Mutational Analysis of Roles for Extracellular Cysteine Residues in the Assembly and Function of Human α7-Nicotinic Acetylcholine Receptors†

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ABSTRACT: Nicotinic acetylcholine receptors (nAChR) containing α7 subunits self-assemble into simple, homopentameric complexes. However, successful heterologous expression of functional α7-nAChR has only been achieved in a few host cell types, such as the SH-EP1 human epithelial cell line. All ionotropic glycine receptor, $GABA_A$ receptor, $5\text{-}HT_3$ receptor, and nAChR subunits contain a pair of highly conserved cysteine residues (C150 and C164 for α7 subunits) in their N-terminal extracellular domain. These residues are thought to be involved in the formation of a conserved cystine loop that is critical to the proper folding and assembly of subunits. However, nAChR α7 (and α8) subunits also contain a third cysteine residue, C138, N-terminal to the conserved cysteine pair. Using SH-EP1 cells as a host for heterologous expression, we evaluated the roles of C138, C150, and C164 in subunit folding, assembly, and cell surface expression and function of α7-nAChR. Results indicate that mutation of C138, but not of C150 or C164, yields an nAChR that can assemble to form ^{125}I-labeled α-bungarotoxin binding sites expressed on the cell surface. Further, whole-cell patch clamp recordings demonstrate that mutation of C138 to alanine does not alter the function of the fully assembled α7-nAChR. These results indicate that C150 and C164 are required for surface expression, but that C138 is neither necessary for nor inhibitory toward the surface expression and function of human α7-nAChR. These results suggest that disulfide bond formation between C138 and either C150 or C164, if it occurs, has no significant effect on α7-nAChR assembly or function.

Nicotinic acetylcholine receptors (nAChRs)[1] are members of the neurotransmitter-gated ion channel superfamily. There are at least 16 different genes that encode nAChR subunits. These individual subunits assemble in various combinations to yield a diverse set of functional, pentameric receptor complexes. nAChRs are implicated in numerous important cellular and physiological processes, including memory processing (*1*), neurotransmitter release (*2*−*4*), cell survival (*5*, *6*), and synaptic plasticity (*7*−*9*). It is therefore important to understand the mechanisms whereby these receptors assemble and function.

The nAChR α7 subunit represents one of the most ancient nAChR subunits. It assembles into functional, homopentameric receptor complexes, making the resultant α7-nAChR the simplest possible model for studies of nAChR structure and function. However, α7-nAChRs have been heterologously expressed successfully in only a few cell types that appear to be capable of guiding proper receptor assembly and membrane trafficking (*10*, *11*). Reasons for inefficient heterologous expression of nAChRs in most cells are poorly understood.

All nicotinic cholinergic, ionotropic glycine, γ-aminobutyric acid type A, and ionotropic serotonin receptor subunits contain two cysteine residues in their extracellular domains that are 14 amino acids apart. These cysteines are thought to form a conserved disulfide loop. In the muscle-type nAChR from electric tissue of *Torpedo californica* or from vertebrate muscle, these cysteines have been demonstrated through mutational analyses to be required for binding of α-bungarotoxin and for proper subunit assembly and expression (*12*, *13*). In addition to these conserved cysteine residues, nAChR α7 and α8 subunits contain a third cysteine in their N-terminal extracellular domain (C138 for α7 subunits counting the translation initiation methionine as residue 1). In this work, we use site-directed mutagenesis and heterologous expression of the human mutant α7-nAChR to assess the role of C138 in the assembly and function of the α7-nAChR. Specifically, we test the hypothesis that the additional cysteine at position 138 may form alternative disulfide bonds with either conserved cysteine of the disulfide loop, thereby inhibiting the functional expression of the α7-nAChR.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The human nAChR α7 subunit cDNA [originally obtained as a generous gift from S. Leonard, University of Colorado, Boulder, CO (*14*)] was subcloned from pCEP4 (*15*) into pGEM11Zf+ using the *Not*I and *Bam*HI restriction sites from the pCEP4 multiple-cloning

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[1] Abbreviations: Bgt, α-bungarotoxin; I-Bgt, ^{125}I-labeled α-bungarotoxin; nAChR, nicotinic acetylcholine receptor; SE, standard error.

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site. A FLAG epitope was then introduced at the C-terminus by PCR. The resulting plasmid was sequenced to confirm introduction of the FLAG epitope. This plasmid was then used as the template for site-directed mutagenesis using the Quickchange mutagenesis system (Stratagene, La Jolla, CA). Resulting plasmids were isolated and sequenced to confirm that only the desired point mutations (C138A, or C150A and C164A for residues in the conserved disulfide loop) were present. The mutant α7 cDNAs were then subcloned into pCEP4 using the *Not*I and *Bam*HI restriction sites to re-enable heterologous expression in mammalian cells.

Transfection of SH-EPI Cells. SH-EPI cells routinely maintained using techniques already established (*11*, *15*) were grown to 70% confluence and transfected using Superfect reagent (Qiagen, Valencia, CA) following the manufacturer's protocol. Two days following transfection, positive selection of transfectants was initiated by maintenance in standard culture medium (*11*, *15*) supplemented with hygromycin (130 μg/mL). Individual colonies of transfectants evident after selection for 2 weeks were isolated and expanded for subsequent binding and functional assays. For transient transfections, cells were selected for 1 week using hygromycin (130 μg/mL), trypsinized, and simultaneously plated on 10 cm plates for whole-cell ^{125}I-labeled α-bungarotoxin (I-Bgt) binding assays, on six-well plates for cell surface I-Bgt binding assays and RNA isolation, or on 22 mm slide coverslips for immunostaining. As a reference cell line, the SH-EP1-pCEP4-hα7 clonal line previously established (*15*) was used in some studies.

Radioligand Binding Assays. Cell membranes and I-Bgt were prepared as previously described (*16*). Whole-cell membrane I-Bgt binding assays were performed as previously described (*16*). To assess cell surface I-Bgt binding, cells were grown in standard six-well plates to confluence. I-Bgt was added to a concentration of 10 nM in samples used to determine the level of total binding. Nonspecific binding was evaluated using samples containing 10 nM I-Bgt and 1 μM α-cobratoxin, and specific binding was taken to be the difference between total and nonspecific binding. Protein quantitation was performed using BCA reagent (Pierce, Rockford, IL).

Immunocytochemistry. For immunostaining, cells were grown on 22 mm × 22 mm glass coverslips, rinsed once in PBS, and fixed with 4% paraformaldehyde at room temperature for 10 min. Cells were then rinsed again once with PBS for 5 min and permeabilized with 0.2% Triton X-100 in 10% normal serum for 5 min. A monoclonal antibody directed against the cytoplasmic loop of the α7 subunit (mAb306, Sigma, St. Louis, MO) was then added in 10% normal serum. Cells were incubated with the antibody overnight at 4 °C. Cells were then rinsed three times sequentially with PBS for 10 min each time and incubated with a biotinylated rabbit anti-rat secondary antibody (Vector Labs, Burlingame, CA) at a 1:2000 dilution in 10% normal serum at room temperature for 1 h. Cells were again rinsed three times with PBS for 10 min each time. Alexa488-avidin (Molecular Probes, Eugene, OR) was then added at a 1:2000 dilution in 10% normal serum, incubated for 30 min in the dark, and subsequently rinsed three times in PBS for 10 min each time. Cells were post-fixed in cold methanol for 5 min and dry-mounted before being examined using epifluorescence microscopy (Olympus i70, Olympus, Melville, NY).

Quantitative, Real-Time Polymerase Chain Reaction. RNA was isolated using TriZol reagent (Invitrogen, Carlsbad, CA). Total RNA was then reverse-transcribed using the SuperscriptII-RnaseH kit (Invitrogen, Carlsbad, CA) with oligo-(dT) as the primer. PCRs were performed in real time using a LightCycler (Roche, Indianapolis, IN). The number of cycles required to produce a detectable signal above background was recorded for each sample. These cycle numbers were then used to calculate fold differences in starting α7 subunit mRNA levels for each sample using the following method. First, the cycle number difference for vimentin, a housekeeping gene, was determined in both the established, wild-type α7 subunit-expressing cell line and the appropriate cell line expressing the mutant subunit (Δ*H*). Next, the cycle number difference for the α7 subunit PCR from each cell line was determined (Δ*I*). The cycle number difference for α7 subunits was then corrected for slight differences in the amount of total RNA in cells expressing wild-type or α7 mutant subunits by subtracting Δ*H* from Δ*I*, yielding a new value Δ*K*. The expression ratio for the α7 message in the polyclonal cell lines expressing mutant α7 subunits relative to levels in the established monoclonal cell line expressing wild-type α7 subunits was then calculated as $-2^{\Delta K}$.

Patch-Clamp Whole-Cell Recordings. Conventional whole-cell current recording coupled with techniques for fast application and removal of agonist was applied in this study. Briefly, cells plated on polylysine-coated 35 mm culture dishes were placed on the stage of an inverted microscope (Olympus iX7, Olympus, Lake Success, NY) and continuously superfused with a standard external solution [120 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM D-glucose, and 10 mM HEPES (pH 7.4) with Tris base]. Glass microelectrodes (3−5 MΩ resistance between pipet and extracellular solutions) were used to form tight seals (>1 GΩ) on the cell surface until suction was applied to convert to conventional whole-cell recording. Cells were then voltage-clamped at holding potentials of −60 mV, and ion currents in response to application of nicotinic receptor ligands were measured (200B amplifier, Axon Instruments, Foster City, CA). Both pipet and whole-cell current capacitance were minimized, and the series resistance was routinely compensated to 80%. Whole-cell access resistance of less than 20 MΩ was accepted. All experiments were performed at room temperature (22 ± 1 °C). In experiments using acetylcholine as an agonist, 1 μM atropine sulfate was routinely added to the standard solution to exclude any possible influences of muscarinic receptors, but results were the same as those obtained in the absence of atropine (data not shown). For conventional whole-cell recording, the KCl pipet solution contained 140 mM KCl, 4 mM $MgSO_4$, 0.1 mM EGTA, 4 mM ATP, and 10 mM HEPES (pH 7.2) with Tris base. To initiate whole-cell current responses, nicotinic drugs were rapidly delivered into the bath medium by a nine-channel multibarrel system, in which the applied drug completely surrounds the recorded cell in less than 30 ms (i.e., having an upper bound defined by the slowest 10−90% rise time for responses to 1 mM acetylcholine of 26.7 ms). Times between drug applications (~3 min) were adjusted specifically to ensure the stability of nAChR responsiveness. All experimental data were recorded with a 200B amplifier (Axon Instruments), typically using data filtered at 2 kHz, acquired at 5 kHz, displayed and digitized

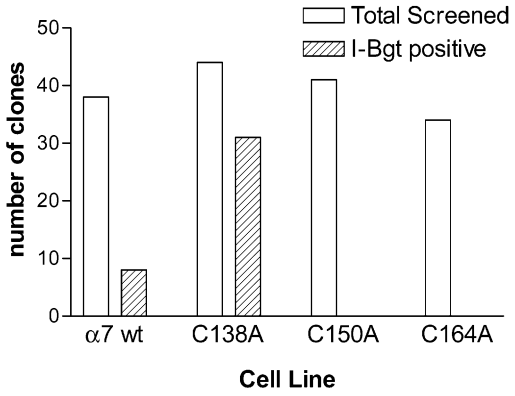

FIGURE 1: Transfection of SH-EP1 cells with human wild-type or C138A mutant α7 subunits yields cell clones capable of binding I-Bgt. Cells were transfected with either wild-type, C138A, C150A, or C164A α7 subunits as indicated (*x*-axis labels) and processed as described in Materials and Methods to obtain monoclonal lines, which were assayed for specific I-Bgt binding to total membrane fractions. Of the indicated total number of clones that were screened (ordinate, white bars), those that were positive for specific I-Bgt binding were tallied (ordinate, cross-hatched bars).

on-line (Axon Instruments Digidata 1200 series A/D board), and stored on a hard drive and videotape. Data acquisition and analyses were carried out using Pclamp8 (Axon Instruments), and results were plotted using Origin 5.0 (Microcal, North Hampton, MA).

RESULTS

The nAChR α7 subunit contains a highly conserved pair of cysteine residues in its extracellular domain (C150 and C164), which are thought to form a conserved disulfide loop. In addition to this highly conserved disulfide loop, nAChR α7 and α8 subunits are the only currently identified nAChR subunits that contain a third cysteine residue in the extracellular domain (C138 in the α7 subunit). The presence of this additional cysteine residue raises the possibility that alternative disulfide bonds could form between it and one of the two conserved members of the disulfide loop, thereby altering the assembly or function of the α7-nAChR. One consequence may be a low efficiency of subunit assembly and expression of the functional, cell surface α7-nAChR in heterologous expression systems.

To determine the roles of extracellular cysteines in the assembly and function of the α7-nAChR, we transfected the SH-EP1 human epithelial cell line with a plasmid containing cDNA corresponding to either the human wild-type α7 subunit, a human α7 subunit in which C138 had been changed to alanine (C138A), or human α7 subunits that contained either a C150A or C164A mutation. We screened monoclonal cell lines from these transfections for the ability to bind to I-Bgt using total membrane fractions, which include cell surface as well as internal pools of the α7-nAChR. Approximately 20% of the clones transfected with the wild-type α7 subunit were found to express I-Bgt binding sites (Figure 1), which were taken to represent the α7-nAChR. Interestingly, 70% of the clones transfected with the C138A mutant α7 subunits expressed α7-nAChRs that were capable of binding to I-Bgt. This difference was validated across multiple transfections and different vector DNA preparations. Thus, when C138 is changed to alanine, there is an increase in the efficiency of isolation of cloned transfected cells in which α7 subunits assemble to form I-Bgt

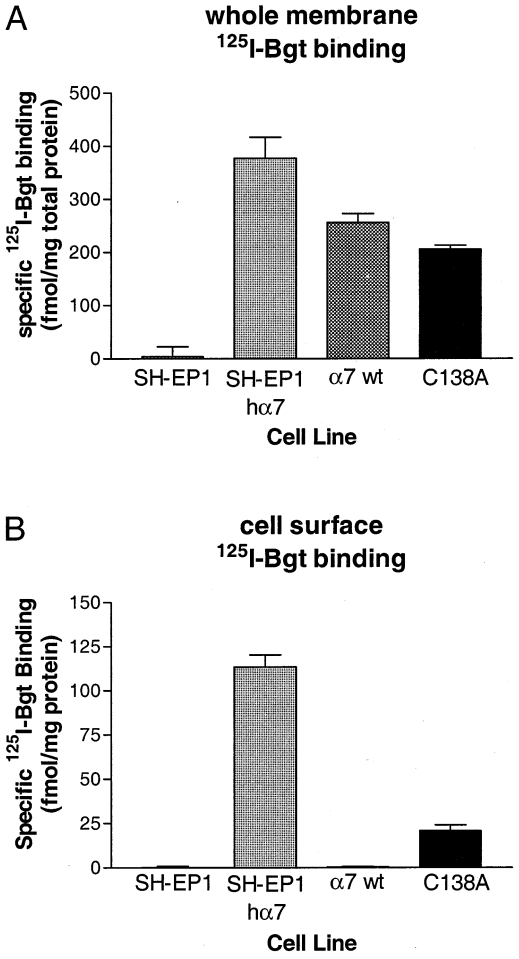

FIGURE 2: C138A mutant α7 subunits are expressed as I-Bgt binding sites on the cell surface. Untransfected SH-EP1 cells (SHEP, white bars), cells of the previously established SH-EP1-pCEP4-hα7 line [SH-EP1 hα7 (*15*), lightly shaded bars], or monoclonal lines positive for total membrane I-Bgt binding sites after transfection with FLAG-tagged wild-type (α7 wt, heavily shaded bars) or C138A mutant (C138A, black bars) human α7 subunits were used to determine (A) total membrane or (B) cell surface levels of specific I-Bgt binding (ordinates, femtomoles of specific I-Bgt binding per milligram of total cell protein ± SE; $n = 3$).

binding sites. However, the absolute levels of I-Bgt binding in the C138A mutant clones, when normalized to the total amount of protein present, were not significantly different from those in the wild-type α7 clones (Figure 2A and data not shown). Last, consistent with prior observations with *Torpedo* and muscle nAChRs, we obtained no clones of cells transfected with either the C150A or C164A α7 subunit mutant that exhibited I-Bgt binding capacity (Figure 1), suggesting that these mutant subunits were not able to assemble into a state capable of interacting with toxin.

α7 Subunits Containing the C138A Mutation Assemble as Surface α7-nAChRs. We screened all of the transfected cell clones that were positive for I-Bgt binding to total membrane fractions for their ability to express cell surface I-Bgt binding sites. Two of the 44 C138A α7 subunit clones expressed I-Bgt binding sites on the cell surface (Figure 2B and data not shown). In contrast, no wild-type α7 subunit clones (of 38 total) expressed cell surface I-Bgt binding sites, suggesting that none of these clones expressed the fully assembled α7-nAChR on the cell surface. This difference between wild-type and C138A mutant subunits could reflect an effect of

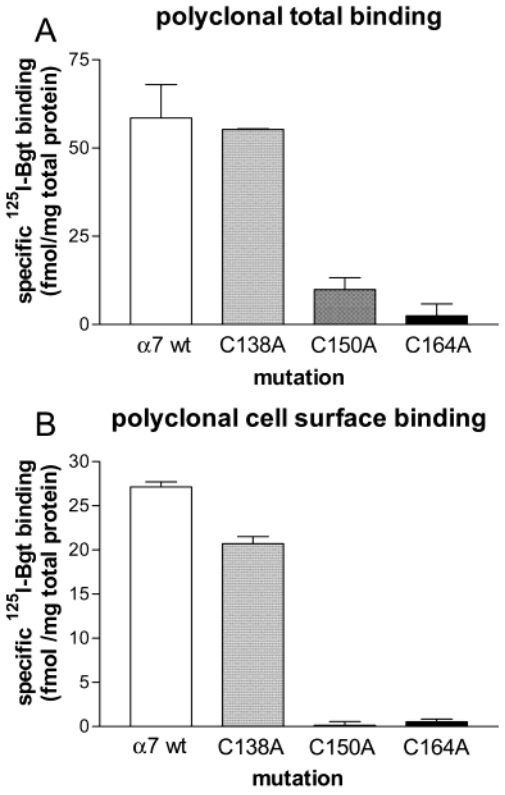

FIGURE 3: I-Bgt binding assays on polyclonal, transiently transfected cell populations verify that C138A mutant α7 subunits, but not C150A or C164A mutant subunits, are expressed as fully assembled, cell surface nAChRs. Polyclonal pools of cells transfected with either FLAG-tagged wild-type (α7 wt, white bars), C138A (lightly shaded bars), C150A (heavily shaded bars), or C164A (black bars) human α7 subunits were used to determine (A) total membrane or (B) cell surface levels of specific I-Bgt binding (ordinates, femtomoles of specific I-Bgt binding per milligram of total cell protein ± SE; $n = 3$).

C138A on cell surface expression. However, results from transient transfection experiments wherein polyclonal populations of cells transfected with wild-type α7 subunits were examined show that wild-type α7 subunits assemble to form cell surface I-Bgt binding sites (see below). Thus, the lack of cell surface expression of I-Bgt binding sites in any of the clonal lines that were transfected with wild-type α7 subunits seems to result simply from the low number of I-Bgt binding positive clones that were obtained.

α7 Subunits Containing a C150A or C164A Mutation Do Not Assemble as Surface α7-nAChRs Capable of I-Bgt Binding. Consistent with prior results using nAChR from *T. californica* or vertebrate muscle, none of the cell lines isolated after transfection with C150A or C164A α7 subunits expressed any I-Bgt binding sites. There are two possibilities for explaining this result. First, the mutant proteins may be rapidly degraded in SH-EP1 cells. Second, C150 or C164 may be directly involved in the assembly of the α7-nAChR to form I-Bgt binding sites. To distinguish between these possibilities, we repeated the transfection experiments described above and assayed the polyclonal transfectant populations for transient expression of both I-Bgt binding sites and α7 mRNA and protein expression.

As expected, polyclonal populations of cells transfected with either wild-type α7 subunits or C138A α7 subunits expressed both total I-Bgt binding sites and cell surface I-Bgt

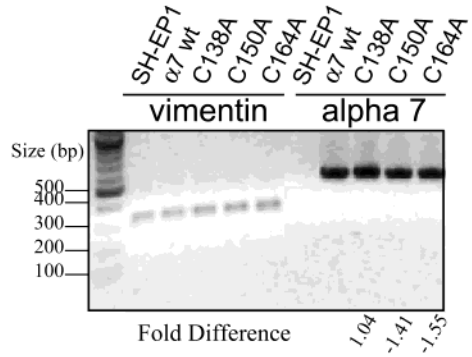

FIGURE 4: Wild-type or mutant α7 subunit cDNAs are all expressed as mRNA. Shown is an inverted image of an agarose gel. Real-time reverse transcription PCR was conducted as described in Materials and Methods to quantify α7 subunit message-derived products following 23 cycles of amplification or vimentin message-derived products after 23 cycles of amplification. Sizes of DNA standards are shown on the left, and values reported below the lanes for α7 samples (fold differences) represent the levels of mRNA expressed in each polyclonal cell population calculated relative to the wild-type α7 sample. The lanes shown are as follows: SH-EP1 (untransfected host cell), α7 wt (cells transfected with wild-type α7 cDNA), C138A (cells transfected with C138A mutant α7 cDNA), C150A (cells transfected with C150A mutant α7 cDNA), and C164A (cells transfected with C164A mutant α7 cDNA).

binding sites (Figure 3A,B). This result with cells expressing wild-type α7 subunits suggests that the previous inability to obtain monoclones that expressed cell surface I-Bgt binding sites (see Figure 2B) likely resulted from insufficient numbers of I-Bgt binding positive clones obtained for screening. Moreover, this result, when combined with earlier results from I-Bgt binding studies using clonal lines of cells transfected with the C138A mutant subunit, confirms the ability of C138A α7 subunits to assemble as stable α7-nAChRs. In contrast to cells expressing wild-type or C138A mutant α7 subunits, cells expressing either the C150A or C164A mutation showed minimal total membrane I-Bgt binding and no transient cell surface I-Bgt binding (Figure 3A,B), consistent with our previous observations.

Reverse transcription polymerase chain reaction (RT-PCR) experiments demonstrated that all transient transfectants expressed α7 mRNA (Figure 4). Real-time quantitation of the PCRs (see Materials and Methods) showed that wild-type and C138A subunit mRNAs were expressed at approximately equal amounts (Figure 4). C150A and C164A transfectants expressed about half the level of α7 mRNA as either wild-type or C138A transfectants (Figure 4B). In addition, immunostaining of these transfectants with the α7 specific antibody demonstrated that all of the polyclonal populations contained individual cells that were expressing α7 protein (Figure 5). However, the percentage of α7 subunit-expressing cells varied between populations. The C150A and C164A polyclones showed the fewest number of α7-expressing cells (Figure 5). The critical observation implicating C150 and C164 directly in I-Bgt binding is that both the C150A and C164A mutant α7 proteins are expressed yet the cells expressing them fail to bind to I-Bgt. This result suggests that the C150A and C164A mutations likely block I-Bgt binding either through disruption of the I-Bgt binding site or through the failure of those mutant subunits to assemble into a state capable of binding I-Bgt, rather than by nonspecifically producing subunits that are more susceptible to an increased level of α7 protein degradation.

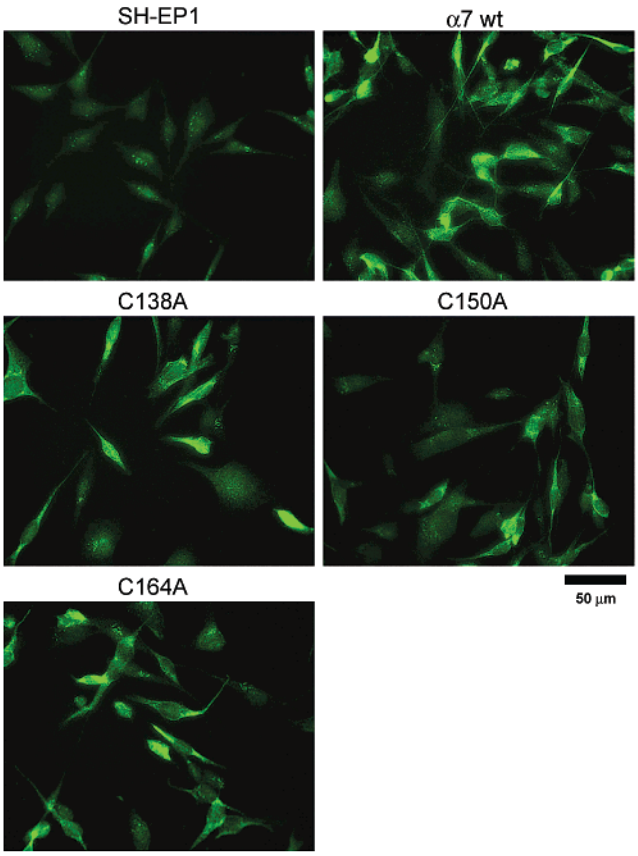

FIGURE 5: Wild-type or mutant α7 subunit cDNAs are all expressed as α7 subunit proteins. Untransfected SH-EP1 cells (top left) or polyclonal pools of cells transfected with wild-type (top right), C138A (middle left), C150A (middle right), or C164A (bottom) α7 subunits were processed as described in Materials and Methods for immunofluorescence using a monoclonal antibody specific for α7 protein. Each polyclonal population contains cells with positive staining, indicating expression of α7 protein, whereas staining of untransfected cells is absent.

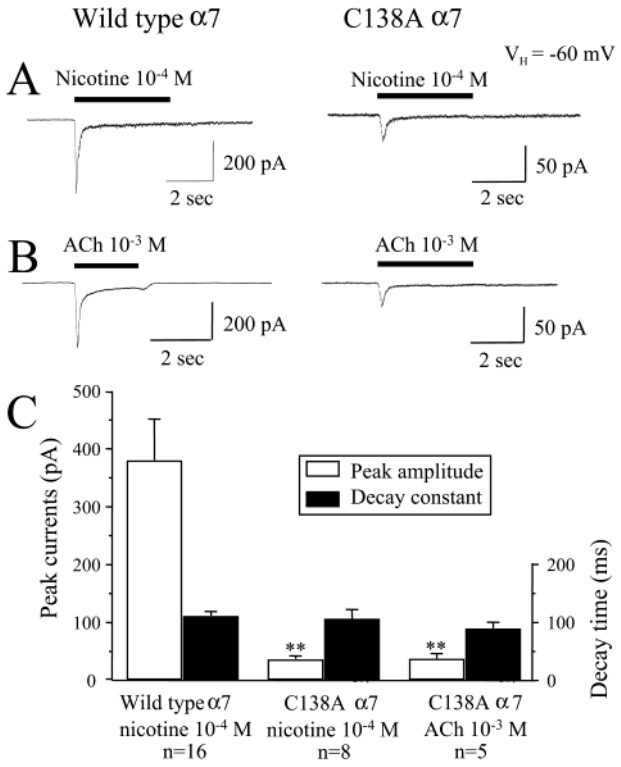

FIGURE 6: Mutation of cysteine-138 to alanine does not alter the channel kinetics of α7-nAChRs. The established SH-EP1-pCEP4-hα7 cell line expressing wild-type α7 subunits (wild-type α7) or a representative, monoclonal, transfected cell line expressing C138A α7 subunits (C138A α7) were subjected to whole-cell current recording as described in Materials and Methods. (A and B) Representative nicotine-induced (A, 0.1 mM) or acetylcholine (ACh)-induced (B, 1 mM) inward currents in cells expressing the wild-type (left panels) or C138A mutant (right panels) α7-nAChR. Horizontal bars above traces represent periods of agonist exposure. Time and current magnitude scales are shown to the lower right of each trace. (C) Bar graph that compares peak currents (left ordinate, picoamperes ± SE, white bars) and desensitization decay time constants (right ordinate, milliseconds ± SE, black bars) for wild-type α7-nAChR responses to 0.1 mM nicotine, C138A α7-nAChR responses to 0.1 mM nicotine, or C138A α7-nAChR responses to 1 mM acetylcholine. The number of cells that are tested is indicated under the columns, and asterisks indicate statistical significance relative to wild-type α7-nAChR values ($p < 0.01$). Peak currents that are illustrated are not normalized to cell surface α7-nAChR expression, which is 6-fold lower for the C138A α7-nAChR than for the wild-type α7-nAChR (see Figure 2B).

Mutation of C138 Does Not Alter the Functional Kinetics of the α7-nAChR. To test whether the C138A mutation altered the function of the assembled, cell surface α7-nAChR, we performed whole-cell patch clamp recordings using both nicotine and acetylcholine as agonists. Peak whole-cell current responses of the C138A mutant α7-nAChR to either nicotine or acetylcholine were approximately 10-fold lower than peak current responses of the wild-type α7-nAChR expressed by the established SH-EP1-pCEP4-hα7 cell line (Figure 6A,B). The amplitudes of peak currents induced by 0.1 mM nicotine or 1 mM acetylcholine for the C138A mutant α7-nAChR were 36.9 ± 5.3 pA (mean ± SE, $n = 8$; Figure 6C) and 38.1 ± 8.3 pA ($n = 5$), respectively. Peak current amplitudes for 0.1 mM nicotine-induced responses for the wild type α7-nAChR were 380.6 ± 5.3 pA ($n = 16$; Figure 6C). However, the established wild-type α7-expressing cell line expresses approximately 6-fold more cell surface nAChR than the cell line expressing the C138A mutant α7 (Figure 2B). Correction of the peak currents relative to the amount of cell surface nAChR expressed suggests that the C138A mutation does not significantly alter the peak current response to either nicotine or acetylcholine. Further, the C138A mutation did not alter the rapid desensitization of the α7-nAChR. As shown in Figure 6C, the desensitization decay constant (τ) for 0.1 mM nicotine-induced currents mediated by the C138A mutant α7-nAChR was 107.1 ± 15.2 ms ($n = 8$), whereas τ for the response of the wild-type α7-nAChR to nicotine was 111.7 ± 7.3 ms ($n = 8$). Combined, these results indicate that the C138A mutation does not significantly affect the function of the fully assembled α7-nAChR.

DISCUSSION

Cysteine 150 and Cysteine 164 Are Both Required for I-Bgt Binding to the α7-nAChR. All nicotinic acetylcholine, ionotropic glycine, $GABA_A$, and $5\text{-}HT_3$ receptors contain two cysteine residues in their extracellular domains that are 14 amino acids apart. In the muscle-type nAChR from *T. californica* electric tissue or from vertebrate muscle, these cysteines have been demonstrated through mutational analyses to be required for binding of Bgt and for proper subunit assembly and expression (*12*, *13*). Our observations indicate that both C150 and C164 are required for proper assembly

of the human α7-nAChR as well. There are no cell surface and minimal total membrane sites that bind I-Bgt in cells transfected with C150A or C164A mutant forms of α7 subunits even though C150A and C164A mRNAs and mutant proteins are expressed. Thus, our findings indicate that both members of the conserved disulfide loop are required for maturation of α7-nAChR to the point of being capable of binding Bgt.

Cysteine 138 of the nAChR α7 Subunit Does Not Interfere with Receptor Assembly. In addition to the conserved C150 and C164 residues, nAChR α7 and α8 subunits contain a third cysteine in their N-terminal extracellular domains (C138 for α7 subunits). This additional cysteine residue could potentially form alternative disulfide bonds with either member of the conserved disulfide loop, thereby perturbing the assembly or function of the α7-nAChR. Some heterologously expressed nAChR α7 subunits have been found in high-molecular mass aggregates in some previous studies when cells were lysed and processed unconventionally in the absence of alkylation agents and then subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis under nonreducing conditions (*17*). The participation of C138 in intermolecular disulfide bond formation may partially account for this observation. However, the in vivo roles of this residue in nAChR subunit assembly have not been addressed previously.

Two key observations indicate that C138 does not interfere with either assembly of α7 subunits to form Bgt binding sites, transit of assembled subunits to the cell surface, or formation of functional α7-nAChRs. First, mutation of C138 to alanine does not prevent the expression of cell surface receptors that bind I-Bgt (Figures 2B and 3B). Second, these cell surface receptors are functionally responsive to nicotinic agonists (Figure 6). For example, peak whole-cell current responses normalized to cell surface α7-nAChR expression levels are similar for cells heterologously expressing wild-type or C138A α7 subunits. The SH-EP1 human epithelial cell line used in this study is permissive for expression of wild-type or mutant forms of the α7-nAChR (*11*, *15*, *18*), as are cells of the SH-SY5Y human neuroblastoma cell line (*10*, *18*). The formal possibility remains that other cell types resistant to expression of functional α7-nAChRs, such as HEK293 cells and their derivatives, may be nonpermissive for such expression due to faulty processing of α7 subunits containing the extra C138. However, the current studies suggest that other factors are more likely to explain host cell specificity in the fidelity of functional α7-nAChR expression.

Cysteine 138 of the nAChR α7 Subunit Is Not Required for Receptor Assembly. Another possibility addressed in this study is that C138 is required for expression of functional cell surface, I-Bgt-binding α7-nAChRs. However, because mutation of C138 to alanine fails to alter the patterns of α7-nAChR expression, C138 is not essential for the production of functional α7-nAChRs. Interestingly, the C138A mutation did appear to increase the efficiency of obtaining I-Bgt binding positive, clonal transfected cell lines. This finding was observed over multiple transfections utilizing different DNA preparations each time. Reasons for this difference are currently obscure. Nevertheless, such a circumstance does not bear relation to levels of expression of α7-nAChRs across a polyclonal pool of positively selected transfectants because they are comparable for cells expressing wild-type or C138A α7 subunits.

Formation of Disulfide Bonds between C138 and either C150 or C164 Is Not Involved in the Functional Response of the α7-nAChR to either Nicotine or Acetylcholine. As allosteric macromolecules, nAChRs mutated at selected sites could potentially have functional properties different from those of the wild-type nAChR. These functional differences may result not only from local effects of the mutation on ligand binding or channel function but also from effects occurring at a distance from mutations. For example, mutation of an amino acid residue thought to line the ion channel (V274T in α7 subunits) alters functional affinities for nicotinic agonists and can lead to changes in the ability of specific drugs to act as agonists or antagonists (*18*) even though the sites involved in ligand recognition are thought to be remote from this mutation. Thus, there was a formal possibility that the C138A mutation could affect some functional properties of the α7-nAChR ion channel. We obtained two C138A clones that expressed the fully assembled α7-nAChR on the cell surface as measured by whole-cell I-Bgt binding. Whole-cell patch clamp recordings using these cell lines yielded two important observations. First, the peak current response for C138A did not significantly differ from that of the wild-type α7-nAChR when normalized to the amount of cell surface nAChR present. This result suggests that the C138A mutation does not disrupt ion flow through the activated nAChR. Second, the rate of nAChR desensitization was unchanged between the C138A mutant α7-nAChR and the wild-type α7-nAChR. These combined results indicate that alteration of C138 does not change channel kinetics for α7-nAChRs. Further, this implies that alternative disulfide bond formation between C138 and either C150 or C164, if it occurs, does not affect the function of α7-nAChRs. This again suggests that this third cysteine residue found in α7 and α8 subunits does not efficiently form incorrect disulfide bonds with either cysteine of the conserved disulfide loop.

nAChRs continue to be useful as prototypes of the ligand-gated ion channel superfamily of neurotransmitter receptors. α7-nAChRs, in part because of the simplicity of their structures as homopentamers, are important models for studies of structure−function relationships (*19*). The current findings demonstrate that the presence of an extra cysteine residue in the N-terminal extracellular domain, a unique feature of α7 and α8 subunits, appears to have neither positive nor negative consequences in the formation of functional, cell surface receptors.

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