We also did not find changes in the surface expression of $\alpha 3\beta 4^*$ receptors. These findings suggest that the D398N polymorphism probably does not have an impact on the interaction between the large intracellular loop and intracellular chaperone proteins that would affect the numbers of [¹²⁵I]-epibatidine binding sites or surface receptors.

The EC₅₀ value for activation of $\alpha 3\beta 4^*$ nAChRs by nicotine is relatively high (approximately 4.6 μ M) and, therefore, $\alpha 3\beta 4^*$ nAChRs may not be activated by nicotine levels that smokers sustain in the plasma for most of their awake hours (0.2 μ M; Benowitz et al., 1996). Despite the reduced probability of direct activation by tobacco-derived nicotine, $\alpha 3\beta 4^*$ receptors are likely to be activated by endogenous ACh during the processes involved in the development of nicotine addiction. The medial habenula and the $\beta 4$ and $\alpha 5$ nAChR subunits within it have been linked with nicotine self-administration and aversion to nicotine (Frahm et al., 2011; Fowler et al., 2011; Glick et al., 2011) as well as nicotine withdrawal symptoms (Salas et al., 2004, 2009; Jackson et al., 2008). In rats, $\alpha 3\beta 4$ receptor antagonists have been shown to reduce the self-administration of cocaine (Glick et

al., 1996), ethanol (Rezvani et al., 1997), methamphetamine (Glick et al., 2000, 2002b), morphine (Glick et al., 1996, 2002b), and nicotine (Glick et al., 2000, 2002a), as well as withdrawal symptoms of morphine (Rho and Glick, 1998). This effect is evidently mediated through the medial habenula (Glick et al., 2011, 2006, 2008) and, in the case of opioids and stimulants, IPN (Glick et al., 2006, 2008). Nicotine self-administration, instead, is increased if $\alpha 3\beta 4^*$ receptors in IPN are blocked (Glick et al., 2011). Peripheral $\alpha 3\beta 4^*$ receptors residing in autonomic ganglia also may be involved in somatic withdrawal symptoms. Therefore, alterations in the normal functioning of these receptors may affect proneness to addiction as well as severity of withdrawal symptoms, making it more easy to initiate smoking and more difficult to quit. Taken collectively, these findings suggest that $\alpha 3\beta 4^*$ -mediated mechanisms are involved in drug self-administration as well as withdrawal symptoms. Since a large number of $\alpha 3\beta 4^*$ receptors also contain an $\alpha 5$ subunit, the D398N polymorphism may affect self-administration as well as withdrawal symptoms of nicotine and possibly also other substances of abuse.

In conclusion, the D398N polymorphism affects the maximal agonist-induced calcium response from $\alpha 3\beta 4\alpha 5$ nAChRs expressed in HEK293 cells under conditions of high external calcium. However, the mechanism through which the polymorphism affects receptor function remains unclear. Nonetheless, the data reported here do indicate that the *CHRNA5* D398N polymorphism can affect the function of $\alpha 3\beta 4\alpha 5$ nAChRs which could contribute to the role of this polymorphism in nicotine dependence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACh	acetylcholine
cDNA	complementary DNA
<i>CHRNA5</i>	human nicotinic acetylcholine receptor subunit $\alpha 5$ gene
DMEM	Dulbecco's modification of Eagle's medium
EC₅₀	half-maximal effective concentration
HBSS	Hank's balanced salt solution
HEK293 cells	human embryonic kidney cells
IC₅₀	half-maximal inhibitory concentration
IP₃	inositoltrisphosphate
IPN	interpeduncular nucleus
nAChR	nicotinic acetylcholine receptor
$R_{\max}$	maximal response
TMPH	2,2,6,6-tetramethylpiperidin-4-yl heptanoate

Fig. 1. [^{125}I]-Epibatidine binding in HEK293 cells stably expressing human $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$. $n = 16$. Results are given as means $\pm$ SEM of femtomoles of [^{125}I]-epibatidine bound per milligram of protein. Two-way ANOVA did not show a significant difference between cell lines [$F_{2, 47} = 1.018$, $p > 0.05$].

Fig. 2.
Representative calcium traces from aequorin assay in HEK293 cell lines stably expressing human nicotinic acetylcholine receptor subtypes $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$. The cells were stimulated with 1 mM nicotine.

Fig. 3.

Nicotine, acetylcholine and varenicline-induced intracellular calcium response in HEK293 cell lines stably expressing human nicotinic acetylcholine receptor subtypes $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5\text{D}$ or $\alpha 3\beta 4\alpha 5\text{N}$. Stimulation was performed with different concentrations of nicotine, acetylcholine or varenicline in the presence of 10 mM Ca^{2+} . Atropine (200 nM) was included in the assay mix to block muscarinic receptors. Data is normalized to the average maximal response of $\alpha 3\beta 4$ cells. $\alpha 3\beta 4\alpha 5$ receptors showed decreased response to agonists. Two-way ANOVAs for the agonist curves: Nicotine, nAChR subtype effect $F_{2,125} = 114.2$, $p < 0.001$; Acetylcholine, nAChR subtype effect $F_{2,104} = 32.46$, $p < 0.001$; Varenicline, nAChR subtype effect $F_{2,161} = 74.32$, $p < 0.001$. Post-hoc results for $\alpha 3\beta 4$ vs. $\alpha 3\beta 4\alpha 5\text{D}$: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; for $\alpha 3\beta 4$ vs. $\alpha 3\beta 4\alpha 5\text{N}$: # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$; and for $\alpha 3\beta 4\alpha 5\text{D}$ vs. $\alpha 3\beta 4\alpha 5\text{N}$: \$ = $p < 0.05$, \$\$ = $p < 0.01$, \$\$\$ = $p < 0.001$. Results are given as normalized response values $\pm$ SEM.

Fig. 4. Inhibition of acetylcholine-induced intracellular calcium response by mecamylamine, *d*-tubocurarine, bupropion or TMPH in HEK293 cell lines stably expressing human nicotinic acetylcholine receptor subtypes $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$. Cells were stimulated with 1 mM acetylcholine in the presence of 10 mM Ca^{2+} . Atropine (200 nM) was added to block muscarinic receptors. TMPH = 2,2,6,6-tetramethylpiperidin-4-yl heptanoate. $n = 10-12$. Results are given as means of normalized response $\pm$ SEM. Curves are normalized to the response at 0 μM of antagonist within each cell line.

Fig. 5.

Nicotine-induced desensitization in HEK293 cell lines stably expressing human $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$ nicotinic acetylcholine receptor subtypes. Prior to stimulation, the cells were pretreated for 30 min with different concentrations of nicotine. Thereafter, the cells were stimulated with 1 mM acetylcholine in the presence of 10 mM Ca^{2+} . Atropine (200 nM) was included in the assay mix to block muscarinic receptors. $n = 9$. Results are given as means of normalized response $\pm$ SEM. Data is normalized to the response at 0 μM nicotine within each cell line.

Fig. 6.

The effect of the $\alpha 5$ variant on receptor desensitization. (A) Macroscopic traces from HEK293 cells expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$ receptors elicited by 40 s applications of 100 μM ACh. The bath solution contained 2 (“low calcium”) or 20 mM (“high calcium”) CaCl_2 . The membrane potential was -60 mV. The traces were analyzed with respect to the relative current amplitude at the end of the 40 s drug application. (B) Summary of the data. The plot shows mean $\pm$ SEM for the residual currents from 5 to 14 cells. Statistical analysis (t -test) was conducted with respect to the effect of change in external Ca^{2+} concentration. ** = $p < 0.01$; ns = not significant. We also examined the effect of the presence of the $\alpha 5D$ or $\alpha 5N$ subunit in the receptor-complex within the 2 mM and 20 mM Ca^{2+} groups. No significant differences (ANOVA with Bonferroni post hoc correction) were observed.

Fig. 7.

Effect of CdCl₂, ryanodine and xestospongine C on acetylcholine-induced calcium response in HEK293 cell lines stably expressing human α3β4, α3β4α5D or α3β4α5N nicotinic acetylcholine receptors. Cells were stimulated with 1 mM acetylcholine in the presence of 10 mM Ca²⁺ and 200 nM atropine. IP₃ receptor blocker, xestospongine C, has a differential effect in cells expressing α3β4 receptors and α3β4α5D/N receptors. More precisely, the IP₃-mediated intracellular calcium release is more pronounced in cells expressing α5* receptors. Two-way ANOVA results for the data presented in this figure: treatment effect: $F_{3, 130} = 77.275$, $p < 0.001$; subtype effect: $F_{2, 130} = 23.128$, $p < 0.001$; treatment × subtype interaction: $F_{6, 130} = 3.286$, $p < 0.01$. XeC = xestospongine C. ** = $p < 0.01$, *** = $p < 0.001$ vs. corresponding treatment in α3β4. $n = 8-12$. Data is presented as means of percentages of the corresponding control ± SEM. Data is normalized to the response of corresponding control cells to 1 mM acetylcholine within each receptor subtype.

Fig. 8.

Effects of varying extracellular calcium (Panel A) and absence of sodium (Panel B) on ACh-induced intracellular calcium response in HEK293 cell lines stably expressing human $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5D$ or $\alpha 3\beta 4\alpha 5N$. The cells were stimulated with 1 mM acetylcholine in the presence of varying Ca^{2+} and Na^{+} concentrations. Atropine (200 nM) was included in the assay mix to block muscarinic receptors. Panel A: Ca^{2+} concentration effect: $F_{7, 227} = 116.2$, $p < 0.001$; subtype effect: $F_{2, 227} = 11.08$, $p < 0.001$; concentration $\times$ subtype interaction: $F_{14, 227} = 2.095$, $p < 0.05$; Bonferroni post hoc comparisons: $\alpha 3\beta 4$ vs. $\alpha 3\beta 4\alpha 5N$ $p < 0.001$, $\alpha 3\beta 4\alpha 5D$ vs. $\alpha 3\beta 4\alpha 5N$ $p < 0.05$. Panel B: subtype effect: $F_{2, 41} = 35.73$, $p < 0.001$. ** = $p < 0.01$, *** = $p < 0.001$, $\alpha 3\beta 4$ vs. $\alpha 3\beta 4\alpha 5N$, # = $p < 0.05$, ### = $p < 0.001$ $\alpha 3\beta 4\alpha 5D$ vs. $\alpha 3\beta 4\alpha 5N$. $n = 12-14$. Results are given as means of normalized response $\pm$ SEM. Data is normalized to the average maximal response of $\alpha 3\beta 4$ subtype to 1 mM ACh in the presence of 10 mM Ca^{2+} .

Table 1

EC₅₀ values for agonist-induced stimulation of intracellular calcium response and IC₅₀ values for antagonist-induced inhibition of acetylcholine-induced intracellular calcium response in HEK293 cells stably expressing human α3β4, α3β4α5D or α3β4α5N nicotinic acetylcholine receptor subtypes. Stimulation was performed in the presence of 10 mM Ca²⁺.

Compound	<i>n</i>	α.3β4	α.3β4α.5D	α.3β4α.5N
<i>Agonists</i>				
Nicotine	8	EC ₅₀ (μM)	149 ± 11	531 ± 251 #
		<i>R</i> _{max} %	88 ± 5	29 ± 3 ***
Acetylcholine	6	EC ₅₀ (μM)	112 ± 28	53 ± 21
		<i>R</i> _{max} %	100 ± 10	24 ± 6 *
Varenicline	12	EC ₅₀ (μM)	24 ± 12	36 ± 26
		<i>R</i> _{max} %	110 ± 9	32 ± 5 ***
<i>Antagonists</i>				
Mecamylamine	12	IC ₅₀ (μM)	1.18 ± 0.17	0.44 ± 0.09 **
D-Tubocurarine	12	IC ₅₀ (μM)	1.01 ± 0.24	0.64 ± 0.15
Bupropion	12	IC ₅₀ (μM)	0.67 ± 0.07	0.38 ± 0.09 *
TMPH	12	IC ₅₀ (μM)	0.83 ± 0.10	0.45 ± 0.04 *
<i>Desensitization</i>				
Nicotine	10	IC ₅₀ (μM)	0.7 ± 0.2	0.8 ± 0.3

Results of the statistical tests: Kruskal–Wallis tests for EC₅₀, IC₅₀ and R_{max} values: nicotine EC₅₀ H = 8.405; p < 0.05, R_{max} H = 15.77; p < 0.001; acetylcholine EC₅₀ H = 2.558, p > 0.05, R_{max} H = 7.797, p < 0.05; varenicline EC₅₀ H = 0.4230; p > 0.05, R_{max} H = 23.42; p < 0.001; mecamylamine IC₅₀ H = 12.53; p < 0.01; d-tubocurarine IC₅₀ H = 1.891; p > 0.05; bupropion IC₅₀ H = 6.444; p < 0.05; TMPH IC₅₀ H = 14.77; p < 0.001; nicotine desensitization IC₅₀ H = 4.025; p > 0.05.

* p < 0.05,

** p < 0.01,

*** p < 0.001 α3β4 vs. α3β4α5D or α3β4α5N;

[#] p < 0.05 α3β4α5D vs. α3β4α5N. Data are given as means ± SEM.