ROLES FOR NICOTINIC ACETYLCHELINE RECEPTOR SUBUNIT LARGE CYTOPLASMIC LOOP SEQUENCES IN RECEPTOR EXPRESSION AND FUNCTION

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Running Title: The Large Cytoplasmic Loop in Nicotinic Receptor Subunits

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Number of text pages = 40 excluding tables and figures
Number of tables = 3
Number of Figures = 7
Number of References = 37
Number of words in abstract = 215
Number of words in Introduction = 644
Number of words in Discussion = 1,492

Abbreviations used: C2, large, second cytoplasmic loop of subunits in the four-transmembrane domain superfamily of neurotransmitter receptors; DHβE, dihydro-β-erythroidine; DMEM, Dulbecco’s modified Eagle’s medium; DMPP, 1,1-dimethyl-4-phenyl-piperazinium; EBDN, epibatidine; H-EBDN, [3H]-epibatidine; M3 or M4, third or fourth transmembrane domains, respectively, in subunits of the four transmembrane domain superfamily of ionotropic neurotransmitter receptors; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptors; PCR, Polymerase Chain reaction; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; β2χ, nAChR β2 subunit-based chimeric subunits containing 5-HT3A subunit sequences as specified; 5-HT3, 5-hydroxytraptamine or serotonin type 3 receptor subunit.

Recommended Section: Neuropharmacology
ABSTRACT

To evaluate possible physiological roles of the large cytoplasmic loops (C2) and neighboring transmembrane domains of nicotinic acetylcholine receptor (nAChR) subunits, we generated novel fusion constructs in which human nAChR α4, β2 or β4 subunit C2 or C2 and neighboring sequences were replaced by corresponding sequences from the mouse serotonin type 3A (5-HT3A) receptor subunit. Following stable expression in human SH-EP1 cells, we found that extensive sequence substitutions involving third and fourth transmembrane domains and neighboring “proximal” C2 sequences (e.g., β2 H322-V335 and V449-R460) did not allow functional expression of nAChR containing chimeric subunits. However, expression of functional nAChR was achieved containing wild-type α4 subunits and chimeric β2 (β2χ) subunits whose “nested” C2 domain sequences K336-S448 were replaced with the corresponding 5-HT3A subunit sequences. Whereas these findings suggested indispensable roles for M3/M4 transmembrane and/or proximal C2 sequences in α4β2-nAChR function, nested C2 sequences in the β2 subunit are not essential for functional receptor expression. Ligand-binding analyses also revealed only subtle differences in pharmacological profiles of α4β2-nAChR compared to α4β2χ-nAChR. Nevertheless, there was heightened emergence of agonist-mediated self-inhibition of α4β2χ function, greater sensitivity to functional blockade by a number of antagonists, and faster and more complete acute desensitization of α4β2χ-nAChR than for α4β2-nAChR. These studies are consistent with unexpected roles of nested C2 sequences in nAChR function.
INTRODUCTION

Nicotinic acetylcholine receptors (nAChR) are members of a ligand-gated ion channel superfamily, each comprised of a homo- or hetero-pentameric assembly of distinct subunits (Lindstrom, 1996; Lukas, 1998; Karlin, 2002). All nAChR subtypes mediate transmembrane ion flux upon activation by interaction with the endogenous neurotransmitter, acetylcholine (ACh), or the tobacco alkaloid, nicotine. However, each nAChR subtype possesses unique channel properties dictated in part by the subtle diversity of its constituent subunits.

Each of the 17, genetically-distinct, vertebrate nAChR subunits identified to date shares a common topology, containing: a large, extracellular, N-terminal domain; four transmembrane domains; a short, cytoplasmic domain between the first and second transmembrane segments; a short, extracellular domain between second and third transmembrane segments; a large, second cytoplasmic loop (C2) situated between the third (M3) and fourth (M4) transmembrane domains; and a short C-terminal, extracellular tail. The N-terminal domain contains key elements for ligand-binding/recognition (Sine, 2002), and the transmembrane domains anchor the proteins in the plasma membrane and contribute to channel kinetics and ion selectivity (Corringer et al., 2000). These structural domains are well conserved among different subunits and have been studied extensively. On the other hand, the less-studied, C2 domain of each subunit contains unique sequences that are distinguishing fingerprints for each subunit. These C2 domains have been suggested to play potential roles in regulation of nAChR trafficking (Williams et al., 1998), mediation of cytoskeletal interactions (Bencherif and Lukas, 1993; Colledge and Froehner, 1997; Shoop et al., 2000), receptor assembly (Yu and Hall, 1994b), functional desensitization of nAChR (Fenster et al., 1999), and as targets of phosphorylation (Yu and Hall, 1994a; Colledge and Froehner, 1997), perhaps affecting and being affected by intracellular signaling cascades.
However, aside from studies focused on homomeric α7-nAChR (Valor et al., 2002), examination of functional roles of C2 has been scant.

5-hydroxytryptamine (serotonin) type 3 (5-HT3) receptors also are members of the four transmembrane domain, ligand-gated ion channel superfamily, and the relevant subunits share topological features of nAChR subunits. Receptors made of 5-HT3A subunits can form functional channels as homomers, although combination with a newly identified 5-HT3B subunit allows assembly of heteromers that have altered biophysical properties relative to 5-HT3A receptor homomers (Brady et al., 2001). Nevertheless, recombinant heteromers display pharmacological features, large single-channel conductance and low calcium permeability like those of native 5-HT3 receptors (Davies et al., 1999). Chimeric subunit models constructed from partial replacement of nAChR subunit sequences with 5-HT3A subunit sequences have been used to advantage in assessing nAChR structure-function relationships. For example, recombinant, chimeric subunits containing the N-terminal domain of nAChR α7 (Eisele et al., 1993) or α8 (Cooper and Millar, 1998) subunits fused to more C-terminal regions of the 5-HT3A receptor subunit (that include all four of the transmembrane segments, the intervening cytoplasmic and extracellular domains, and the C-terminal domain) have been successfully used in studies aimed to reveal regions of physiological significance engaged in toxin- or ligand-binding or in ion channel selectivity. Nevertheless, the ability of 5-HT3A receptor subunits to form homomers and their topological similarities to nAChR subunits suggests that they also would be good fusion partners for studies of other domains in the subunit superfamily.

To evaluate possible physiological roles of C2 and neighboring transmembrane domains of nAChR subunits, we generated novel fusion constructs in which human nAChR α4, β2 or β4 subunit C2 sequences or both C2 and neighboring transmembrane sequences were replaced by
corresponding sequences from the mouse 5HT3A receptor subunit. We find evidence for different roles in nAChR assembly and function of M3 and M4 transmembrane domains and immediately flanking “proximal” C2 sequences (i.e., C2 domain residues just C-terminal to M3 or N-terminal to M4 domains; see Fig. 1) or for “nested” C2 sequences (i.e., C2 domain residues between the conserved “proximal” residues immediately flanking M3 and M4 transmembrane domains; see Fig. 1). A preliminary report of some of these findings has appeared (Kuo et al., 2002).

METHODS

Materials. Unless otherwise noted, all compounds used, including (-)-nicotine ditartrate, were analytical grade and products of Sigma Chemical Company (St. Louis, MO). Whether for ion efflux, binding or electrophysiological studies, drug solutions of desired molar strengths were made based on compound formula weights, and buffered solutions containing drugs were at neutral pH. Dulbecco’s modified Eagle’s medium (DMEM), trypsin, glutamine-penicillin/streptomycin solution, sodium pyruvate, amphotericin B, and horse sera were purchased from GIBCO BRL (Gaithersburg, MD), and fetal calf sera were obtained from Hyclone (Logan, UT).

Construction and Subcloning of Human nAChR - Mouse 5-HT3A Subunit Chimeric cDNAs. Human nAChR α4 subunit cDNA was excised as a KpnI-XhoI fragment from a pcDNA3.1-zeo (Invitrogen, Carlsbad, CA)-based expression construct and subcloned into the pCEP4 (Invitrogen) plasmid vector, and human nAChR β2 or β4 subunit cDNAs were subcloned into pcDNA3.1-zeo or pcDNA3.1-hygro as described (Eaton et al., 2003). The mouse 5-HT3A subunit cDNA carried by pcDNA1 vector was a gift from Dr. Philippe Séguela (Montreal...
Neurological Institute). Similar strategies employing successive polymerase chain reactions (PCRs) were used for generation of human nAChR α4, β2, or β4 - mouse 5-HT3A subunit chimeric cDNAs. Details are provided here for construction of the nAChR β2 - 5-HT3A subunit chimera studied [see Fig. 1 for the schematic illustration of the constructs (Fig. 1A) and their sequences (Fig. 1B)]. First, three partial chimeric cDNA fragments were synthesized: one coding for the N-terminus to the 335th amino acid residue (counting from the initiation methionine; M1-V335) of the human nAChR β2 subunit, another coding for part of the putative C2 of the mouse 5-HT3A subunit (amino acids R347-A448), and the third coding for amino acid residues 449 to the C-terminus of the human nAChR β2 subunit (V449-K502; Fig.1A; Fragments I, II and III). Chimeric PCR primers were designed so that the amplified partial cDNA fragments carried 18- or 17-bp of sequence overlap at the intended nAChR β2/5-HT3A cDNA sequence junctions. Sequences of the primers are as follow: sense 5’-taatacgactcactatataggg-3’ (located within the pcDNA3.1-hygro T7 promoter) and chimeric anti-sense 5’-gtctaggaccaagtgtgcccaacctcggcccagctgg-3’ (italicized fonts, 5-HT3A sequence; regular font, nAChR β2 sequence) for fragment I; chimeric sense 5’-ccatggcgcctgggttaggcagcttgctgtctagac-3’ and chimeric anti-sense 5’-acgtacttccagtcctgccacctceccgcatct-3’ for fragment II; chimeric sense 5’-gagatgcgggaggtgaggctgaggctgtacg-3’ and anti-sense 5’-tagaaggcacagtcgagg-3’ (3’ to the multiple cloning site in pcDA3.1-hygro vector) for fragment III. Primary PCRs were carried out using 10 ng of template DNA (e.g., pcDNA3.1-hyrgo-hβ2), 10 pmoles of sense and antisense primers, and 2.5 units of PLATINUM® Taq DNA polymerase (Invitrogen™ Life Technologies, Carlsbad, CA) in 50 µl reactions for 30 cycles at 94°C for 1 min each, 55°C for 90 sec, and 72°C for 90 sec, followed by a 4-min extension at 72 °C to generate fragments with complementary
overlaps. Fragments II and III were then ligated in the secondary PCR followed by gel purification (Prep-A-Gene™; Bio-Rad, Hercules, CA), and a tertiary PCR was used to fuse fragments I and II/III to complete the chimeric cDNA. The secondary and tertiary PCRs used approximately 100 ng of a mixture of the two DNA templates with one template in an approximately ten-fold molar excess over the other. For each secondary and tertiary PCR, the first 5 cycles of reaction (94°C for 1 min 30 sec, 55°C for 90 sec, and 72°C for 2 min) were carried out in the absence of PCR primers, allowing extension of ligated template fragments, followed by addition of primers selecting for the extended DNA templates in the next 30 cycles of amplification reactions. The final chimeric cDNA [\(\beta_2\chi, \beta_2(M1-V335)-5-HT_3A(R347-A448)-\beta_2(V449-K502)\)] was then digested with EcoRI and XbaI and subcloned into the pCDNA3.1-zeo plasmid and their sequences verified. Similar strategies were used to generate cDNAs for other chimeric subunits [\(\beta_2\chi(M3-E3-\text{FLAG}), \beta_2(M1-V294)-5-HT_3A(P306-S487)-\text{LEDYKDDDDK}\) including the indicated C-terminal \(\text{FLAG}\)-tag; \(\beta_4\chi(M3-E3-\text{FLAG}), \beta_4(M1-V292)-5-HT_3A(P306-S487)-\text{LEDYKDDDDK}\); \(\alpha_4\chi(M3-E3-\text{FLAG}), \alpha_4(M1-I303)-5-HT_3A(P306-S487)-\text{LEDYKDDDDK}\); \(\beta_2\chi(M3-E3), \beta_2(M1-V294)-5-HT_3A(P306-S487); \beta_4\chi(M3-E3), \beta_4(M1-V292)-5-HT_3A(P306-S487); \beta_2\chi(nC2-E3), \beta_2(M1-V335)-5-HT_3A(R347-S487); \) illustrated in Figure 1A], which were then digested with appropriate restriction enzymes and cloned into pCDNA3.1-zeo (\(\beta_2\) or \(\beta_4\) wild-type or chimeric subunits) or pCEP4-hygro (\(\alpha_4\) wild-type or chimeric subunits) followed by sequence verification.

**Model Cell Lines, Cell Culture, and Transfection.** The native nAChR-null SH-EP1 cell line (Lukas et al., 1993) was used as the host for generating model cell lines stably expressing nAChR composed of wild-type or chimeric subunits. SH-EP1 cells were grown in DMEM (high...
glucose, bicarbonate-buffered, with 1 mM sodium pyruvate and 8 mM L-glutamine) supplemented with 10% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B plus 5% fetal bovine serum on 100-mm diameter plates in a humidified atmosphere containing 5% CO₂ in air at 37°C. The SH-EP1-hα4β2-nAChR cell line expressing functional and radioligand binding human α4β2-nAChR was generated earlier in our laboratory (Eaton et al., 2003). For constructing SH-EP1-hα4β2γ or other transfected cell lines, low-passage (less than 15) SH-EP1 host cells were grown to approximately 50% confluence on 100-mm plates on the day of transfection and transfected with 10 µg of pCEP4 containing human wild-type α4 subunit DNA or pcDNA3.1-hyg containing chimeric α4 subunit cDNA using Superfect™ (Qiagen, Valencia, CA) transfection reagent following the manufacturer’s protocol. Forty-eight hours after transfection, the cells were split into four 100-mm plates, and the selection process began by supplementing the culture medium with hygromycin B (130 µg/ml; Calbiochem, San Diego CA). Two weeks later, polyclonal SH-EP1-hα4 or SH-EP1-hα4γ(M3-E3-FLAG) cells selected by hygromycin-resistance were then pooled and used as host cells for secondary transfection with pCDNA3.1-zeo construct containing wild-type or chimeric β2 or β4 subunit cDNAs, and zeomycin and hygromycin dual-drug resistant monoclonal cells were isolated. To obtain monoclonal cells, the transfected cells were split 1:20 onto fresh 100-mm plates to start dual drug selection two days after the transfection. Growth of the cells was monitored until single and well-isolated colonies (≤ 20-30 colonies per plate) reached the size of approximately 2-3 mm in diameter. The clones were then transferred to a fresh dish using cloning discs (Fisher Scientific, Tustin, CA), expanded, and then screened for function using ⁸⁶Rb⁺ efflux assays (see below). The candidate transfected cell lines identified by their ability to exhibit ⁸⁶Rb⁺ efflux in response to nicotinic agonists were further verified for their wild-type or chimeric α4 and β2 or β2 subunit RNA expression by reverse
transcription (RT)-PCR, split once weekly, and maintained in low-passage (less than 26) cultures to ensure stable expression of phenotype. In some cases, $^3$H-labeled epibatidine (H-EBDN) binding assays (see below and Eaton et al., 2003) were also done to evaluate expression of nAChR containing wild-type or chimeric subunits as radioligand binding entities.

**RNA Preparation, Reverse Transcription (RT) and Polymerase Chain Reaction.** To isolate total RNA from wild-type or transfected SH-EP1 cell lines, 2 ml of TRIZOL® reagent (Life Technologies) was added to cells growing at approximately 80% confluence in a 100-mm dish. RNA was then immediately isolated, precipitated, washed, and resuspended in RNase-free water as described by the manufacturer. Prior to the RT-PCR experiment, the RNA preparations were treated with RNase-free DNase I (Ambion, Austin, TX) to remove residual genomic DNA contamination, and the added DNaseI was inactivated at 65°C for 10 minutes following addition of 25 mM EDTA. For first-strand cDNA synthesis, we used 2 µg of the DNA-free total RNA, oligo d(T) primer, and the Superscript II™ Preamplification system (Life Technologies). At the end of the RT reaction, reverse transcriptase was deactivated by incubating the reaction at 75°C for 10 min, and RNA was removed by adding 1 unit of RNaseH to the mixture followed by incubation at 37°C for 30 min. An RT-negative control was also carried out in the absence of reverse transcriptase to check for residual genomic DNA contamination in the RNA samples. Each downstream PCR was performed using 1/20th of cDNA template, 1 µl of each 10 µM sense and anti-sense gene-specific primers, 1 µl of 10 mM dNTP, and 2.5 units of RedTaq™ (Sigma, St. Louis, MO) in a 50 µl reaction. The amplifications were carried out for 35 cycles at 95°C for 1 min, 55°C for 90 sec, and 72°C for 90 sec, followed by a 4-min extension at 72°C. PCR primers used in these reactions are: sense 5’- cgtatggtggcccctgtaccag-3’, anti-sense 5’-
gtcctgcccacagcttgccgac-3’ for GAPDH used as a positive control (predicted product size of 624 bp); sense 5’- gaatgtcacctctcgccatatc-3’, anti-sense 5’-ccggca(a/g)tggtc(c/t)tggaccac-3’ (a human-rodent “universal” primer) for the human nAChR α4 subunit (predicted product size of 790 bp); sense 5’-cggctcccttcacaaccaca-3’, anti-sense 5’-gcacctcggtggctgctgctgca-3’ for the nAChR β2 subunit (predicted product size of 754 bp); and sense 5’-ccatggcgccctgggtgagg cacctggtcctagac-3’ (chimeric primer), anti-sense 5’-tagaaggcacagtcgagg-3’ (3’ to the multiple cloning site in pcDNA-3.1hygro) for the nAChR β2χ chimera subunit (predicted product size of 420 bp). RT-PCR products were electrophoretically resolved on 1% agarose gel containing ethidium bromide, and digital photography under ultraviolet illumination was used to document results.

Immunoprecipitation and Western Blotting Analysis. Preparation of solubilized membranes for immunoprecipitation began with medium removal from 10-mm dishes harboring cells at confluence, rinsing of dishes 3 times with sodium phosphate buffer (100 mM NaCl, 25 mM NaPO₄, pH 7.4), mechanical harvesting of cells, and centrifugation of healthy cells at 1000g for 5 min. The cell pellets were then resuspended and homogenized in ice-cold sodium phosphate buffer (200 µl per confluent plate) supplemented with Complete Protease Inhibitor cocktail (1 mini tablet per 10 ml; Roche Diagnostics, Indianapolis, IN) followed by centrifugation at 10,000g for 10 minutes at 4°C. After centrifugation, the supernatant was discarded and the pellet resuspended in sodium phosphate buffer supplemented with 1% Triton X-100 (200 µl per ml; supplemented with protease inhibitor cocktail) by incubating at room temperature for 30 min. The preparation was then centrifuged at 12,500g for 10 min at 4°C. The supernatant fraction containing the solubilized membrane protein was collected, and the receptor
protein was immunoprecipitated using H133 (sc-5591; rabbit anti-nAChR α4 subunit targeting amino acids 342-474) or H92 (sc-11372; rabbit anti-nAChR β2 subunit targeting amino acids 342-433) antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA; 5 µg per 1 mg of solubilized total membrane protein) and protein G (reactive with IgG from rabbits or many other species)-agarose beads (50 µl; Calbiochem, San Diego, CA). After an overnight incubation at 4°C, the mixture was washed 3 times in detergent-supplemented sodium phosphate buffer, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing β-mercaptoethanol (20 µl per sample; ICN Biomedicals, Irvine, CA), heated to 95°C for 3 min, and centrifuged briefly to remove the agarose beads. The protein samples were then subjected to 10% SDS-PAGE separation and transferred to nitrocellulose membrane for Western analysis. Membranes were blocked in phosphate-buffered saline containing 5% skim milk powder and incubated with primary antibodies: mAb 299 (rat anti-nAChR α4 subunit targeting the N-terminal extracellular domain; 1:1000; Sigma, St. Louis, MO) and/or polyclonal antisera s-1724 (rabbit ant-nAChR β2 subunit targeting the N-terminal extracellular domain; 1:50; a gift from Dr. J. Patrick of the Baylor College of Medicine) for 1 hr. After three 5-min washes in phosphate-buffered saline containing 0.05% Tween 20, horseradish peroxidase-conjugated anti-rat and/or anti-rabbit secondary antibodies (1:1000; Santa Cruz Biotechnologies) were added to the reaction. The antibody-labeled bands were visualized by using enhanced substrate (Opti-4CN, Bio-Rad). For quantification, the blots were scanned and the signal intensity was analyzed by using Kodak 1-D gel analysis software (Kodak, New Haven, CT).

**Epibatidine-Binding Competition Studies.** Membrane preparations for binding studies were prepared according to our previously described method (Lukas et al., 2002; Eaton et al.,
2003). Briefly, transfected SH-EP1 cells were mechanically dislodged using a polypropylene policeman, pelleted by low-speed centrifugation, and resuspended in 3 ml of ice-cold 5mM Tris (pH 7.4). The cells were then homogenized with a Polytron (45 sec; Brinkmann model 10/35 with a PTA10S generator), and homogenized membranes were centrifuged at 45,000g for 10 min at 4°C, washed twice in 6 ml of Ringer’s solution supplemented with 0.1 mg/ml of sodium azide before being resuspended in the same buffer. The total membrane protein was quantified using the BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Typically, 0.24 mg of membrane protein could be obtained from a confluent 100-mm dish culture, and 1-3 µg protein yielded ~25 fmol of binding sites. For H-EBDN (New England Nuclear, Boston, MA) binding competition assays (Eaton et al., 2003), 800 µl reactions consisting of 400 pM H-EBDN, competing ligand at various concentrations, and an aliquot of membrane preparation containing ~25 fmol binding sites were incubated for 2 hr at room temperature. Reaction mixtures were then filtered with an Inotech 1H-201-A sample processor (Rockville, MD) using glass fiber filters with 1.0-1.5 µm retention and pretreated with 0.2% polyethylenimine. After three rinses, filters were transferred to 96-well plates and quantified for radioligand binding by scintillation counting (Wallac Microbeta Trilux 1450, Perkin Elmer/Wallac Inc., Boston, MA). Data for binding, ion flux, and electrophysiological studies (see below) were plotted, analyzed, and tested for statistical significance using Prism (GraphPad Software, Inc., San Diego, CA)

**Assay of nAChR Function by 86Rb+ Efflux.** Function properties of nAChR channels in the model cell lines were measured by using 86Rb+ efflux assays with the “flip-plate” technique developed in our laboratory (Lukas et al., 2002; Eaton et al., 2003). Briefly, cells
grown to confluence on two 100-mm plates were harvested by mild trypsinization, resuspended in complete medium, and seeded onto a 24-well plate. After cells had adhered overnight, medium was removed and replaced with 250 µl complete medium supplemented with ~300,000 cpm of $^{86}$Rb$^+$ (NEN) per well. Following a minimum of a 4-hr incubation, cells in each well were rinsed three times with 2 ml of efflux buffer (130 mM NaCl, 5.4 mM KCl, 2 mM CaCl$_2$, 5 mM glucose, 50 mM HEPES, pH 7.4), and introduced to drugs of choice at indicated final concentrations in efflux buffer for a 3-min incubation, all utilizing the “flip-plate” technique. The drug solution was then “flipped” back into the efflux/drug plate and subjected to Cerenkov counting (Wallac Micobeta Trilux 1450). Normalization and quality control for each experiment were accomplished using measurements of non-specific $^{86}$Rb$^+$ efflux in samples containing efflux buffer alone (background subtraction; 0% of control) and of total $^{86}$Rb$^+$ efflux in samples containing a fully efficacious concentration of 1 mM carbamylcholine (100% of control). Specific $^{86}$Rb$^+$ efflux for each drug concentration was expressed as a percentage of specific $^{86}$Rb$^+$ efflux for 1 mM carbamylcholine.

**Patch-Clamp Whole-Cell Current Recordings and Data Analysis.** Conventional whole-cell current recordings combined with use of a U-tube for rapid drug application have been previously described (Wu et al., 2002). Briefly, cells plated on 35-mm culture dishes were continuously superfused with standard external solution (120 mM NaCl, 3 mM KCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 25 mM D-glucose, 10 mM HEPES, pH 7.4, with Tris base; 2 ml/min). Glass microelectrodes (3-5 MΩ resistance between pipette and extracellular solutions) filled with solution containing 140 mM KCl, 4 mM MgSO$_4$, 0.1 mM EGTA, 4 mM Na-ATP, and 10 mM HEPES with Tris base (pH 7.2) were used, and cells were voltage-clamped at holding potentials of
-60 mV. To induce whole-cell current responses, 1 μM nicotine was delivered into the bath medium in close proximity to the targeted cells via the U-tube system, and ionic currents were measured using a 200B amplifier (Axon Instruments; Foster City, CA). Both pipette and whole cell current capacitance was minimized, and the series resistance was routinely compensated to 80%. Whole-cell access resistance less than 20 MΩ was accepted. The interval between drug applications was 3 min, which was adjusted specifically to eliminate receptor functional rundown. All experiments were performed at room temperature (22 ± 1°C). Data were typically filtered at 2 kHz and acquired at 5 kHz using Pclamp8 (Axon Instruments) and were displayed and digitized on-line (Axon Instruments Digidata 1200 series A/D board). The results were plotted using Origin 5.0 software (Microcal; North Hampton, MA). The decline in whole-cell current amplitude from peak to steady-state values during agonist application was fit to a single exponential decay function [current = (peak current * e^(t/τ)) + steady-state current; Clampfit 8.0, Axon Instruments] using either the data from 90% to 10% of the peak current amplitude when it decays to a zero steady-state current or the data from the middle 80% of the duration of drug application. These fits allowed determination of the decay constant, τ [i.e., the time required for an e-fold reduction (to 37% of the peak current amplitude) in whole-cell current amplitude], even when extrapolating beyond the actual data if current amplitude did not fall during agonist exposure to 37% of peak amplitude.

RESULTS

Generation and Initial Characterization of SH-EP1 Cell-Based Model Cell Lines. C2 domain sequences “proximal” to M3 and M4 transmembrane domains are highly conserved across nAChR subunits, but diverge from proximal C2 domains of analogous sequences in serotonin 5-
HT3A receptor subunits (Fig. 1B) or GABA\(_A\) or glycine receptor subunits from the same, four-transmembrane domain, ligand-gated ion channel superfamily, thus distinguishing nAChR subunits as a subfamily. Perhaps proximal C2 sequences constitute radiating tunnels from the central channel suggested by Unwin’s images (Miyazawa et al., 1999), through which ion flux could occur, forming additional structures that could help define channel kinetics and ion permeability. Matching of proximal C2 sequences across subunits within a closed assembly may be required for formation of functional ligand-gated ion channels. On the other hand, nAChR subunit C2 domain sequences “nested” between the conserved, proximal domains (Fig. 1B) are absolutely unique, nAChR subunit-specific fingerprints that nevertheless must carry signatures for interactions of nAChR with cytoplasmic or cytoskeletal proteins. These sequences may be involved in any downstream signal transduction through nAChR (Dajas-Bailador et al., 2002; Shaw et al., 2002). They also may be important in trafficking of nAChR through precursor pools and to the cell surface (Williams et al., 1998) and perhaps in recycling at the plasma membrane. In addition, post-translational modification of cytoplasmic domain residues, such as subunit phosphorylation, may help regulate subunit assembly into pentamers, trafficking of nAChR, and/or functional activation/inactivation of nAChR (Huganir and Greengard, 1992; Fenster et al., 1999; Guo and Wecker, 2002; Wecker and Rogers, 2003). However, few previous studies have investigated structure and function of nAChR subunit C2 sequences and neighboring transmembrane domains.

To critically examine the physiological role of the putative C2 domain and neighboring transmembrane sequences of nAChR subunits, we generated chimeric subunits based on the human nAChR \(\alpha_4\), \(\beta_2\) or \(\beta_4\) subunit backgrounds, substituting selected sequences from the mouse 5-HT\(_{3A}\) subunit analog (Fig. 1). We reasoned that the ability of the 5-HT\(_{3A}\) subunit to assemble
into a functional homomer and the relatively short length of its C2 domain minimized the likelihood that substitution with its sequences would hinder co-assembly of chimeric subunits with other wild-type or chimeric nAChR subunits. We thought that substitutions with 5-HT3A sequences would be more likely to produce properly folded subunits than would substitutions with, for example, green fluorescent protein sequences. We also anticipated that we could create a library of chimeras of different nAChR subunits, having in common the 5-HT3A subunit C2 signature, allowing clearer interpretation of effects of those substitutions on nAChR expression and function.

In our initial studies, we found that nAChR chimeric α4 subunits containing 5-HT3A subunit sequences spanning from the beginning of M3, through all of C2, and including M4 and C-terminal extracellular domains, as well as a C-terminal FLAG-tag [α4χ(M3-E3-FLAG); Fig. 1], were unable to assemble with wild-type β2 or β4 subunits to form stably functional nAChR in appropriately transfected SH-EP1 cells (data not shown). Some specific binding of H-EBDN to presumptive α4χ(M3-E3-FLAG)β2-nAChR in membrane preparations was observed (data not shown). Similarly, β2χ(M3-E3-FLAG) or β4χ(M3-E3-FLAG) subunits in combination with wild-type α4 subunits could not form functional nAChR, although small amounts of specific H-EBDN binding to presumptive α4β2χ(M3-E3-FLAG)-nAChR were evident (data not shown). Doubly chimeric nAChR containing α4χ(M3-E3-FLAG) subunits plus β2χ(M3-E3), β4χ(M3-E3), β2χ(M3-E3-FLAG), or β4χ(M3-E3-FLAG) subunits failed to form functional nAChR (data not shown). Chimeric β2χ(nC2-E3) subunits contained a slightly shorter stretch of 5-HT3A subunit sequences beginning ~22 amino acids C-terminal to M3 and extending through the rest of the C2, M4 and E3 domains (Fig. 1). These β2χ(nC2-E3) subunits were able to combine with chimeric α4χ(M3-E3-FLAG) subunits to form some H-EBDN binding sites, but not functional nAChR,
and no nAChR-like function was observed in cells expressing chimeric β2χ(nC2-E3) and wild-type α4 subunits (data not shown).

A different outcome was obtained when an alternative sequence substitution scheme was used to generate a chimera based on the human nAChR β2 subunit. Rather than the substitution of most or all of the C2 sequence, the M4 (+/-M3) domain, and the short C-terminal E3 tail, as was done in generation of (M3-E3(+/-FLAG)) or (nC2-E3) chimeras, the natural β2 subunit sequences in M3, M4, and E3 domains were retained along with C2 domain sequences “proximal” to M3 and M4, with only “nested” C2 sequences bounded by the proximal C2 sequences exchanged (Fig. 1). That is, the 5’- and 3’-“switch sites” of the C2 domain in the resultant β2χ gene were located at sequences encoding amino acids K336-S448 (making reference to the translation initiation methionine; Fig. 1), thus, preserving the relatively conserved, proximal linkers of ~14 or ~12 residues situated near M3 or M4, respectively.

After transfection of SH-EP1 cells with β2χ subunits along with either wild-type α4 subunits or chimeric α4χ(M3-E3-FLAG) subunits, candidate clonal lines, isolated by hygromycin- and zeocin-resistance (hyg^R/zeo^R), were expanded and assessed for channel function in response to nicotine using ^86Rb^+ ion efflux assays. Cells expressing β2χ and chimeric α4χ(M3-E3-FLAG) subunits were positive for H-EBDN binding, but not for functional responses to nicotinic agonists (data not shown). However, a clone displaying stable nicotine concentration-dependent functional responses was obtained from cells transfected with β2χ and wild-type α4 subunits, and RT-PCR analysis confirmed expression of transgenes (Figure 2). We have previously shown that SH-EP1 human epithelial cells do not express any endogenous nAChR subunits detectable by Northern analysis or any radioligand binding or functional sites corresponding to nAChR (Lukas et al., 1993). Results of the more sensitive RT-PCR approach used in the present study...
again confirmed the absence of nAChR α4 and β2 subunit messages in wild-type SH-EP1 cells, whereas expression of α4 and β2χ subunit messages in the transfected SH-EP1 clone displaying $^{86}\text{Rb}^+$ ion efflux in response to nicotine was verified (Fig. 2). For reasons that were not investigated, SH-EP1-hα4β2χ cells did not grow to confluence as did SH-EP1-hα4β2 cells and tended to lift off from the plate once the cell culture became confluent. Nevertheless, this SH-EP1-α4β2χ-nAChR clone assumed the normal SH-EP1 cell morphology and replicated at the same rate as SH-EP1-α4β2 cells generated earlier in our laboratory, having a doubling-time of approximately 30 hr.

**nAChR α4 and β2χ Subunit Proteins are Expressed and Assemble Efficiently in SH-EP1-hα4β2χ cells.** To address whether the transgenes were properly expressed at the protein level and whether the β2χ subunit assembled efficiently with wild-type human α4 subunits in the SH-EP1-hα4β2χ model cell line, we performed immunoprecipitation on solubilized membrane samples from SH-EP1-hα4β2χ, SH-EP1-hα4β2, or SH-EP1 cells followed by Western blot analyses (Fig. 3). Since one purpose of these experiments was to determine subunit assembly efficiency, we intended to quantify signals derived from both α4 and either β2 or β2χ subunits from immunoprecipitated samples on the same Western blot and identified during the immunoblot stage by simultaneous reaction with both anti-α4 and anti-β2 antibodies. Therefore, the specificities of mAb 299 (rat anti-α4) and polyclonal antisera s-1724 (rabbit anti-β2) as probes for Western analysis were first examined with H133 (rabbit anti-α4)- or H92 (rabbit anti-β2)-immunoprecipitated samples. Our results (data not shown) indicated that neither Western probe antibody cross-reacted with the other subunit. Western analysis using both rat anti-α4
mAb 299 and rabbit anti-β2 polyclonal s-1724 of H133-immunoprecipitated samples isolated based on reaction toward human α4 subunits (Fig. 3, left 3 lanes) showed that whereas the host SH-EP1 cell line did not express nAChR α4 or β2 subunit proteins, the SH-EP1-hα4β2χ and SH-EP1-hα4β2 cell lines abundantly expressed each of the two expected subunits. Western analysis of H92-immunoprecipitated samples targeting C2 domain sequences of the wild-type β2 subunit (Fig. 3, right 3 lanes) showed isolation of nAChR assemblies containing both α4 and β2 subunits from SH-EP1-hα4β2 cells, but not from wild-type (untransfected) SH-EP1 cells or from SH-EP1-hα4β2χ cells. The faint signals present in H92-immunoprecipitated SH-EP1 or SH-EP1-hα4β2χ cell samples, migrating slightly above the 52.9kD molecular mass marker, were not observed in replicate studies and therefore appear to represent sporadic, non-specific staining of primary antibody heavy chain. To assess nAChR subunit assembly efficiency in SH-EP1-hα4β2 and SH-EP1-hα4β2χ cells, we quantified α4 and either β2 or β2χ subunit signal densities on the Western blot. Ratios for β2:α4 subunit staining in the SH-EP1-hα4β2 sample and for β2χ:α4 subunit staining in the SH-EP1-hα4β2χ sample were ~1 for H133-immunoprecipitated preparations, and absolute levels of staining for α4 and either β2 or β2χ subunits were very similar across samples, suggesting comparable efficiencies of nAChR assembly and comparable levels of expression of relevant subunits in either transfected cell line. Moreover, samples immunoprecipitated from SH-EP1-hα4β2 cells using either the α4 or β2 subunit-targeted antibodies had comparable amounts of α4 and β2 proteins, suggesting that immunoprecipitation was not executed under conditions where sample recovery was limited by reactive antibody. Thus, tandem immunoprecipitation-Western analyses indicated that replacement of the β2 subunit nested C2 sequence does not appear to compromise α4β2χ-nAChR assembly.
\[ \alpha 4 \beta 2 \chi \cdot nAChR \] Display Subtle Differences in Ligand-Binding Properties Compared to \[ \alpha 4 \beta 2 \cdot nAChR. \]

H-EBDN binding competition studies were executed to assess the ligand-binding properties of \[ \alpha 4 \beta 2 \chi \cdot nAChR \] compared to \[ \alpha 4 \beta 2 \cdot nAChR \] (Fig. 4, Table 1). Unlabeled epibatidine (EBDN) is the most potent ligand for blocking specific H-EBDN binding for both \[ \alpha 4 \beta 2 \chi \cdot \] and \[ \alpha 4 \beta 2 \cdot nAChR. \] In addition, as previously shown for \[ \alpha 4 \beta 2 \cdot nAChR \] (Eaton et al., 2003), all nicotinic agonists blocked H-EBDN-binding to \[ \alpha 4 \beta 2 \chi \cdot nAChR \] fully and with relatively high potency (Fig. 4A-B). Generally, with the exception of cytisine, carbamylcholine and hexamethonium (obviating systematic differences in IC\textsubscript{50} determinations between the two nAChR subtypes), ligands showed higher potency blockade of H-EBDN binding to \[ \alpha 4 \beta 2 \chi \cdot nAChR \] than \[ \alpha 4 \beta 2 \cdot nAChR. \] The rank order of binding inhibition potency and IC\textsubscript{50} values for agonists acting at \[ \alpha 4 \beta 2 \chi \cdot nAChR \] were: 390 pM EBDN >> 10 nM cytisine > 65 nM nicotine >> 460 nM acetylcholine (ACh) ≥ 720 nM 1,1-dimethyl-4-phenyl-piperazinium (DMPP) >> 6.0 µM carbamylcholine. Agonists tested in ligand-binding competition displayed a wider range of binding inhibition potency than agonists (Fig. 4C-E) with rank order: 27 nM lobeline >> 280 nM suberyldicholine >> 2.3 µM dihydro-β-erythroidine (DHβE) > 35 µM decamethonium ≥ 37 µM methyllycaconitine (MLA) ≥ 66 µM d-tubocurarine ≥ 160 µM trimethaphan > 490 µM pancuronium > 1.9 mM hexamethonium. In addition, mecamylamine did not appear to compete with H-EBDN binding and exhibited no inhibition up to 100 µM (data not shown). Generally, there were larger differences in H-EBDN binding competition potency for antagonists than agonists in comparisons between \[ \alpha 4 \beta 2 \cdot \] and \[ \alpha 4 \beta 2 \chi \cdot nAChR. \] Differences (≥ 0.3 log units in log IC\textsubscript{50} values, translating into factors of two or more in molar concentrations) in inhibition potency between \[ \alpha 4 \beta 2 \chi \cdot nAChR \] and \[ \alpha 4 \beta 2 \cdot nAChR \] were observed for three antagonists: lobeline,
suberyldicholine and pancuronium (Table 1), and these as well as smaller differences observed for EBDN, ACh, DHβE, decamethonium and MLA were significant at the 95% confidence limit. Pancuronium also subtly fell out of rank order established for α4β2-nAChR when assessed for H-EBDN binding competition properties at α4β2χ-nAChR.

**Function of α4β2χ-nAChR Assessed Using ⁸⁶Rb⁺ Efflux Assays.** Function of expressed α4β2χ-nAChR was evaluated using ⁸⁶Rb⁺ efflux assays, and results were compared to findings using α4β2-nAChR (Figs. 5-6, Tables 2-3). Agonist concentration-response profiles did not differ dramatically at sub-maximally efficacious concentrations of selected ligands for α4β2χ-nAChR (Fig. 5, solid lines and filled symbols) and α4β2-nAChR (Fig. 5, dashed lines and open symbols). At the 95% confidence level, nicotine and DMPP had lower EC₅₀ values, and carbamylcholine had a higher EC₅₀ value when acting at α4β2χ-nAChR compared to action at α4β2-nAChR. However, none of these differences were more than 0.3 log units or a factor of two, nor were they larger than between-study differences observed (compare Table 2 entries for α4β2-nAChR to those in Eaton et al., 2003). Nevertheless, self-inhibition of α4β2χ-nAChR function occurred at higher concentrations of EBDN, DMPP and carbamylcholine, which did not exhibit self-inhibition in actions at α4β2-nAChR, and nicotine showed more self-inhibition of α4β2χ-nAChR than of α4β2-nAChR. Cytisine had comparable potency and comparable, sub-maximal efficacy without showing evidence of self-inhibition at α4β2χ- and α4β2-nAChR.

Rank order agonist potency from the appropriate fits to agonism only or to agonism with self-inhibition profiles and corresponding EC₅₀ values (Table 2) for actions at α4β2χ-nAChR (compared to EC₅₀ values following in parentheses for actions of agonists at α4β2-nAChR)
were: 12 nM (17 nM) EBDN >> 1.5 µM (1.0 µM) nicotine ≥ 1.8 µM (1.7 µM) ACh > 3.3 µM (3.7 µM) cytisine > 9.8 µM (5.1 µM) DMPP > 17 µM (32 µM) carbamylcholine. Note that the rank order was the same for agonists acting at α4β2χ-nAChR or α4β2-nAChR. In addition, self-inhibitory IC\textsubscript{50} values (Table 2) were also determined for EBDN (93 µM), DMPP (1 mM), nicotine (2.0 mM), carbamylcholine (32 mM) acting at α4β2χ-nAChR, and for nicotine (9.1 mM) acting at α4β2-nAChR.

As is the case for actions at α4β2-nAChR, antagonist log concentration-response profiles showed full inhibition of α4β2χ-nAChR function stimulated by 1 mM carbamylcholine (Fig. 6; Table 3). There were insignificant differences in the abilities of suberyldicholine, trimethaphan, lobeline, MLA or DHβE to inhibit function of α4β2χ-nAChR compared to α4β2-nAChR (Fig. 6A, Table 3). Nevertheless, α4β2χ-nAChR, relative to α4β2-nAChR, displayed increased sensitivity (Table 3) to functional blockade by mecamylamine, which is a non-competitive inhibitor of α4β2-nAChR function, decamethonium, which has a competitive inhibition signature at α4β2-nAChR, or d-tubocurarine, pancuronium, or hexamethonium, which show mixed mechanisms of functional block of α4β2-nAChR (Fig. 6B-C; see also Fig. 9 in Eaton et al., 2003). Rank order antagonist potencies and IC\textsubscript{50} values for α4β2χ-nAChR were: 200 nM mecamylamine >> 2.0 µM DHβE > 6.5 µM MLA > 9.1 µM hexamethonium > 18 µM lobeline ≥ 21 µM d-tubocurarine ≥ 25 µM trimethaphan > 36 µM pancuronium > 110 µM decamethonium ≥ 150 µM suberyldicholine. Furthermore, succinylcholine at concentrations as high as 1 mM did not display antagonist activity. Relative to actions at α4β2-nAChR, antagonist functional potency for hexamethonium, d-tubocurarine, decamethonium, and pancuronium at α4β2χ-nAChR subtly fell out of rank order.
Accelerated Decay of Nicotine-Induced, Inward, Whole-Cell Currents in SH-EP1-α4β2χ-nAChR. Patch-clamp electrophysiological recording revealed that peak whole-cell current responses to nicotinic agonists were not different for cells expressing α4β2- or α4β2χ-nAChR and responding to either 1 µM nicotine (Fig. 7A) or 1 mM ACh (Fig. 7B). Peak current amplitudes are 631.3 ± 142 pA for α4β2-nAChR and 577 ± 100 pA for α4β2χ-nAChR in response to 1 µM nicotine (Fig. 7C, left panel). However, compared with α4β2-nAChR-mediated currents, or to agonist-induced currents mediated by wild-type 5-HT3A receptors (Choi et al., 2003), α4β2χ-nAChR-mediated responses exhibited faster acute desensitization (diminished inward current during the course of nicotinic agonist application) represented as a significant decrease in the current decay constant and reflected in a smaller steady-state current relative to peak current for α4β2χ-nAChR (Fig. 7A-B). The ratios of steady-state current to peak current (I_s/I_p, as a % of peak current) are 41.9 ± 5.2 % for α4β2-nAChR and 26.7 ± 4.4% for α4β2χ-nAChR (Fig. 7C middle and right panels). Transitions from peak to steady-state currents were characterized by decay constants (τ) for an e-fold (63%) reduction in inward current amplitude of 3508 ± 512 ms for α4β2-nAChR and 1721 ± 123 ms for α4β2χ-nAChR [compare to τ ~ 3 sec also for 5-HT3A receptor whole cell current responses to agonist; e.g., see Choi et al., 2003].

DISCUSSION

One of the principal findings of this study is that functional and ligand-binding nAChR are formed as a combination of human nAChR wild-type α4 subunits with chimeric β2χ subunits in which “nested” C2 domain sequences are replaced by corresponding sequences from...
the 5-HT$_{3A}$ receptor subunit. This indicates that native, nested C2 sequences in β2 subunits are not essential for formation of functional α4β2χ-nAChR. Radiolabeled agonist binding competition properties and functional agonist potencies varied no more than subtly between α4β2-nAChR and α4β2χ-nAChR, as one might expect given preservation of the E1 and E2 domains thought to compose the agonist binding site. Nevertheless, and quite unexpectedly, compared to α4β2-nAChR, α4β2χ-nAChR displayed novel or heightened functional self-inhibition by agonists at higher concentrations and higher sensitivity to functional blockade by several antagonists. Furthermore, α4β2χ-nAChR displayed faster and more complete acute desensitization of function. For these or any other mutagenesis or chimera study, there is a formal possibility that effects are non-specific and due to molecular distortion imposed by sequence alterations. However, a reasonable interpretation of the current findings is that changes in sensitivity to blockade by antagonists, sensitivity to self-inhibition, and rates and extents of desensitization of nAChR correlate with changes in β2 subunit nested C2 sequences not previously implicated in nAChR function.

When more extensive substitutions were made involving 5-HT$_{3A}$ subunit M3 and/or M4 transmembrane domains and their neighboring “proximal” C2 sequences, chimeric α4, β2, or β4 subunits are unable to form functional nAChR. When coupled with observations from previous reports concerning similar lines of study (see below), the new findings suggest that native M3, M4, and/or proximal C2 domains are essential for formation of functional α4β2- or α4β4-nAChR. However, the ability of some of the nAChR containing M3-E3 chimeric subunits to exhibit low levels of specific H-EBDN binding implies that subunit-subunit interactions allowing formation of closed assemblies to create ligand-binding interfaces does occur. Further studies including substitutions of just proximal C2 sequences and not the transmembrane domains but
also of more extensive, M3, M4 and proximal C2 sequence substitutions are warranted to ascertain whether any inability to form functional entities is due to failure to traffic subunit assemblies to the cell surface or is due to some other functional flaw in cell surface-expressed receptors.

How do the present findings relate to other existing literature? Williams et al. (1998) noted differences, using the *Xenopus* oocyte heterologous expression system, in agonist functional potency and/or channel kinetics in their whole-cell current studies of wild-type, chick \( \alpha 7 \)-nAChR compared to nAChR composed of chimeric \( \alpha 7 \) subunits containing \( \alpha 3 \) or \( \alpha 5 \) subunit proximal-plus-nested C2 loop sequences. The close similarities between nAChR subunit proximal C2 sequences makes it likely that nested C2 sequences account for the differences observed. When coupled with our findings also indicating differences in agonist effects and in whole-cell current response profiles for \( \alpha 4 \beta 2 \chi \)-nAChR compared to \( \alpha 4 \beta 2 \)-nAChR, functional consequences of nested C2 domains are underscored.

Based on sequence deletion studies, Valor et al. (2002) identified regions within C2 essential for the expression of functional, rat \( \alpha 7 \)-nAChR. One of these corresponds to 14 residues of the human \( \alpha 7 \) subunit in C2 proximal to M3 (H319-R332; Fig. 1), and another corresponds to 11 residues in C2 proximal to M4 (human \( \alpha 7 \) E459-R469; Fig. 1). Our chimera/substitution analyses based on nAChR \( \alpha 4 \), \( \beta 2 \) and \( \beta 4 \) subunits are consistent with indispensable roles for these highly conserved, proximal C2 sequences in formation of functional nAChR, whether or not neighboring M3 and M4 domains also are indispensable for functional nAChR formation.

The deletion studies of Valor et al. also suggested essential roles in formation of functional, rat \( \alpha 7 \)-nAChR expression for the most N-terminal amino acids within the nested C2
domain (corresponding to human α7 V333-R347; Fig. 1) including a three residue MKR signal (corresponding to human α7 M345-R347) that would not tolerate substitution. Perhaps because the human nAChR β2 subunit does not have the α7 subunit C2 domain MKR signature (having MQQ instead), substitution of the nested C2 sequence from the 5-HT$_{3A}$ subunit did not compromise functional expression of α4β2χ-nAChR. Substitutions of rat nAChR α7 C339-R344 in the N-terminal, nested C2 region were tolerated in the studies of Valor et al., perhaps suggesting a role merely in maintaining a structural requirement in the region. The ability to substitute for this part of the nAChR subunit nested C2 sequence was also observed in our studies of chimeric β2 subunits.

Valor et al. reported that substitutions for rat nAChR α7 subunit nested C2 sequences (117 amino acids corresponding to human α7 P348-E454; Fig. 1) with rat nAChR α4 (226 amino acids) or α5 (45 amino acids) subunit sequences reduced heterologous expression as radioligand binding sites and fully or largely eliminated function of resultant, chimeric α7-nAChR. However, this could be due to steric incompatibilities if α7 subunit C2 sequences are substituted with sequences from subunits that do not seem to assemble as homomers. Substitution instead with green fluorescent protein (239 amino acids) or SNAP-25 (206 amino acids) also sharply attenuated or abolished expression of functional nAChR, even though minimal substitution with an artificial, seven amino acid insert or with a natural EGM or mutated EAA or AGA sequences allowed for higher functional expression of mutant α7-nAChR expression than observed for wild-type α7-nAChR (Valor et al., 2002). However, our SH-EP1-α4β2χ cells, substituting the mouse 5-HT$_{3A}$ subunit sequence for an essentially equivalent stretch of nested C2 sequence in nAChR β2 subunits, express ligand-binding and functional nAChR at levels like that seen in SH-EP1-α4β2 cells containing wild-type subunits. Thus, the
incompatibility with functional expression of the substitutions made for nested C2 sequences in
the rat α7 subunit are specific to either nAChR subunit/subtype or substituting sequence, or the
incompatibility can be overcome by some compensating feature of the wild-type α4 subunits
expressed in our SH-EP1-α4β2χ cells. Continuing work investigating roles of nested C2
domains in α4, β2, and α7 subunits seeks to further elucidate our understanding in this area.

Several studies have been conducted addressing sequence elements required for muscle-
type nAChR [(α1)2β1γδ-nAChR or α1*-nAChR] assembly. First, the inability to form
functional nAChR from chimeric α4 and/or β2 subunits containing substituted M3, M4, and
proximal C2 domains is consistent with earlier studies showing that M1, M2 and M3 domains of
α1 subunits are important for efficient expression of Torpedo nAChR on the cell surface in the
Xenopus oocyte heterologous expression system (Tobimatsu et al., 1987), even though
extracellular, N-terminal domains of α1, δ, and γ subunits appear to contain information
sufficient for initial, specific, subunit association (Verrall and Hall, 1992). However, a 17-amino
acid sequence at the C-terminus of the large cytoplasmic loop in the α1 subunit is essential in the
late stage of receptor assembly with β1 subunits, as chimeric α1 proteins containing the
corresponding β1 sequence can not properly assemble with β1 subunits (Yu and Hall, 1994b).
The equivalent I434-S448 in the C-terminal part of the human β2 subunit nested C2 domain is
replaced by the 5-HT3A subunit sequence, but V449 in the beginning of the M4-proximal C2
sequence is preserved in our chimeric β2χ subunit without an apparent compromise in efficiency
of α4β2χ-nAChR assembly. Perhaps the sequence requirement is specific to an α subunit for α-
β subunit assembly and will be revealed in studies of roles for α4 subunits in formation of
functional α4β2- or α4β4-nAChR, or it may only be relevant to formation of higher order complexes such as (α1)_2β1γδ-nAChR.

Using the oocyte expression system, Morgado-Valle et al. reported a sequence motif conserved across nAChR α4, β2, β4, and other subunits, RXPXTH(X)_{14}P (corresponding to human β4 R324-P344; Fig. 1), located in the C-terminus of the nAChR β2 subunit C2 domain, that is necessary for function of α4β2-nAChR (Morgado-Valle et al., 2001). However, our findings differ, in that α4β2χ-nAChR function is evident even though human β2 P344 is replaced by an alanine in our chimeric β2χ subunit. Nevertheless, note that none of the nAChR subunits capable of forming homomeric nAChR, namely α7, α8 and α9, nor 5-HT_{3A} subunits, contains this motif, having instead an alanine residue at the equivalent position to β2 P344 followed by a tryptophan (Fig. 1). Perhaps the AW signature present in the β2χ subunit adequately substitutes for P344 in influencing subunit folding and assembly.

Although both α and β subunits in heteromeric, αnβn-nAChR can contribute to ligand binding and functional properties and coupling of drug binding to channel opening, several studies have suggested that β subunits may play a dominant role in controlling the rate and extent of nAChR functional inactivation after chronic agonist exposure (Fenster et al., 1997; Kuryatov et al., 2000; Gentry et al., 2003). Results from the current study suggest, quite unexpectedly, that functional inactivation or desensitization of α4β2-nAChR is regulated in part by nested C2 sequences. Nevertheless, there are other, recently reported indications that C2 domain determinants can influence single channel conductance of 5-HT_{3A} receptors (Kelly et al., 2003) or α4β2-nAChR (Lambert et al., 2004), consistent with the current findings suggesting active roles of the nested cytoplasmic loop in nAChR function.
REFERENCES


FOOTNOTES

Financial support: This project, part of which was conducted in the Charlotte and Harold Simensky Neurochemistry of Alzheimer's Disease Laboratory, was funded by grants from the Arizona Disease Control Research Commission (9730 and 9615), the National Institute of Health (NIH NS40417) and Targacept, Inc, by the Roberta and Gloria Wallace Foundation, and by endowment and/or capitalization funds from the Men's and Women's Boards of the Barrow Neurological Foundation.
LEGENDS FOR FIGURES

Figure 1. A. Strategy for construction of the chimeric nAChR β2γ subunit and schematic diagram illustrating wild-type and chimeric nAChR subunit protein structures. The four putative transmembrane domains, M1-M4, are displayed by cross-hatched boxes. Open boxes represent the three extracellular domains, E1, E2, and the C-terminal E3, the small C1 cytoplasmic loop, and the large C2 cytoplasmic domain located M3 and M4 regions. The regions indicated by roman numerals and arrows above the block schematic drawing indicate protein sequence regions coded by the PCR fragments that were generated and ligated to form chimeric subunit cDNA, with fragments I and III derived from the human nAChR β2 subunit sequence and fragment II derived from the murine 5-HT3A subunit sequence as described in details in Materials and Methods. The grey area in the chimeric subunit drawing marks regions of nAChR subunits replaced by the 5-HT3A sequences. The Flag sequence motif (LEDYKDDDK) is represented by ♦. B. Alignment for human nAChR α4, β2, β4 and α7 subunits, and human or mouse 5-HT receptor 3A subunits (amino acid numbering for all starting at the translation initiation methionine; single letter code) for sequences beginning at the start of the M3 domain and ending at the C-terminus. M3 and M4 transmembrane domains are indicated, as are “proximal” C2 domain sequences close to M3 or M4 and the intervening “nested” C2 sequence between those proximal C2 sequences (italicized, boldface type above the relevant sequences). Amino acids in the nAChR α4, β2, or β4 subunit sequences indicated in boldface are among those substituted with 5-HT3A subunit sequences and characterized in the study. Amino acids in the human nAChR α7 subunit sequence in boldface or italics aid identification of regions mentioned in the Discussion that correspond to those suggested to be essential or not essential,
respectively, for function of rat α7-nAChR as studied by Valor et al. (2002). Note the similarities across some nAChR subunits for regions corresponding to M3, proximal C2, and M4 domains but the differences compared to 5-HT3A subunits (differences are especially marked when comparing across nAChR, 5-HT3, GABA and ionotropic glycine receptor subunit families). The nested C2 sequences are absolutely unique to each subunit, although the high similarity and identity between human and murine 5-HT3A subunits in this region is also evident.

Figure 2. Confirmation of human nAChR α4 and β2χ subunit transcript expression in SH-EP1-α4β2χ cells. Reverse transcription-polymerase chain reactions were executed as described in Materials and Methods using wild-type SH-EP1 cells or transfected SH-EP1-α4β2χ cells and the α4, β2χ, gapdh, or β2 primer sets as indicated on the image. Products of expected size [determined by reference to mass standards indicated in the left lane; MW (bp)] were generated in SH-EP1 cells for gapdh or in SH-EP1α4β2χ cells for α4 and β2χ subunits. The minus-RT control was negative, and faint bands evident were not reproducibly observed.

Figure 3. Protein expression and subunit assembly of nAChR α4 and β2 or β2χ subunits. Solubilized membrane protein from SH-EP1, SH-EP1-α4β2 or SH-EP1-α4β2χ cell lines was immunoprecipitated using H133 (anti-nAChR α4 subunit; α4-IP) or H92 (anti-nAChR β2 subunit; β2-IP) antibodies and analyzed by Western analysis for immunoblots probed simultaneously with rat anti-α4 mAb 299 and rabbit-anti-β2 polyclonal antisera s-1724. Labeled arrows on the left side of the image indicate positions for immunoreactive α4 and β2 subunits, and molecular masses (kD) of standards (middle lane) are labeled to the right of the image. Ratios of α4 vs. β2 signals in both SH-EP1-α4β2 and SH-EP1-α4β2χ cell samples
immunoprecipitated with the anti-α4 subunit antibody were approximately 1 as was the ratio of α4:β2 subunit in SH-EP1-α4β2 cell samples immunoprecipitated with the β2 subunit antibody targeting native β2 subunit C2 loop sequences, although that antibody failed to isolate α4β2χ complexes from the SH-EP1-α4β2χ cell line. Faint signals from SH-EP1-α4β2χ and SH-EP1 cell samples immunoprecipitated with the anti-β2 subunit antibody with apparent masses of ~50-70 kD likely are due to cross-reactivity with heavy chains from primary immunoprecipitating antibody carried over into the sample and were not observed in replicate studies.

Figure 4. Drug competition for specific H-EBDN binding to sites on SH-EP1-α4β2 and SH-EP1-α4β2χ cells. Reaction mixtures containing the indicated competing ligand at the specified concentrations (abscissa; molar, log scale) were used to compete for specific binding (ordinate; % of control) of 400 pM H-EBDN to membrane preparations containing ~25 fmol binding sites from SH-EP1-α4β2 cells [dashed lines indicate curve fits to data points not displayed to enhance clarity, see Eaton et al., 2003] or SH-EP1-α4β2χ cells [solid lines indicating curve fits to data for: A - epibatidine (EBDN; ◆), nicotine (Nic; ▽), dimethyl-phenyl-piperazinium (DMPP; ●); B - cytisine (Cyt; ■), acetylcholine (ACh; △), carbamylcholine (Carb; ▲); C - suberyldicholine (Sub; ◊), dihydro-β-erythrodine (DHβE; ▲), methyllycaconitine (MLA; ▽); D - lobeline (Lob; ▲), decamethonium (Deca; □), hexamethonium (Hexa; ▼); E - d-tubocurarine (d-TC; ■), trimethaphan (Trim; △), or pancuronium (Panc; ▼)]. Results are the averages of 3 separate experiments (mean ± SEM).

Figure 5. Functional assessment of agonist action at SH-EP1-α4β2χ and SH-EP1-α4β2 cells. Measurements of specific $^{86}$Rb$^+$ efflux (ordinate; percentage of 1 mM carbamylcholine control)
were made in the presence of selected agonists at the indicated concentration (abscissa; molar; log scale) in parallel experiments using SH-EP1-α4β2χ (solid lines fit to filled symbols) or SH-EP1-α4β2 (dashed lines fit to open symbols) cell lines. Results are the averages of 4-7 separate experiments (mean ± SEM) for: A - epibatidine (Epi; □, □), acetylcholine (ACh: ▲, △), or dimethyl-phenyl-piperazinium (DMPP; ●, ○); B - nicotine (Nic; ●, ○), carbamylcholine (Carb; ▲, △), or cytisine (Cyt; □, □).

Figure 6. Antagonist effects on function of α4β2χ-nAChR and α4β2-nAChR. Measurements of specific 86Rb+ efflux (ordinate; percentage of 1 mM carbamylcholine control) were made in the presence of 1 mM carbamylcholine and selected antagonists at the indicated concentration (abscissa; molar log scale) in parallel experiments using SH-EP1-α4β2χ (solid lines fit to filled symbols) or SH-EP1-α4β2 (dashed lines fit to open symbols) cell lines. Results are the averages of 3-7 separate experiments (mean ± SEM) for: A - suberyldicholine (Sub; ●, ○), trimethaphan (Trim; ▲, △), lobeline (Lob; ◊, ◊), methyllycaconitine (MLA; ■, □), or dihydro-β-erythroidine (DHβE; ▽, ▼); B - d-tubocurarine (d-TC; ●, ○), mecamylamine (Meca; ■, □), or pancuronium (Panc; ▲, △); or C - decamethonium (Deca; ●, ○), or hexamethonium (Hexa; ▲, △).

Figure 7. Whole-cell current responses in α4β4-nAChR and α4β2χ-nAChR. A,B: Typical whole-cell current traces recorded at -70 mV holding potential in response to 4 sec applications (2 sec horizontal scale) of 1 μM nicotine (A; 100 pA vertical scale) or 1 mM acetylcholine (B; 500 pA vertical scale) for α4β2-nAChR (left) or α4β2χ-nAChR (middle) with traces superimposed on the right. Current responses desensitize faster and more completely for α4β2χ-nAChR than for α4β2-nAChR. C: Bar graphs show no significant difference in peak current
amplitudes (631.3 ± 142 pA for α4β2-nAChR, 577 ± 100 pA for α4β2χ-nAChR; p = 0.51; left panel), but that there are differences in ratios of steady-state current to peak current (I_s/I_p as a % of peak current; 41.9 ± 5.2 % for α4β2-nAChR, 26.7 ± 4.4 % for α4β2χ-nAChR; p = 0.03; middle panel) and in decay constants for transition from peak to steady state currents (τ = 3508 ± 512 ms for α4β2-nAChR, τ = 1721 ± 123 ms for α4β2χ-nAChR; p = 0.009; right panel) for results obtained from 8 cell measures for α4β2-nAChR or 12 cells for α4β2χ-nAChR in response to 1 μM nicotine.
TABLE 1. Drug competition toward specific H-EBDN binding to α4β2-nAChR and α4β2χ-nAChR in transfected SH-EP1 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>log IC50 ± SEM</th>
<th>α4β2</th>
<th>α4β2χ</th>
</tr>
</thead>
<tbody>
<tr>
<td>epibatidine *</td>
<td>-9.31 ± 0.04</td>
<td>-9.41 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>cytisine</td>
<td>-8.04 ± 0.04</td>
<td>-7.99 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>lobeline **</td>
<td>-7.10 ± 0.05</td>
<td>-7.57 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>nicotine</td>
<td>-7.09 ± 0.04</td>
<td>-7.19 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>suberyldicholine **</td>
<td>-6.26 ± 0.03</td>
<td>-6.56 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>acetylcholine *</td>
<td>-6.12 ± 0.02</td>
<td>-6.34 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>dimethyl-phenyl-piperazinium</td>
<td>-6.09 ± 0.04</td>
<td>-6.14 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>dihydro-β-erythroidine *</td>
<td>-5.49 ± 0.04</td>
<td>-5.64 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>carbamylcholine</td>
<td>-5.26 ± 0.04</td>
<td>-5.22 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>decamethonium *</td>
<td>-4.28 ± 0.04</td>
<td>-4.45 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>methyllycaconitine *</td>
<td>-4.21 ± 0.04</td>
<td>-4.43 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>d-tubocurarine</td>
<td>-4.12 ± 0.03</td>
<td>-4.18 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>trimethaphan</td>
<td>ND</td>
<td>-3.79 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>hexamethonium</td>
<td>-2.73 ± 0.15</td>
<td>-2.71 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>pancuronium **#</td>
<td>-2.63 ± 0.11</td>
<td>-3.31 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>mecamylamine</td>
<td>&lt;&lt; - 4</td>
<td>&lt;&lt; - 4</td>
<td></td>
</tr>
</tbody>
</table>
Radioligand binding competition assays were conducted as described in Materials and Methods. Results were fit to the logistic equation to determine log IC\textsubscript{50} values (± S.E.M). Overlap in the range of values at the 95% confidence interval was used as a cut-off in tests of statistical significance for any differences.

ND – not determined.

* higher potency for interaction at $\alpha_4\beta_2\chi$-nAChR than at $\alpha_4\beta_2$-nAChR at the 95% confidence level.

** higher potency for interaction at $\alpha_4\beta_2\chi$-nAChR than at $\alpha_4\beta_2$-nAChR at the 95% confidence level with differences in IC\textsubscript{50} values also greater than 0.3 log units.

# rank order in activity at $\alpha_4\beta_2\chi$-nAChR falls out of rank order when compared to activity at $\alpha_4\beta_2$-nAChR.
### TABLE 2. Agonist function at α4β2-nAChR or α4β2χ-nAChR as assessed using 86Rb+ efflux assays in transfected SH-EP1 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>log EC50 ± SEM (log IC50 ± SEM)</th>
<th>α4β2</th>
<th>α4β2χ</th>
</tr>
</thead>
<tbody>
<tr>
<td>epibatidine</td>
<td>-7.77 ± 0.07</td>
<td>-7.92 ± 0.07 (-4.03 ± 0.26)</td>
<td></td>
</tr>
<tr>
<td>nicotine #</td>
<td>-5.98 ± 0.05 (-2.04 ± 0.18)</td>
<td>-5.81 ± 0.03 (-2.69 ± 0.04)</td>
<td></td>
</tr>
<tr>
<td>acetylcholine</td>
<td>-5.76 ± 0.08</td>
<td>-5.75 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>cytisine</td>
<td>-5.43 ± 0.10</td>
<td>-5.48 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>dimethyl-phenyl-piperazinium #</td>
<td>-5.29 ± 0.07</td>
<td>-5.01 ± 0.11 (-3.01 ± 0.12)</td>
<td></td>
</tr>
<tr>
<td>carbamylcholine *</td>
<td>-4.50 ± 0.05</td>
<td>-4.77 ± 0.06 (-1.50 ± 0.12)</td>
<td></td>
</tr>
</tbody>
</table>

86Rb+ efflux assays were conducted as described in Materials and Methods. Results were fit to the logistic equation to determine log EC50 and log IC50 (for self-inhibition of function) values (+ S.E.M). Overlap in the range of values at the 95% confidence interval was used as a cut-off in tests of statistical significance for any differences.

# higher potency for interaction at α4β2-nAChR than at α4β2χ-nAChR at the 95% confidence level.

* higher potency for interaction at α4β2χ-nAChR than at α4β2-nAChR at the 95% confidence level.
**TABLE 3.** Antagonist action at α4β2-nAChR and α4β2χ-nAChR as assessed using 86Rb⁺ efflux assays in transfected SH-EP1 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>log IC50± SEM</th>
<th>α4β2</th>
<th>α4β2χ</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecamylamine **</td>
<td>-5.96 ± 0.05</td>
<td>-6.71 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>dihydro-β-erythroidine</td>
<td>-5.83 ± 0.07</td>
<td>-5.69 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>methyllycaconitine</td>
<td>-5.28 ± 0.08</td>
<td>-5.19 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>lobeline</td>
<td>-4.64 ± 0.06</td>
<td>-4.75 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>trimethaphan</td>
<td>-4.48 ± 0.07</td>
<td>-4.57± 0.06</td>
<td></td>
</tr>
<tr>
<td>hexamethonium ** #</td>
<td>-4.27 + 0.07</td>
<td>-5.04 + 0.06</td>
<td></td>
</tr>
<tr>
<td>d-tubocurarine ** #</td>
<td>-4.17 + 0.04</td>
<td>-4.68 + 0.05</td>
<td></td>
</tr>
<tr>
<td>suberyldicholine</td>
<td>-3.76 + 0.06</td>
<td>-3.83 + 0.08</td>
<td></td>
</tr>
<tr>
<td>decamethonium ** #</td>
<td>-3.47 + 0.06</td>
<td>-4.00 + 0.06</td>
<td></td>
</tr>
<tr>
<td>pancuronium ** #</td>
<td>-3.41 + 0.07</td>
<td>-4.44 + 0.08</td>
<td></td>
</tr>
</tbody>
</table>

86Rb⁺ efflux assays were conducted as described in Materials and Methods. Results were fit to the logistic equation to determine log IC50 values (± S.E.M). Overlap in the range of values at the 95% confidence interval was used as a cut-off in tests of statistical significance for any differences.

** higher potency for interaction at α4β2χ-nAChR than at α4β2-nAChR at the 95% confidence level.
# rank order in activity at $\alpha 4\beta 2\chi$-nAChR falls out of rank order when compared to activity at $\alpha 4\beta 2$-nAChR.
PCR fragments for β2χ chimeric subunit construction:

Chimeric Subunits
α4/β2/β4 WT
α4χ/β2χ/β4χ(M3-E3-FLAG)
α4χ/β2χ/β4χ(M3-E3)
β2χ (nC2-E3)
β2χ

Fig. 1A
Fig. 1B

---M3---/proximal----/nested→

h nAChR α4 304 PLIGYELFTMIFTVLSIVTVFVNLVHHRSPRTHTPFWRTWRRFVLDVPRLLM
h nAChR β2 295 PLVGKYMFTMVLTSTVTSCLVNLVHHRSPTHHTMAPWVKVFLEKLPALLFM
h nAChR β4 293 PLIGYKLMFTMVLTSTVTSCLVNLVHHRSPWVKKCFLHKLTPFTLM
h nAChR α7 291 PLIAQYPASTMIVGSLVVTVTIVLQYHHDGDGPDKMPKWTGRVILLNWWCAWFLRM
h 5-HT3A 303 PLIGVFVFVMALLVSLAETIFVTLVHDQDQFPFPWPWLHRVLERIAWLLCL
m 5-HT3A 306 PLIGVFVFVMALLVSLAETIFVTLVHDQDQFPFPWPWLHRVLERIAWILCL

h nAChR α4 359 KRPSVVKDNCRLIESMVKMASAPFRWPEPEGPPATSTGQLHFPSPSFVPLDVPAEPPGS
h nAChR β2 350 QQQRRHHCARQR.LRLRRQRSREGALLFREAPGADSCFTCVNRASVQOLAGAFAEPVA
h nAChR β4 348 KRPGPDSSPARRFPPSKSCVKTPEATATSTSFSNYYSSFYNPASAAKSPA.GSTPVAIP
h nAChR α7 346 KRPGEDKVRPACLEQHKQRCRSLASVEMSAVAPPPASNGULLYIGFRGLGVCVTPDSHVCVCG
h 5-HT3A 358 R............EQSTSQRPPATSTQATKTDDCS.......AMNHCS.HMGGPQDFEKS.PDRR
m 5-HT3A 361 G............EQQMAHRPPATFQANKTDDCSGSDLPAMNHCS.HVVGPQDFLEKT.PGR

h nAChR α4 422 CKSPDQLPPQQPLEAEKASPHPSPGCRPHGTQPAGLAKARLSVQHMSPPGEVEGVRRC

h nAChR α4 485 RSRISIQYCVPRDDAAPEADGQAAGALASRNTHSAELPPPD

←nested/----M4---/proximal收到了

h nAChR α4 548 QPSPPCKCTCKEPSSVSATVKSTRSKAPPPLPLSAPALTAVEGVQYIADLKAEADTDGPSV
h nAChR β2 411 ..................GP...GRSGEFCGC...GLREAVGVRIPFADHMRSEDDQVS
h nAChR β4 410 .................. RDFWLRMSGRFRQ........DVQEALEGVSFIAQHMKNDDEDQSVV
h nAChR α7 409 ..................RMACSPTHDEHLLHGGQPPEDD...PDIAKILEEVRITANRFQDESEAVC
h 5-HT3A 402 CSP..................PPPPREAS...........LAVC.GLLQEELSRQFLEKRDEIREVA
m 5-HT3A 411 GSP..................LPREAPREAS...........LAVR.GLLQELSSRHFFLEKREDEIREVA

---proximal----M4---

h nAChR α4 589 EDWKYVAMVIDRLFLMVFPVCVMGFLQPLFQNTTTTPFLHSDHSAPSSK 502
h nAChR β2 449 VDKYVAMVIDRLFLMVFPVCVMGFLQPLFQNTTTTPFLHSDHSAPSSK 502
h nAChR β4 449 EDWKYVAMVIDRLFLMVFPVCVMGFLQPLFQNTTTTPFLHSDHSAPSSK 498
h nAChR α7 458 SEWKFACVVDRLCLMNASFPVTICTGILMSAPNFVEAVSKDFA 502
h 5-HT3A 440 RDWLRGVSVDKLLFHHYLLAVAYSITLVMWLSIQYA 478
m 5-HT3A 449 RDWLRGVSVDKLLFHHYLLAVAYSITLVMWLSIWYS 487
Fig. 2

RT-PCR

<table>
<thead>
<tr>
<th>MW (bp)</th>
<th>SH-EPI</th>
<th>SH-EPI-α4β2χ</th>
<th>α4 β2χ</th>
<th>α4 β2χ β2−RT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α4 β2χ</td>
<td>gapdh</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 3

IP-Western

MW (kD)

- 115
- 92
- 52.9
- 35.4
- 29

SH-EP1-α4β2
SH-EP1-α4β2
SH-EP1
Marker
SH-EP1-α4β2
SH-EP1-α4β2
SH-EP1
Fig. 4

**[3]H-Epibatidine Binding Competition**

- **A**: EBDN, Nic, DMPP
- **B**: Cyt, ACh, Carb
- **C**: Specific 86Rb⁺ efflux (% of control)
  - Sub, MLA, DHβE
- **D**: Deca, Lob
- **E**: Panc, Trim, d-TC

**Log [Drug] (M)**

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Fig. 5

Functional Assay: Agonist Dose-Response

- Epi
- ACh
- DMPP
- Nic
- Carb
- Cyt

Specific $^{86}$Rb$^+$ efflux (% of control)

Log [Drug] (M)

$\alpha_4\beta_2\chi$
$\alpha_4\beta_2$
Fig. 6

Functional Assay: Antagonist Dose-Response

Specific $^{86}\text{Rb}^+$ efflux (% of control)

- Sub
- Trim
- Lob
- MLA
- DHβE

- dTc
- Meca
- Panc

Log [ Drug ] (M)

0 25 50 75 100

-9 -8 -7 -6 -5 -4 -3 -2

α4β2χ

α4β2
Fig. 7

A

Wild-type $\alpha_4\beta_2$

Niacine 1 $\mu$M

$100 \, pA$

2 sec

$\alpha_4\beta_2\chi$

Niacine 1 $\mu$M

$V_r = -70 \, mV$

B

ACH 1 mM

$500 \, pA$

2 sec

C

$P = 0.51$

Peak currents (pA)

$P = 0.03$

Is (p%) (%)

$P = 0.009$

Decay constant (ms)

$\alpha_4\beta_2$

$\alpha_4\beta_2\chi$

$\alpha_4\beta_2$

$\alpha_4\beta_2\chi$