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Roles of nicotinic acetylcholine receptor β subunit cytoplasmic loops in acute desensitization and single channel features

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Abstract

To evaluate physiological roles of the large, second cytoplasmic loops (C2) situated between the M3 and M4 transmembrane domains of nicotinic acetylcholine receptor (nAChR) subunits. We have constructed chimeric $\beta 2$ ($\beta 2\chi$) and $\beta 4$ ($\beta 4\chi$) subunits in which the “nested” C2 domains (but not the “proximal” sequences of ~14 residues immediately adjacent to the M3 or M4 domains) of these β subunits were replaced by the corresponding sequence from the serotonin 5-HT_{3A} receptor subunit. We previously reported that heterologously expressed nAChR containing $\alpha 4$ and $\beta 2\chi$ subunits displayed a faster whole-cell current decay in its agonist response compared to responses of all-wild-type $\alpha 4\beta 2$ -nAChR. This suggests an unexpected, functional role for the C2 domain of the $\beta 2$ subunit in $\alpha 4\beta 2$ -nAChR acute desensitization. Here we report that there also is faster desensitization of $\alpha 4\beta 4\chi$ -nAChR relative to $\alpha 4\beta 4$ -nAChR stably and heterologously expressed in the human SH-EP1 cell-line. In addition, cell-attached, single channel recording shows that both acetylcholine-activated $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR have a significantly lower mean open probability, shorter mean open-time, and a longer mean closed-time than their fully wild-type counterparts while not having different conductance amplitudes. These findings reveal microscopic bases for the faster desensitization of $\alpha 4^*$ -nAChR containing chimeric instead of wild-type β subunits. Our findings also remain consistent with novel and unexpected roles of β subunit nested C2 domains in modulation of $\alpha 4^*$ -nAChR function.

Keywords

nicotinic acetylcholine receptor; cytoplasmic loops; desensitization; patch clamp; single channel

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Introduction

Nicotinic acetylcholine receptors (nAChR) are ligand-gated cation channels composed as homopentameric or heteropentameric combinations of α and β subunits (Lindstrom, 1996, Lukas, 1998). The most abundant heteromeric nAChR in the brain contain $\alpha 4$ subunits, bind nicotine with high affinity, and have been implicated in conditions including Parkinson's disease, Alzheimer's disease, epilepsy, and drug dependence (Picciotto et al., 1995, Cordero-Erausquin et al., 2000, Jensen et al., 2005).

Each nAChR subunit shares a conserved topology, having an extensive extracellular N-terminal domain, four transmembrane domains (M1-M4), short intracellular cytoplasmic loops between M1 and M2 and between M3 and M4, a short extracellular loop between M2 and M3, a large second cytoplasmic loop (C2) between M3 and M4, and a short C-terminal extracellular tail (Sine, 2002, Kuo et al., 2005). The N-terminal domain plays a key role in ligand binding (Sine, 2002), and the transmembrane domains are important for channel kinetics and ion permeability (Corringer et al., 2000). Previous studies suggested that the C2 domain interacts with cytoskeletal or cytoplasmic proteins, and thus could play important roles in nAChR expression and distribution (Bencherif and Lukas, 1993, Yu and Hall, 1994, Colledge and Froehner, 1997, Shoop et al., 2000).

Previously, we sought to investigate functional roles of nAChR subunit C2 domains by generating chimeric nAChR subunits containing C2 domains from serotonin (5-HT) 3A subunits, which also are members of the same ligand-gated ion channel superfamily (Kuo et al., 2005). Interestingly, when they are stably and heterologously expressed in SH-EP1 human epithelial cells, nAChR composed of $\alpha 4$ and chimeric $\beta 2$ ($\beta 2\chi$) subunits display faster whole-cell current response decay during agonist exposure than do all-wild-type $\alpha 4\beta 2$ -nAChR, thus suggesting a novel, functional role for the C2 domain in nAChR acute desensitization (Kuo et al., 2005). Here, we also examined nAChR containing $\alpha 4$ and chimeric $\beta 4$ subunits ($\beta 4\chi$ subunits, which similarly to $\beta 2\chi$ subunits, have their amino acid residues K334-V448 in the C2 domain replaced with R347-R448 from the mouse 5HT_{3A} (Kuo et al., 2005). We determined whole-cell current response and single channel properties of $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR using whole-cell patch-clamp and cell-attached single channel recording techniques. Our results show distinct differences between nAChR containing wild-type as opposed to chimeric β subunits in both whole cell current decay and single channel kinetics. These findings support novel roles of C2 domains of nAChR β subunits in channel kinetics of $\alpha 4^*$ -nAChR.

Materials and Methods

SH-EP1 $\alpha 4\beta 2$ -, $\alpha 4\beta 4$ -, $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR cell lines

Construction of nAChR chimeric $\beta 2$ and $\beta 4$ genes as well as the SH-EP1 cells stably and heterologously expressing $\alpha 4\beta 2$ -, $\alpha 4\beta 4$ -, $\alpha 4\beta 2\chi$ -, or $\alpha 4\beta 4\chi$ -nAChR has been described previously in detail (Kuo et al., 2005) (Eaton et al., 2003). In brief, gene fragments corresponding to human nAChR subunit N termini amino acids up to V335 ($\beta 2$) or V333 ($\beta 4$), amino acids from V449 ($\beta 2$) or E449 ($\beta 4$) to C termini, or mouse 5HT_{3A} receptor subunit residues R347-R448 were amplified from cDNA using polymerase chain reactions

(PCR) and primers spanning the chimeric subunit junctions (Figure 1). The PCR products with chimeric sequence overhangs were then joined together by successive PCR reactions and then cloned into pCDNA3.1-zeo expression vectors (Invitrogen). After sequencing validation, the chimeric cDNA constructs were then delivered into the SH-EP1 host cell lines already expressing wild-type, human nAChR $\alpha 4$ subunits, using Superfect (QIAGEN, Valencia, CA) as the transfection reagent. Monoclonal cell lines stably expressing chimeric $\alpha 4\beta 2\gamma$ -nAChR or $\alpha 4\beta 4\gamma$ -nAChR were identified using $^{86}\text{Rb}^+$ efflux assays of receptor function. Transfected SH-EP1 cells were grown in Dulbecco's modified Eagle's medium (high glucose, bicarbonate-buffered with 1 mM sodium pyruvate and 8 mM l-glutamine) supplemented with 10% horse serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B plus 5% fetal bovine serum on 100-mm diameter plates in a humidified atmosphere containing 5% CO_2 in air at 37°C .

Solutions and drug application

The standard external solution contained (mM): 120 NaCl, 3 KCl, 2 MgCl_2 , 2 CaCl_2 , 25 d-glucose, 10 HEPES, pH adjusted to 7.4 with Tris-base. In whole-cell and single channel recordings using acetylcholine (ACh) as an agonist, 1 μM atropine was added to the standard solution to exclude any possible influences of muscarinic receptors, but the results were the same as those obtained in the absence of atropine (data not shown). Electrodes containing standard external solution with 100 nM ACh and 1 μM atropine were used for cell-attached single channel recording. For lifted whole-cell recording, the internal solution contained (mM): 110 Tris phosphate dibasic, 28 Tris-base, 11 EGTA, 2 MgCl_2 , 0.1 CaCl_2 , and 4 Mg-ATP; pH 7.3. Drug solutions (at the indicated concentrations of nicotine or of ACh with 1 μM atropine) for whole-cell recording were applied to cells of interest. ACh (Sigma, MO) was first dissolved in distilled water to make a stock solution and further diluted with external solution before each use.

Electrophysiological recording

For conventional whole-cell recording, cells plated on polylysine-coated 35 mm culture dishes were placed on the stage of an inverted microscope (Olympus iX70, Lake Success, NY, USA) and continuously superfused with standard external solution (2 mL/min). Glass microelectrodes (3–5 $\text{M}\Omega$ resistance between the pipette and extracellular solutions) were used to form tight seals ($>1\text{ G}\Omega$) on the cell surface until suction was applied to convert to conventional whole-cell recording. The cells were then voltage-clamped at holding potentials of -60 mV , and ion currents in response to application of ligands were measured. Whole-cell access resistance was less than 20 $\text{M}\Omega$ before series resistance compensation. Both pipette and whole-cell current capacitances were minimized, and series resistance was routinely compensated to 80%. Cells were lifted during recording to facilitate rapid solution exchange (Wu *et al.*, 2006). To initiate whole-cell current responses, nicotinic agonists were delivered into the bath medium near to the cell being recorded via a computer-controlled two-barrel system so that solution exchange occurred within 20 ms (based on 10%–90% peak current rise times). Intervals between drug applications (2 min) were adjusted specifically to ensure the stability of nAChR responsiveness (without functional rundown). Signals were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 5 kHz, and stored in a computer via analog-to-digital conversion (Digidata

1200; Molecular Devices, Sunnyvale, CA). Pclamp 9.2 software (Molecular Devices, Sunnyvale, CA) and a 4-sec drug perfusion mode were used for whole-cell patch clamp recordings.

Cell-attached, patch-clamp, single-channel recording was used to define single-channel properties of $\alpha 4^*$ -nAChR containing either wild-type $\beta 2$ or $\beta 4$ or chimeric $\beta 2\chi$ or $\beta 4\chi$ subunits. Patch pipettes typically contained 100 nM ACh. In cell-attached recordings, the applied holding current is opposite to the resting membrane potential. Recordings were done at holding potentials between +20 mV and +60 mV, in part to eliminate voltage-gated potassium channel responses that could be activated under negative holding potentials. The resistance of single channel recording electrodes was 7-8 M Ω when filled with internal solution. Single channel currents were recorded at room temperature. The gap-free recording mode was used for single channel recording. Periods of channel activity that persisted for >10 min were used for analysis. Data was acquired while being filtered at 1 kHz (8-pole Butterworth filter and digitized at 10 kHz; Digidata interface 1322A). The results were analyzed with Clampfit 9.2 (Axon Instruments, CA, USA) and QuB Software Suite (Qin et al., 1997, Qin and Li, 2004). Single channel current-voltage curves were fit (Prism 3.0; GraphPad Software, Inc.) to the equation $I = \gamma (V - V_r)$. Open probabilities were estimated from all point histograms fit to a sum of Gaussians using Clampfit routines. Open or closed channel duration analyses were performed using QuB on records with a dead time of 0.1 ms and idealized by the half amplitude threshold method.

Results

Expression of functional $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR in SH-EP1 cells

We employed a strategy, honed by results of preliminary experiments examining other chimeric subunits, to create chimeric $\beta 2\chi$ or $\beta 4\chi$ subunits permissive for nAChR function. “Nested” C2 domains in nicotinic receptor β subunits that are bordered by “proximal” C2 segments of ~14 residues immediately adjacent to M3 or M4 domains were replaced by the corresponding domains from the serotonin receptor 5-HT_{3A} subunit (Fig. 1). Function of $\alpha 4\beta 2\chi$ - or $\alpha 4\beta 4\chi$ -nAChR expressed in SH-EP1 cell lines and containing chimeric subunits, and of all-wild-type $\alpha 4\beta 2$ or $\alpha 4\beta 4$ -nAChR, was characterized first using whole cell patch-clamp recording (Fig. 2A, B).

Accelerated decay of ACh- or nicotine-induced whole-cell currents in $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR

As evident from examination of whole-cell current response traces and corroborated by more detailed analysis of data pooled from many studies, peak whole-cell current responses to either 1 μ M nicotine or 1 mM ACh are not different for cells expressing $\alpha 4\beta 2\chi$ - or $\alpha 4\beta 4\chi$ -nAChR compared to responses mediated by all-wild-type $\alpha 4\beta 2$ - or $\alpha 4\beta 4$ -nAChR, respectively (Fig. 2). However, macroscopic current decay from peak to steady state levels are faster and more complete for $\alpha 4\beta 2\chi$ - or $\alpha 4\beta 4\chi$ -nAChR compared to $\alpha 4\beta 2$ - or $\alpha 4\beta 4$ -nAChR, respectively. The ratios for steady state to peak current amplitude (I_s/I_p , normalized to the peak current response to 1 μ M nicotine are $26.3 \pm 5.2\%$ for $\alpha 4\beta 2\chi$ -nAChR and $55.2 \pm 4.3\%$ for $\alpha 4\beta 2$ -nAChR and are $40.7 \pm 8.9\%$ for $\alpha 4\beta 4\chi$ -nAChR and $83.6 \pm 9.1\%$ for $\alpha 4\beta 4$ -

nAChR ($n=7$; $p < 0.05$, t -test). Decay constants for transition from peak to steady state currents are 1920 ± 140 ms for $\alpha 4\beta 2\chi$ -nAChR and 3610 ± 530 ms for $\alpha 4\beta 2$ -nAChR and are 2210 ± 320 ms for $\alpha 4\beta 2\chi$ -nAChR and 4960 ± 510 ms for $\alpha 4\beta 2$ -nAChR ($n=7$; $p < 0.01$, t -test). However, the peak amplitudes recorded from cells expressing $\alpha 4\beta 2\chi$ - or $\alpha 4\beta 4\chi$ -nAChR are not significantly different when compared to all-wild-type $\alpha 4\beta 2$ - or $\alpha 4\beta 4$ -nAChR, respectively (Fig. 1C a and b; $n=7$; $p > 0.05$, t -test). These data show that rates and extents of desensitization of nAChR are increased when nAChR $\beta 2$ or $\beta 4$ subunit nested C2 sequences are replaced with corresponding domains from the serotonin receptor 5-HT_{3A} subunit, suggesting an unexpected role for nested C2 sequences in modulating nAChR function.

Single channel properties of $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR

Single-channel patch-clamp recording was performed using 100 nM ACh plus 1 μ M atropine in the patch pipette to elicit less frequent single channel openings and to avoid too-robust channel activity elicited by ACh at higher concentrations. Traces revealed single channel events that vary in current amplitude (Fig. 3A, 4A). Histograms (Fig. 3B, 4B) of single channel events fit to Gaussian distributions indicate that there are two classes of events for each nAChR subtype. Based on current amplitudes at +60 mV holding potential, estimated low and high conductances are 19 ± 2 and 37 ± 3 pS for $\alpha 4\beta 2\chi$ -nAChR, 20 ± 2 and 44 ± 3 pS for $\alpha 4\beta 4\chi$ -nAChR, 18 ± 2 and 40 ± 3 pS for $\alpha 4\beta 2$ -nAChR, and 22 ± 2 and 42 ± 3 pS for $\alpha 4\beta 4$ -nAChR (Fig. 3B and 4B). Thus, there are no significant differences in channel event conductances between $\alpha 4^*$ -nAChR containing wild-type or chimeric $\beta 2$ subunits or between wild-type or chimeric $\beta 4$ subunits ($n=8$; $p > 0.05$, t -test). These findings are consistent with our whole-cell recording indications that macroscopic peak current amplitudes also are not affected by substitution of chimeric for wild-type β subunits.

Single channel kinetics of $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR

On-cell patch-clamp recordings also were analyzed to characterize single channel kinetics. Both open (Fig. 5A a and b, Fig. 6A a and b) and closed (Fig. 5B a and b, Fig. 6B a and b) time histograms are fit by the sum of multiple exponential components. The two values for mean open times are 3.5 ± 0.6 and 1.3 ± 0.3 ms for $\alpha 4\beta 2$ -nAChR and are 8.3 ± 1.3 and 2.9 ± 1.2 ms for $\alpha 4\beta 4$ -nAChR. Significantly shorter mean open times were observed for $\alpha 4\beta 2\chi$ -nAChR ($1.2 \pm 0.2^{**}$ and $0.5 \pm 0.1^*$ ms) and for $\alpha 4\beta 4\chi$ -nAChR ($4.3 \pm 0.5^{**}$ and $1.1 \pm 0.3^*$ ms; $n=8$; $^*p < 0.05$, $^{**}p < 0.01$). Three closed time components are observed for each nAChR subtype. Mean closed times are 0.2 ± 0.1 , 2.6 ± 1.1 and 7.8 ± 2.5 ms for $\alpha 4\beta 2$ -nAChR and $1.6 \pm 0.5^*$, $9.5 \pm 3.8^*$ and $25.6 \pm 9.2^*$ for $\alpha 4\beta 2\chi$ -nAChR ($n=8$; $^*p < 0.05$). Mean closed times are 0.5 ± 0.2 , 0.9 ± 0.2 and 5.9 ± 1.3 ms for $\alpha 4\beta 4$ -nAChR and 0.4 ± 0.1 , $1.6 \pm 0.4^*$ and $11.2 \pm 2.3^*$ for $\alpha 4\beta 4\chi$ -nAChR ($n=8$; $^*p < 0.05$). The significantly shorter mean open times and longer mean closed times are consistent with our findings in whole-cell recordings and indicate that nested C2 domains of β subunits affect desensitization of $\alpha 4^*$ -nAChR by altering single channel kinetics.

Discussion

The major findings from this study are that substitution of 5-HT_{3A} subunit nested C2 domain sequences for those of nAChR $\beta 2$ or $\beta 4$ subunits leads to production of functional

$\alpha 4^*$ -nAChR that have faster and more complete whole-cell current response decays from peak levels during exposure to agonists (defined as acute desensitization). There also are shorter mean single channel open times and longer closed durations between single channel events for $\alpha 4^*$ -nAChR that contain chimeric $\beta \chi$ rather than wild-type β subunits. These effects occur without there being effects on peak whole-cell current levels or significant differences in single channel current conductance levels between $\alpha 4^*$ -nAChR containing a given wild-type or chimeric β subunit. Our earlier studies indicated that it was essential to preserve highly conserved, proximal C2 sequences of $\beta 2$ and $\beta 4$ subunits in order to maintain nAChR function (Kuo et al., 2005). Those studies plus the results reported here also indicate that even the divergent, nested C2 sequences of nAChR $\beta 2$ and $\beta 4$ subunits play important roles in modulating $\alpha 4^*$ -nAChR desensitization and channel kinetics.

Analysis of single-channel events induced by ACh shows that there are two amplitude levels for both $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR stably expressed in SH-EP1 cells, just as is the case for all-wild-type subunit $\alpha 4\beta 2$ - and $\alpha 4\beta 4$ -nAChR. Our findings pertaining to $\alpha 4\beta 2$ -nAChR that are consistent with other reports of the existence of two conductance states (Buisson and Bertrand, 2001, Curtis et al., 2002, Nelson et al., 2003, Zuo et al., 2004). Zuo et al. found conductance values very similar to those described here (Zuo *et al.*, 2004); however, many other studies described various conductance states of $\alpha 4\beta 2$ -nAChR different from our findings here. One conductance of 20 pS was described for the chick $\alpha 4\beta 2$ nAChR (Ballivet et al., 1988, Cooper et al., 1991), whereas two different conductances (15 and 34 pS) were observed for the rat $\alpha 4\beta 2$ nAChR (Papke *et al.*, 1989) and Pereira et al. reported two conductance states (18 and 30 pS) from chick $\alpha 4\beta 2$ nAChR (Pereira *et al.*, 1994). Charnet et al. reported three different conductances (12, 22 and 34 pS) for the rat $\alpha 4\beta 2$ nAChR (Charnet *et al.*, 1992). Buisson et. al recorded human $\alpha 4\beta 2$ and showed a predominant conductance of ~46 pS (Buisson *et al.*, 1996). Although one would think that single channel conductance states would be the same for specific nAChR subtypes irrespective of the expression system, Buisson et. al used a different host cell line than we used (Buisson *et al.*, 1996), and this could account for differences observed from ours. On the other hand, although mammalian nAChR subunit sequences are very similar for a given subunit across species, there can be subtle differences of even conserved amino acid substitutions, and the studies by Papke *et al* (Papke *et al.*, 1989) concerned rat nAChR whereas our work concerned human nAChR. Thus, differences between studies could reflect subunit sequence differences. Additionally, most above studies used outside-out patch recording, whereas we used the cell-attach technique, and there is the formal possibility that interactions with cytoplasmic or cytoskeletal elements could alter channel properties (Janmey, 1998, Akk and Steinbach, 2000, Amici et al., 2012), especially given that our studies show effects of substitution of nested C2 sequences expected to be quite remote from channel and nicotinic ligand binding domains but are candidates for cytoskeletal/cytoplasmic protein interactions. Consequently, we can attribute differences between studies to differences in the potential for such interactions due to the patch-clamp recording configuration used.

Substitution of serotonin 5-HT_{3A} subunit amino acids for those in the nested C2 domain of nAChR $\beta 2$ or $\beta 4$ subunits does not alter single channel conductance amplitudes. This is not very surprising given that channel lining, ligand binding, and transmembrane domains are

not altered by these changes. These findings are consistent with single channel conductances reflecting intrinsic properties of $\alpha 4^*$ -nAChR.

The findings reported here that $\alpha 4^*$ -nAChR single channel events display two levels of conductance would suggest that there are two classes of channel openings if the high conductance state had an amplitude that was different than about double the amplitude of the low conductance events. In general, interpretations of findings when there are two or more, distinct conductance levels have been that the different (in this case, two) conductance states reflect openings of singly-liganded or doubly-liganded $\alpha 4^*$ -nAChR. Another possibility is that two classes of channel openings reflect openings of distinctive, low sensitivity or high sensitivity $\alpha 4^*$ -nAChR isoforms. For $\alpha 4\beta 2$ -nAChR, it is known that such isoforms exist and have the presumed subunit makeups of $(\alpha 4)_3(\beta 2)_2$ - or $(\alpha 4)_2(\beta 2)_3$ -nAChR, respectively (Eaton et al., 2014). ACh interacts at lower concentrations at $\alpha 4:\beta 2$ subunit interfaces present in both receptor isoforms, but only at higher concentrations at $\alpha 4:\alpha 4$ subunit interfaces unique to the “low sensitivity” $(\alpha 4)_3(\beta 2)_2$ -nAChR. Concentration-dependence studies in principle could be used to address this issue, but if the ratio between high and low conductance states changes with a function of agonist concentration, it would be difficult to determine whether this is because there is a difference in frequency of opening of channels in high sensitivity vs. low sensitivity receptors or because there are changes in receptor occupancy altering ratios between openings of doubly- as opposed to singly-liganded receptors. The fact that our data were collected using a relatively low concentration of ACh (100 nM) might argue against actions involving the low sensitivity $\alpha 4:\alpha 4$ subunit interface.

However, because the high conductance state events have amplitudes that are approximately double those for low conductance events, it is possible that the former reflect coinciding openings of two channels rather than just one (i.e., double openings). The records for $\alpha 4\beta 4$ - or $\alpha 4\beta 4\chi$ -nAChR single channel responses show that events with higher conductance amplitude almost always are found in clusters of openings, which could be explained by double openings (i.e., a second channel opening when there is a flurry of first channel openings). By contrast, the records for $\alpha 4\beta 2$ - or $\alpha 4\beta 2\chi$ -nAChR do not have this feature, as high conductance events are almost always isolated from low conductance events. As mentioned above, studies of agonist concentration dependence of the ratio between low and high conductance events could be used to evaluate different possibilities, but such studies still could not cleanly differentiate between effects due to single or double ligand occupancy, activity of high vs. low sensitivity isoforms, or isolation from or coincidence with low conductance state events of high conductance responses. Further work is underway to define single channel responses to agents such as AMOP-H-OH (sazetidine) or cytosine, respectively, which appear to be selective agonists for high sensitivity or low sensitivity $\alpha 4\beta 2$ -nAChR (Kozikowski et al., 2009), as this potentially presents a way pharmacologically to make such discriminations. Work also is in progress on tethered pentameric $\alpha 4\beta 2^*$ -nAChR having just a single subunit ratio and arrangement also to illuminate bases for different single channel conductance states.

Evaluation of single channel kinetics data can be with regard to the issues raised above pertaining to single channel conductance states. The findings that any of the $\alpha 4^*$ -nAChR have single channel events characterized by two mean channel open times means that $\alpha 4^*$ -

nAChR may both be caused by receptor stoichiometry to form low and high sensitivity $\alpha 4^*$ -nAChR. Our observations that any of the $\alpha 4^*$ -nAChR enter phases of inactivity characterized by three channel closed times is significant and support our interpretation that may be attributed to the existence of two different channel composition (low and high sensitivity $\alpha 4^*$ -nAChR). Most interestingly, the facts that nAChR containing chimeric β subunits instead of the matching wild-type β subunit have shorter mean open times and longer mean closed times are not only consistent with there being faster desensitization for chimeric β subunit-containing receptors, but also suggest that the chimeric intracellular loop may interact with intracellular elements to affect channel gating of nAChRs.

The findings that any of the $\alpha 4^*$ -nAChR have single channel events characterized by two mean channel open times could be explained if they reflect different mean open times for $\alpha 4^*$ -nAChR isoforms having different subunit stoichiometry or by differences in open time reflecting occupancy at one or both high sensitivity agonist binding sites. However, and most interestingly, the facts that nAChR containing chimeric β subunits instead of the matching wild-type β subunits have shorter mean open times and longer mean closed times are not only consistent with there being faster desensitization for chimeric β subunit-containing receptors, but also suggest that the chimeric intracellular loop may interact with intracellular elements to affect channel gating of nAChRs. This suggests that such interactions rather than intrinsic properties of the receptor may influence gating kinetics, and work using excised patches should be illuminating in that regard.

Furthermore, it provides the microscopic basis for the observed quickening of macroscopic whole-cell current response desensitization attributable to substitution of chimeric for wild-type β subunits. For example, nested C2 intracellular loops may be targets for phosphorylation that could affect or be affected by intracellular signaling cascades. However, consensus sequences for phosphorylation are sparse except in $\alpha 4$ subunits, which haven't been altered in this study. Nevertheless, the possibility remains that inter-subunit interactions might differentially expose or reveal sites for C2 intracellular loop interactions with kinases or phosphatases or other cytoskeletal or cytoplasmic modulators. Once again, excised patch studies are warranted to simplify the experimental system and reveal mechanisms beyond intrinsic channel properties that can influence single channel open and closed times.

In conclusion, our findings indicate that changes in nested C2 domains of nAChR $\beta 2$ or $\beta 4$ subunits influence acute desensitization of $\alpha 4^*$ -nAChR-mediated macroscopic whole-cell current responses due to shortened single channel open times and longer closed times. Thus, nAChR $\beta 2$ or $\beta 4$ subunit nested C2 domains play new and unexpected roles in $\alpha 4^*$ -nAChR function.

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Highlights

- $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR desensitize faster than their wild-type counterparts.
- $\alpha 4\beta 2\chi$ - and $\alpha 4\beta 4\chi$ -nAChR have shortened single-channel kinetics.
- nAChR β subunit nested C2 domains play a role in modulation of $\alpha 4^*$ -nAChR function.

Fig. 1. Alignment for amino acid sequences of human nAChR β2 or β4 subunits or of mouse serotonin (5-HT)_{3A} receptor subunits spanning from the M3 transmembrane domain to the C terminus
The amino acid sequences are from the NCBI data base (NP_000739, β2; CAA69693, β4; P23979.1, 5HT_{3A}). Shading indicates where β2 or β4 subunit sequences transition to that for the 5-HT_{3A} subunit in chimeric β2 (β2_χ) and chimeric β4 (β4_χ) subunits. Amino acid numbering is based on the translation initiation methionine as residue 1.

Fig. 2. Whole-cell current responses for $\alpha 4\beta 2$ -, $\alpha 4\beta 2\gamma$ -, $\alpha 4\beta 4$ -, or $\alpha 4\beta 4\gamma$ -nAChR
(A and B) Typical whole-cell current response traces (pA; see vertical calibration bars) recorded at -60 mV holding potential ($V_H = -60$ mV) during 4 s applications (see horizontal calibration bars) of 1 mM ACh in the presence of 1 μ M atropine (Aa, Ab) or 1 μ M nicotine (Ba, Bb) for SH-EP1 cells heterologously expressing $\alpha 4\beta 2$ -nAChR (leftmost trace) or $\alpha 4\beta 2\gamma$ -nAChR (middle trace) (Aa, Ba) or for cells expressing $\alpha 4\beta 4$ -nAChR (leftmost trace) or $\alpha 4\beta 4\gamma$ -nAChR (middle trace) (Ab, Bb) (the rightmost traces show superimposed responses). Current responses decay faster and more completely for $\alpha 4\beta 2\gamma$ - or $\alpha 4\beta 4\gamma$ -nAChR than for respective, all-wild-type-subunit, $\alpha 4\beta 2$ - or $\alpha 4\beta 4$ -nAChR. (C) Bar graphs for normalized peak responses (Peak Amp) or ratios of steady state to peak currents (Is/Ip) (left ordinates) and for current decay constants (msec, right ordinates) averaged from 7 cells each group show no significant difference in peak current amplitudes, but that nAChR containing $\beta \gamma$ subunits have smaller ratios of steady-state to peak currents and faster rates of decay from peak to steady-state currents (agonist was 1 μ M nicotine).

Fig. 3. Single-channel responses of $\alpha 4\beta 2$ - and $\alpha 4\beta 2\gamma$ -nAChR

(A) Current response traces are shown for single channel recordings from SH-EP1 cells heterologously expressing $\alpha 4\beta 2$ - (Aa) or $\alpha 4\beta 2\gamma$ - (Ab) nAChR (note calibration bars for current magnitude and time). Patch recording pipettes contained 100 nM ACh with 1 μ M atropine at a holding potential of +60 mV. Bars beneath traces indicate response segments represented in the lower set of traces on an expanded time scale, and arrows point to single channel events having low or high conductance. (B) Bar graphs are shown for numbers of events (ordinate) as a function of single event amplitude (pA) and indicate that there are two conductance states for either $\alpha 4\beta 2$ - (Ba) or $\alpha 4\beta 2\gamma$ - (Bb) nAChR.

Fig. 4. Single-channel responses of $\alpha 4\beta 4$ - and $\alpha 4\beta 4\gamma$ -nAChR

(A) Current response traces are shown for single channel recordings from SH-EP1 cells heterologously expressing $\alpha 4\beta 4$ - (Aa) or $\alpha 4\beta 4\gamma$ - (Ab) nAChR (note calibration bars for current magnitude and time). Patch recording pipettes contained 100 nM ACh, and the holding potential was +60 mV. Bars beneath traces indicate response segments represented in the lower set of traces on an expanded time scale, and arrows point to single channel events having low or high conductance. (B) Bar graphs are shown for numbers of events (ordinate) as a function of single event amplitude (pA) and indicate that there are two conductance states for either $\alpha 4\beta 4$ - (Ba) or $\alpha 4\beta 4\gamma$ - (Bb) nAChR.

Fig. 5. Single-channel kinetics for nAChR containing wild-type or chimeric $\beta 2$ subunits
 Histograms are shown for the number of events having the indicated, single channel open response durations (A) or having the indicated periods of single channel closure (B) in the presence of 100 nM acetylcholine in recording pipettes for SH-EP1 cells heterologously expressing $\alpha 4\beta 2$ - (Aa, Ba) or $\alpha 4\beta 2\chi$ - (Ab, Bb) nAChR. Mean open times are best fit to two components, and mean closed durations are fit to three components. nAChR containing $\beta 2\chi$ subunits have shorter open times and longer closed times than nAChR containing $\beta 2$ subunits.

Fig. 6. Single-channel kinetics for nAChR containing wild-type or chimeric $\beta 4$ subunits
 Histograms are shown for the number of events having the indicated, single channel open response durations (A) or having the indicated periods of single channel closure (B) in the presence of 100 nM acetylcholine in recording pipettes for SH-EP1 cells heterologously expressing $\alpha 4\beta 4$ - (Aa, Ba) or $\alpha 4\beta 4\chi$ - (Ab, Bb) nAChR. Mean open times are best fit to two components, and mean closed durations are fit to three components. nAChR containing $\beta 4\chi$ subunits have shorter open times and longer closed times than nAChR containing $\beta 4$ subunits.