Amino Acid Determinants of α7 Nicotinic Acetylcholine Receptor Surface Expression

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Transient transfection has not been a successful method to express the α7 nicotinic acetylcholine receptor such that these receptors are detected on the cell surface. This is not the case for all ligand-gated ion channels. Transient transfection with the 5-hydroxytryptamine type 3 subunit cDNA results in detectable surface receptor expression. Cell lines stably expressing the α7 nicotinic acetylcholine receptor produce detectable, albeit variable, levels of surface receptor expression. α7 nicotinic acetylcholine receptor surface expression is dependent, at least in part, on cell-specific factors. In addition to factors provided by the cells used for receptor expression, we hypothesize that the surface expression level in transfected cells is an intrinsic property of the receptor protein under study. Employing a set of α7–5-hydroxytryptamine type 3 chimeric receptor subunit cDNAs, we expressed these constructs in a transient transfection system and quantified surface receptor expression. We have identified amino acids that control receptor distribution between surface and intracellular pools; surface receptor expression can be manipulated without affecting the total number of receptors. These determinants function independently of the cell line used for expression and the transfection method employed. How these surface expression determinants in the α7 nicotinic acetylcholine receptor might influence synaptic efficacy is discussed.

The α7 nicotinic acetylcholine receptor (nAChR) is a member of the ligand-gated ion channel superfamily of neurotransmitter receptors that includes the nAChRs, the cation-permeable 5-hydroxytryptamine type 3 (5HT3) receptor, and the inhibitory glycine and GABA, receptors. All members possess the conserved subunit topology of a large extracellular amino-terminal domain followed by four transmembrane domains, the third and fourth of which are separated by the cytoplasmic loop, a highly variable stretch of amino acids both in composition and length (for reviews, see Refs. 1–3). α7 subunits are capable of forming homooligomeric receptors for which α7-bungarotoxin is a specific and high affinity label (4, 5). Upon activation, α7 nAChRs conduct a significant amount of Ca2+ (ICa2+=20) (5). Thus, depending on their anatomical location, α7 nAChRs may influence neurotransmitter release and synaptic integration. In the mammalian brain, small populations (compared with predictions from α7-bungarotoxin binding density) of this receptor have been functionally located to presynaptic and postsynaptic sites by recording from individual neurons under conditions of rapid drug application and removal (6–13).

Heterologous expression of the α7 nAChR would facilitate investigations of the structure-function relationships of this receptor; however, transient transfection of neuronal and non-neuronal cell lines with α7 cDNA results in a surface receptor expression level that is below the detection limit of an 125I-α-bungarotoxin binding assay. The failure to detect receptors with this assay indicates that less than 1,000 α7 nAChR toxin binding sites are on the cell surface. It is possible to select for cell clones that express abundant α7 nAChRs. There are numerous examples of surface α-bungarotoxin binding sites on cell lines that stably express the α7 nAChR cDNA as well as on primary neuronal cultures (14–22). The establishment of stably transfected cell lines, however, does not amend itself to mutational analysis of proteins in that rapid evaluation of mutated cDNA phenotypes is not feasible with this method. Recent work by Rakhilin et al. (23) indicates that cells that do express surface α7 nAChRs detectable with 125I-α-bungarotoxin binding express α7 subunits in two disulfide-bonded conformations, whereas cells that do not express surface α-bungarotoxin binding sites predominantly express α7 subunits in a single disulfide-bonded conformation. Apparently, the formation of α-bungarotoxin binding sites from α7 subunits requires cellular factors that are insufficient in many clones of a cell line and types of cells.

An α7–5HT3 chimeric receptor is an example of a receptor that expresses substantial surface receptors in a transient transfection experiment (48). The α7–5HT3 chimeric receptor forms from subunits composed of the extracellular ligand binding domain of the α7 nAChR and the four transmembrane domains and the cytoplasmic loop of the 5HT3 receptor (5HT3R). The junction between the two subunit types occurs at a shared valine residue at position 201 (48), and the receptor is called the V201 chimeric receptor.

The α7 nAChR and the V201 chimeric receptor provide us with tools to study the molecular basis for the differences between the surface expression level of these two receptors. We tested the hypothesis that amino acid residues within the subunits of ligand-gated ion channels function as surface expression determinants by regulating the distribution of receptors between surface and intracellular pools. We constructed a series of α7–5HT3 chimeric subunit cDNAs, expressed them in transfected cells, and measured the level of surface receptor expression with an 125I-α-bungarotoxin binding assay. This approach has identified amino acids that determine the number of α7–5HT3 chimeric surface toxin-binding receptors without a concomitant change in total number of toxin-binding receptors produced by the cell. Analogous surface expression

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The abbreviations used are: nAChR, nicotinic acetylcholine receptor; 5HT3, 5-hydroxytryptamine type 3; 5HT3R, 5HT3 receptor; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.
determinants were identified in the α7 subunit that increase α7 nAChR surface expression, as measured with α-bungarotoxin binding. We have identified an additional structure-function relationship based on the amino acid sequence of ligand-gated ion channel subunits; the amino acid sequence of ligand-gated ion channel subunits regulates the number of toxin-binding receptors on the cell surface.

**EXPERIMENTAL PROCEDURES**

Unless otherwise specified, all chemicals were purchased from Sigma. Cell culture reagents were purchased from Sigma and Life Technologies, Inc. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Roche Molecular Biochemicals. Rat α7 nAChR cDNA was cloned as described (5). Mouse 5HT3A serotonergic receptor subunit cDNA was a gift from D. Julius (24). Purified preparations of ion channel subunits; the amino acid sequence of ligand-toxin binding. We have identified an additional structure-functional determinant based on the amino acid sequence of ligand-gated ion channel subunits; the amino acid sequence of ligand-gated ion channel subunits regulates the number of toxin-binding receptors on the cell surface.

**Chimera Construction and Site-directed Mutagenesis**

α7–5HT3 chimeric subunit cDNAs were constructed by a three-step polymerase chain reaction (PCR) protocol using Pfu polymerase (Stratagene, La Jolla, CA) as follows. Oligonucleotide primers were designed to construct the junction between α7 nAChR and 5HT3R subunit cDNA sequences. These primers were synthesized as forward (5′–3′) and reverse (3′–5′) oligonucleotides comprised of half α7 nAChR subunit sequence and half 5HT3R subunit sequence flanking the junction between the two subunit sequences (as designed for each chimera subunit; Table I and Fig. 2). The α7 nAChR subunit sequence was amplified with the reverse primer of this type in conjunction with an α7 nAChR-specific forward primer (Table I). 5HT3R subunit sequence was amplified with the forward version of the chimeric primer in partnership with a 5HT3R-subunit-specific reverse primer (Table I). The amplicons from these two PCRs were purified and placed into a third PCR in order to amplify the chimeric sequence between the α7 nAChR-specific and 5HT3R-specific oligonucleotides; the amplicons purified from the previous PCR served as template, and the forward α7 nAChR-specific and the reverse 5HT3R-specific oligonucleotides amplify the chimeric sequence. This is possible because the PCR products from the previous reaction contain overlapping sequence (generated during the first PCR) and therefore can anneal and prime for cDNA synthesis. The latest amplicon was digested with SacI and Bst33I restriction enzymes and ligated with a similarly digested and dephosphorylated (U. S. Biochemical Co., Cleveland, OH) chimeric subunit cDNA in an eukaryotic expression vector, pcDNAI/Neo (Invitrogen, Carlsbad, CA).

A chimeric subunit cDNA was constructed in which the first transmembrane domain (transmembrane domain I, amino acid residues 201–235) of the α7 nAChR was replaced with homologous sequence from the 5HT3R. This was achieved using a three-step PCR similar to that described above. Site-directed mutagenesis was carried out using PCR techniques. A three-step PCR protocol was utilized. Mirror image forward and reverse oligonucleotides were designed to contain a mutation of choice (Table II). The forward mutating primer was coupled with the above-mentioned 5HT3R-specific reverse primer in a PCR using the appropriate α7–5HT3 chimeric subunit cDNA as template. Similarly, the reverse mutating primer was coupled with the α7 nAChR-specific oligonucleotide in a PCR with the same α7–5HT3 chimeric template. Each of the mutant amplicons was purified and mixed in a PCR with the forward and reverse primers specific for the α7 nAChR and 5HT3R subunit cDNAs, respectively. The purified product from this amplification was digested with SacI and Bst33I restriction enzymes and then ligated with similarly digested and dephosphorylated α7–5HT3 chimeric subunit cDNA.

**FLAG epitope-tagged 5HT3R (5HT3-FLAG) subunit cDNA in the pcDNAI/Neo (Invitrogen) was a gift from P. Seguela (McGill University, Montreal, Canada). This construct was described previously (5). The subunit cDNA was subcloned into pcDNAI/Amp at HindIII and XbaI restriction sites. A FLAG epitope-tagged V201 chimera cDNA was generated by subcloning.

The sequence of all mutated cDNA constructs was verified by dyeoxy sequencing (26) as performed by SeqWright (Houston, TX).

**Cell Transfection**

**COS Cells—**COS cells were transfected with receptor subunit cDNA by the DEAE-dextran method as described previously (27, 28). Briefly, 1 × 106 cells/150-mm culture dish were transfected with 5 μg of the appropriate cDNA in 10 ml of Dulbecco’s modified Eagle’s medium containing 1% antibiotics, 1% fetal bovine serum, 100 μg/ml chloroquine disulfate, 0.04% DEAE-dextran. Following 4 h of incubation, cells were treated for 2 min with 10% Me2SO in PBS followed by transfer to complete medium (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 1% antibiotics). Twenty-four h following transfection, cells were trypsinized, counted, and plated to 24-well dishes coated with poly-l-lysine (Sigma, M2000 > 300,000) at 25,000 cells per well. Receptor expression was assayed 24 h later.

**Neuroblastoma Cells—**Adenoviral transfection was performed according to a modified version of Cristiano et al. (29). Briefly, complexed adenovirus, plasmid cDNA, and poly(l-lysine) in PBS were incubated for a total of 40 min at room temperature. The mixture was added to wells of a 48-well plate containing 25,000 SK-N-SH neuroblastoma cells per well in a total volume of 400 μl of complete medium. After a 2 h incubation at 37 °C, 5% CO2, the transfection mixture was aspirated and replaced with complete medium. Cells were assayed 48 h later.

**Quantification of Receptors**

**125I-a-Bungarotoxin Binding Assay—**Surface receptors were assayed by binding 125I-a-bungarotoxin. Transfected cells were rinsed twice with PBS and then preincubated in PBS, 1% bovine serum albumin with (nonspecific binding samples, in triplicate) or without (total binding samples, in triplicate) 10 μl cobratoxin for 30 min at room temperature. This incubation was followed by the addition of 125I-a-bungra-
toxin (NIH Life Science Products) to 5 nM and incubation for 1 h at room temperature. Cells were rinsed four times with 1 ml of PBS and dissolved in 0.5 ml of 0.1 N NaOH, and isotope was quantified by liquid scintillation counting. Toxin binding dose-response data were generated by performing 125I-α-bungarotoxin binding using concentrations of toxin ranging from 0.1 to 10 nM as described above.

Total receptors were measured under the same conditions as above, except binding was performed in solubilization buffer (10 mM sodium phosphate, pH 7.4, 50 mM NaCl, 2 mM EDTA/EGTA, 1% Triton X-100), and toxin-bound cell lysates were rinsed over filters. GFC filters (Whatman) pretreated in 3% polyethylenimine, 10 mM sodium phosphate, pH 7.4, followed by 2% bovine serum albumin block in 10 mM sodium phosphate, pH 7.4, were used. Filters were rinsed four times with 4 ml of 10 mM sodium phosphate, pH 7.4, 50 mM NaCl, 1% bovine serum albumin. After drying, filters were treated with 1 ml of Soluene (Packard) for 30 min at 60 °C and then liquid scintillation-counted in the presence of 7 ml of Ultima Gold (Packard).

**FLAG Antibody Binding Assay**—COS cells transfected with FLAG epitope-tagged V201 chimera or 5HT3R subunit cDNA were rinsed twice with PBS and fixed in 4% paraformaldehyde, 4% sucrose in PBS for 10 min at room temperature. Cells were rinsed twice in PBS and blocked in 6% casein in PBS for 30 min at 37 °C. Mouse anti-FLAG for 10 min at room temperature. Cells were rinsed twice in PBS and fixed in 4% paraformaldehyde, 4% sucrose in PBS.

**Table II**

| Primer name | Nucleotide position(s) mutated | Sequence |
|-------------|--------------------------------|----------|
| a7, V219 T202I | C726T | ACCTACAAGTATCATGCAGCCTG |
| a7, V218 M203I | G730C | ACGTAACACCCGCTGTAAGG |
| a7, V219 T207P | A740C, A742T | GCCCGCTGAGGCTCTCTATAT |
| V201 I203T | T782A | TACAGATGCACTCCTGCCGAGG |
| V201 I203M | C786G | ACGTAACTATGCGCGCGAGG |
| V201 P207T | T786A | GCCCGAGGAGGCATATTCTATAT |
| a7 C218T | T788A | CTCAATCTGCTGATCTGCTGAGCC |
| a7 V219F | G776A, A778C | CTCATCCTCTGATCTGCTGAGCC |
| V219 C218S | A779A | CTCATCCTCTGATCTGCTGAGCC |
| V219 V219I | G776A, A778C | CTCATCCTCTGATCTGCTGAGCC |
| V201 S219C | A777T | TTGGTCGCCGCGATCTCCTGAT |
| V201 I219V | G778C, C778A | TTGGTCGCCGCGATCTCCTGAT |

**Sucrose Gradient Fractionation**

Lysates of approximately 1 × 10^7 cells were prepared as above, preincubated with 20 nM 125I-α-bungarotoxin, and applied to a 5–20% linear sucrose gradient prepared in 10 mM sodium phosphate, pH 7.4, 50 mM NaCl, 1% Triton X-100. Gradient and loaded sample were sedimented at 60,000 rpm (SW 60; Beckman Corp., Palo Alto, CA), 4.5 h at 4 °C. An aliquot of purified T. californica nAChR preincubated with 20 nM 125I-α-bungarotoxin was run in parallel as a standard for toxin-bound pentameric and dipentameric receptor. Fractions were collected from the bottom of the centrifuge tube. Odd numbered fractions were assayed for radioactivity by liquid scintillation counting, and even numbered fractions were resolved with SDS-polyacrylamide gel electrophoresis and immunodetected as described.

**Oocyte Expression and Electrophysiological Recordings**

Oocytes were prepared, injected, and recorded according to Chen and Patrick (28). Currents induced by 3 μM agonist application (300 μM nicotine) were recorded. The V201 chimera served as a control, and the oocytes injected with the V201 chimera subunit cDNA had currents of 300 nA or greater when agonist was applied. Recorded currents were filtered at 300 Hz using an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Data were acquired and analyzed using Axodata, Axograph (Axon Instruments), and Igor (Wave Mechanics) software on an Apple Macintosh Ici computer.

**RESULTS**

For much of the data to be described, 125I-α-bungarotoxin binding was utilized to quantify receptor expression level. Because the ligand binding domain of the chimeric receptor is contributed by the α7 subunit and α7 nAChRs and V201 chimeric receptors exhibit equivalent affinity for α-bungarotoxin (33), iodinated α-bungarotoxin is a quantitative label for both α7 nAChRs and α7–5HT3 chimeric receptors. Thus, the term “receptor” is defined as an α-bungarotoxin binding site and “expression level” refers to the number of toxin binding sites produced by a cell.

**Surface Receptor Expression Level Is Low for α7 nAChR and Is High for 5HT3-R and V201 Chimeric Receptors**—We evaluated the surface expression levels of the V201 chimeric receptor, the 5HT3R, and the α7 nAChR. COS cells were transfected with α7 nAChR or V201 chimeric subunit cDNAs, and surface receptor expression was quantified 2 days post-transfection. α7 nAChR surface expression is below the detection limit of a 125I-α-bungarotoxin binding assay (Fig. 1A). In contrast, cells transfected with the V201 chimeric subunit cDNA express tens of thousands of surface toxin binding sites (Fig. 1A). These results demonstrate again that the V201 α7–5HT3 chimeric receptor expresses many more toxin binding sites on the cell surface than the α7 nAChR.
We then determined whether the high surface expression level of the V201 chimera reflects a property of wild type 5HT3R's or is unique to the chimera. We compared the surface expression level of wild type 5HT3R with that of the V201 chimeric receptor. The 3’-end of each subunit cDNA was tagged with a sequence encoding the FLAG peptide, and surface receptor expression was measured on transfected cells with a FLAG antibody-125I-streptavidin binding assay. Surface receptor expression levels were equivalent for the 5HT3R and the V201 chimeric receptor, indicating that the surface expression phenotype of the V201 chimera is conferred by 5HT3 sequence (Fig. 1B). Based on the subunit sequence of the V201 chimera, 5HT3R surface expression determinants are contained within amino acids 201–449 of the subunit.

The Difference between V201 Chimeric Receptor and α7 nAChR Surface Expression Levels Involves a Postreceptor Assembly Mechanism—We measured total α7 nAChR expression and total V201 chimeric receptor expression to determine whether the number of surface receptors is simply a result of higher levels of expression of the V201 chimeric receptor. Cells expressing the V201 receptor express approximately 3.5 times more toxin binding sites than cells expressing the α7 nAChR. 65,000 ± 15,000 toxin binding sites are detected in extracts of cells transfected with α7 cDNA (n = 30), while extracts of cells expressing the V201 chimeric receptor contain 224,000 ± 23,000 toxin binding sites per cell (n = 41; Fig. 1C). Less than 1% of α7 nAChRs expressed are on the cell surface, whereas 30% of V201 chimeric receptors are surface receptors (Fig. 1D). This 30-fold difference demonstrates that the number of surface toxin binding sites produced by the α7 nAChR and the V201 chimeric receptor does not simply result from greater V201 chimeric receptor production. These results indicate that the mechanism underlying the different surface expression levels of these two receptors lies downstream of receptor assembly and processing events that produce a toxin binding site. We propose that the differential surface expression of the two receptor types is determined by the amino acid sequence of their subunits. To state it another way, sequence differences carboxyl-terminal to residue 201 between α7 and V201 chimeric subunits underlie the surface expression differences observed for these receptors.

Mutational Analysis of α7–5HT3 Chimeric Receptors Reveals Five Surface Expression Determinants—We tested the idea that subunit sequence determines the level of surface receptor expression by identifying amino acids in α7–5HT3 chimeric receptor subunits that act as surface expression determinants. Initially we mapped the 5HT3 portion of the V201 chimera necessary for surface receptor expression. Chimeric subunit cDNAs were engineered in which the junction between α7 and 5HT3 sequence moved progressively toward the 3’-end of the cDNA. Based on secondary structure analysis (34), the junction between α7 and 5HT3 amino acid sequence for each of these chimeric subunits was positioned at amino acids 235, 241, 267, 282, and 271 (Fig. 2A). Each cDNA construct was expressed in COS cells, and surface receptor expression was tested by a 125I-bungarotoxin binding assay. Each of the engineered chimeric cDNAs failed to express surface toxin binding sites on transfected COS cells. Receptor protein was produced, since immunoblot of these cell lysates with anti-α7 antibodies detected subunit protein, indicating that α7–5HT3 chimeric receptor

2 K. T. Dineley and J. W. Patrick, unpublished observations.
Surface expression requires part or all of 5HT₃ amino acids 201–235. We searched for surface expression determinants in the region 201–235 by constructing cDNA subunits in which the junction between α7 and 5HT₃ sequence occurs at the amino acids shown in Fig. 2B. Expression of cDNAs encoding these new chimeras in COS cells and subsequent surface ¹²⁵I-a-bungarotoxin binding revealed surface expression determinant domains that we termed domains I and II.

Surface expression determinant domain I lies within the amino acid sequence 201–208. Surface receptor expression is maintained but reduced in the L208 chimera as compared with the V201 chimera (Fig. 2B). No additional surface expression determinants are located within the amino acid sequence 208–216 because surface receptor expression levels are maintained from the L208 to L216 chimeric receptors. Surface expression determinant domain II is located within the amino acid sequence 216–219. Surface expression is lost when the V219 chimera is expressed by COS cells.

The V219 chimeric receptor surface expression phenotype is not the result of a failure in receptor assembly. Sucrose gradient fractionation of lysates from cells transfected with V219 chimeric subunit cDNA demonstrates that pentameric receptors form (Fig. 7, and see below). Therefore, amino acid regions 201–208 and 216–219 of these subunits contain one or more amino acid residues that determine the number of chimeric receptors on the cell surface.

An alignment of α7 and 5HT₃ amino acids 201–235 identifies 5 amino acids that are nonconserved within domains I and II (Fig. 3). We tested these amino acids as candidates for surface expression determinants by point-mutating each within V201 and V219 chimeric receptor subunits. The V201 chimera was chosen because surface expression determinant domains I and II are encoded by 5HT₃ sequence, and in transfected cells, this subunit cDNA expresses high numbers of surface receptors. Conversely, the V219 chimera was used because surface expression determinant domains I and II are encoded by α7 sequence and, when expressed in transfected cells, do not express detectable surface toxin binding sites. The predictions for these experiments are as follows: 1) point mutation of a surface expression determinant from a 5HT₃ amino acid to an α7 amino acid results in a failure to express a surface toxin binding site, and 2) point mutation of an α7 amino acid to a 5HT₃ amino acid results in the expression of a surface toxin binding site.

![Fig. 2. Schematic of chimeric receptor subunits used to map surface expression determinant domains I and II.](http://www.jbc.org/Downloaded from http://www.jbc.org/). 

**Fig. 2.** Schematic of chimeric receptor subunits used to map surface expression determinant domains I and II. α7–5HT₃ chimeric receptor subunit cDNAs were engineered to encode chimeric subunit proteins as illustrated. Open bars, α7 sequence; stippled bars, 5HT₃ sequence. Each chimeric receptor is named according to the amino acid (single letter code) and the position in α7 sequence at which the chimera junction occurs (arrows). Surface receptor expression was evaluated as described under “Experimental Procedures.” N ≥ 6 for each construct tested. TMD, transmembrane domain. A, α7–5HT₃ chimeric subunits identify amino acid region 201–235 from 5HT₃R as necessary for chimeric receptor surface expression. B, measurement of the number of surface toxin binding sites for α7–5HT₃ chimeric receptor subunits within amino acid region 201–235 reveal two surface receptor expression determinant domains. * significance (p < 0.001) comparing the L208, L212, and I216 chimeras with the V201, V219, A223, and L231 chimeras (Tukey multiple comparisons test).
acid will decrease surface expression of the V201 chimera, and 2) mutation of an α7 residue to a 5HT3 residue at a surface expression determinant position will increase V219 chimera surface expression.

Surface expression determinants were identified at two positions in the V201 chimera and at five positions in the V219 chimera. The P207T and S218C mutant V201 chimeric receptors expressed 32 and 68% fewer surface receptors, respectively (Fig. 4A). I202T, M203I, and V219I mutated V201 chimeric receptor surface expression was equivalent to the parent V201 chimera (Fig. 4A). V219 chimera chimeric receptor surface expression was increased by any one of the five point mutations introduced. Surface toxin binding sites were detected for the T202I, M203I, T207P, C218S, and V219I mutated V219 chimeric receptor point mutants (Fig. 4B).

We find that mutation at amino acids amino-terminal to and within the first transmembrane domain of the subunit have a profound effect on α7–5HT3 chimeric receptor expression. Five surface expression determinants have been identified for the V219 chimeric receptor, two of which have a significant influence on V201 chimeric receptor expression as well. Mutation of a surface expression determinant from an α7 amino acid to a 5HT3 amino acid increases chimeric receptor expression, whereas mutation of an expression determinant from a 5HT3 residue to an α7 residue decreases chimeric receptor expression.

**Mutation of Surface Expression Determinants Affects Chimeric Receptor Distribution**—We have shown that the surface expression phenotypes of the α7 nAChR and the V201 chimeric receptor are not the result of a mass action mechanism and are likely to have their effects downstream of receptor processing events that produce a toxin binding site. We hypothesized that the observed surface expression levels are determined by the amino acid sequence of each receptor’s subunits. We have identified five surface expression determinants that significantly alter the surface expression level of α7–5HT3 chimeric receptors. Next, we tested if surface expression determinants defined for α7–5HT3 chimeric receptors function through a mass action mechanism by quantifying the total number of receptors produced by each chimeric subunit cDNA. We performed [125I]α-bungarotoxin binding to lysates of transfected cells. Our results show that the total number of toxin binding sites per cell is equivalent for each set of chimeric receptors (Table III). Therefore, changes in surface expression level are due to receptor redistribution between intracellular and surface pools, and the different surface expression levels measured for the chimera are most likely achieved through a receptor transport mechanism.

The V201 chimeric receptor and the point-mutated forms of this receptor produce approximately 200,000 toxin binding sites per cell. Although the V219 chimeric receptor produces far fewer toxin binding sites per cell, the original and point-mutated forms of this receptor are produced at equivalent levels (Table III). Since the total number of receptors per cell is equivalent for each class of chimeric receptor, one can express the number of surface receptors as a percentage of the total number of toxin binding sites measured (Table III). This quotient describes the extent to which surface receptor expression is affected by mutation of an expression determinant; insertion of a 5HT3 amino acid increases surface expression and insertion of an α7 amino acid decreases surface expression.

**Expression Determinants Control α7 nAChR Surface Expression**—Are the determinants identified for α7–5HT3 chimeric receptors relevant to native receptors? Wild type α7 nAChR subunit cDNA was mutated to a 5HT3 amino acid at each of the five nonconserved positions within surface expression determinant domains I and II, and surface receptor expression was measured as before. In four out of five cases, surface expression of mutated α7 nAChR subunit cDNA increased to levels detectable in a toxin binding assay. On transfected COS cells, α7 T202I, M203I, C218S, and V219I expressed 1,000–3,000 toxin binding sites per cell (Fig. 5A), an expression level that is just above the detection limit of the assay. These residues determine surface expression of wild type α7 nAChRs.

Modest but reproducible and measurable increases in α7 nAChR surface expression can be achieved with point mutations at four surface expression determinant positions in and near the first transmembrane domain. This level of receptor expression is much lower than that of the V201 chimera. One possibility is that more than one α7 nAChR surface expression determinant must be mutated to a 5HT3 amino acid in order to achieve a V201 chimeric receptor-like (or 5HT3R-like) surface expression phenotype. A second possibility is that additional surface expression determinants lie elsewhere in the 5HT3 subunit. These two possibilities were tested by evaluating the surface expression level of a chimeric receptor formed from a subunit in which residues 201–235 (containing surface expression determinant domains I and II) of α7 were replaced with homologous sequence from the 5HT3R. This receptor expresses 2,381 ± 684 surface toxin binding sites per cell, an expression level comparable with the point-mutated α7 nAChRs (Fig. 5B). Therefore, the surface expression level of the V201 chimeric receptor (and wild type 5HT3R) is probably due to a cooperative interaction between the expression determinants identified and additional determinants that lie elsewhere in the subunit.

**Surface Expression Level Is an Intrinsically Property of the Receptor**—We tested whether the surface expression determinants identified for α7–5HT3 chimeric receptors are dependent on the type of cell used for receptor expression. A comparison was made of the surface expression level of V201 receptor, V219 receptor, and point mutants generated from them between COS cells and SK-N-SH neuroblastoma cells. Surface α-bungarotoxin binding sites have been reported for these cells (35). We, however, did not detect any surface receptors on the mock-transfected cells used for these experiments. In all cases, surface receptor expression was lower on SK-N-SH cells than on COS cells (Fig. 6A). When the data are plotted as a percentage of V201 surface expression, the relative level of expression of each receptor is equivalent between COS and SK-N-SH cells (Fig. 6B). The surface expression level of wild-type and α7 point mutants expressed on SK-N-SH cells were also tested. As was the case for the chimeric receptors, receptor surface expression level was reduced compared with COS cells. These results demonstrate that the surface expression phenotype exhibited by these receptors is a structure/function property of the receptor.

**Chimeric Receptor Toxin Binding Unchanged by Point Mutations**—Interpretation of the surface binding data would be confounded if the mutations introduced into V201 and V219 chimeric receptors alter the apparent binding affinity of chimeric receptor for toxin. Toxin binding to COS cells transfected
FIG. 4. Surface expression determinants revealed with point mutants of V201 and V219 chimeric receptors. A, replacement of 5HT₃ amino acid with α7 amino acid at surface expression determinant positions reduces V201 chimeric receptor surface expression. Surface binding sites were measured on COS cells transfected with original or point-mutated V201 chimeric receptor subunit cDNA. The mean number of surface binding sites per cell ± S.E. for the V201 chimera and the I202T, I203M, P207T, S218C, and I219V point mutants generated from V201 were as follows: 68,746 ± 4,350, 59,155 ± 4,373, 67,900 ± 10,507, 47,188 ± 5,297, 22,036 ± 1,950, and 84,999 ± 15,086, respectively. *, significant (p < 0.05) reduction in surface binding sites compared with V201 (two-tailed Student’s t test). B, replacement of α7 amino acid with 5HT₃ amino acid at surface expression determinant positions results in detectable V219 chimera surface toxin binding. Surface receptors were measured on COS cells transfected with original or point-mutated V219 chimeric receptor subunit cDNA. The mean number of surface binding sites per cell ± S.E. for the V219 chimera and the T202I, I203M, P207T, S218C, and I219V point mutants generated from V219 were as follows: 60,607 ± 1,475, 58,558 ± 1,842, 16,040 ± 4,361, 15,000 ± 3,975, and 23,000 ± 3,231, respectively. *, significant (p < 0.001) as compared with V219 (two-tailed Student’s t test). n = 9 for all cDNAs tested.

| Construct | Mutation | Total toxin binding sites per cell | Surface toxin binding sites per cell | Surface as percentage of total |
|-----------|----------|-----------------------------------|-------------------------------------|-------------------------------|
| V201      |          | 224,000 ± 23,000 (n = 41)         | 70,000 ± 4,000                      | 31 ± 2                        |
| V201      | P207T    | 200,000 ± 27,000 (n = 54)         | 45,000 ± 5,000                      | 24 ± 3                        |
| V201      | S218C    | 202,000 ± 32,000 (n = 39)         | 22,000 ± 2,000                      | 10 ± 1                        |
| V219      |          | 30,000 ± 12,000 (n = 27)          | 1,000 ± 1,000                       | 3 ± 3                         |
| V219      | T207P    | 32,000 ± 21,000 (n = 9)           | 16,000 ± 3,000                      | 77 ± 10                       |
| V219      | C218S    | 50,000 ± 15,000 (n = 21)          | 26,000 ± 4,000                      | 52 ± 8                        |

with V201 chimeric subunit cDNA and the point mutants generated from this construct was performed in order to estimate the apparent affinity of each mutant for α-bungarotoxin. Surface toxin binding was performed with concentrations of radiolabeled α-bungarotoxin that ranged from 0.1 to 10 nM. Normalized specific toxin binding was plotted against the log dose of toxin, and Hill coefficient values were determined with Scatchard analysis (Table IV). No significant differences were found for these parameters compared with the V201 chimeric receptor. These results show that mutations at surface expression determinant positions within the receptor subunit do not affect the apparent affinity of these receptors for toxin, only the number of surface receptors capable of binding toxin.

Oligomerized Chimeric Receptor Subunits Bind Toxin—The fact that V219 chimeric receptor expression is not detected with a surface toxin binding assay although receptors are detected in cell lysates raises the issue of whether the V219 chimera is a monomer or an oligomerized receptor. Either V219 chimeric subunits do not associate properly to form receptor, thus preventing transport to the cell surface, or the V219 chimeric receptor, properly folded and assembled, is not transported to the surface. We distinguished between these two possibilities by performing sucrose gradient fractionation of lysates from COS cells transfected with V201 or V219 chimeric receptor subunit cDNA. Thirty fractions (15 drops/fraction) were collected from each gradient, and the radioactivity in each fraction was counted. Purified T. californica nAChR (Fig. 7). In replicates of the experiment, the V201 peak was observed in 1–3 fractions up the gradient, indicating that the chimeric receptors have a lower S value than the T. californica nAChR. The small hump...
Fig. 5. Surface expression determinants function in wild-type α7 nAChR. A, point mutation of wild-type α7 to 5HT7 amino acids results in detectable surface toxin binding. The mean number of surface binding sites per cell ± S.E. for wild-type α7 and the T202I, M203I, T207P, C218S, and V219I α7 point mutants were as follows: $377 \pm 445$, $4630 \pm 362$, $3,506 \pm 785$, $103 \pm 669$, $2,731 \pm 1,399$, and $5,299 \pm 3,457$, respectively. *, significance ($p > 0.05$) as compared with wild-type α7 (two-tailed Student’s t test), $n = 6$ for all cDNAs tested. B, replacement of surface expression determinant domains I and II in wild-type α7 subunit with 5HT7 subunit sequence results in surface receptor expression. Replacement of the wild-type α7 subunit sequence 201–235 with homologous sequence from the 5HT7, R subunit results in increased surface receptor expression but not greater than observed for single point mutations. *, significance ($p < 0.001$) as compared with wild-type α7 (two-tailed Student’s $t$ test), $n = 12$.

The peak current amplitudes for the α7 nACh and α7–5HT7 Chimeric Receptors—Xenopus oocyte expression system were determined and analyzed by quantitative immunoblot (as described under “Experimental Procedures”). Densitometric measurements of the band in the eluate sample indicate that an equivalent amount of V201, V201 I202T, and V201 P207T receptors capable of binding toxin are produced in a transfection experiment (Fig. 8). The three α7–5HT7 chimeric receptors tested exhibit different surface expression phenotypes, yet the total number of receptors capable of binding toxin is equivalent, as is the protein comprising the toxin binding sites.

Toxin-purified receptor protein is approximately 5% of the total. It should be noted that equivalent amounts of receptor protein were immunodetected in each sample’s starting material. It should also be noted that the toxin binding species for these receptors is of a higher $M_r$ than the majority of immunoreactive protein in the starting material. The smaller $M_r$ species in the starting material represents truncated or degraded subunits that do not bind toxin, since these bands are not present in the toxin-purified material.

Purification of V219 and V219 C218S chimeric receptors with this method yielded visually equal amounts of receptor protein ($n = 3$).2 Densitometry quantification was not performed on these samples, because the band intensities on the immunoblot films were below the linear detection range of emulsified film. These observations are in agreement with the finding that the total number of α7–5HT3 toxin binding sites measured in transfected cell lysates is equivalent for each receptor class and supports the hypothesis that surface expression determinants affect receptor transport.

When V201 S218C chimeric receptors were purified with toxin-coupled agarose, much less receptor protein was obtained. The toxin purification results obtained for the V201 S218C chimeric receptor indicate that much less receptor protein is produced when this mutant chimaera is expressed (Fig. 8). These data are inconsistent with the toxin binding data for this receptor (which is equivalent to the V201 chimeric receptor and other mutants; see Table III) and the purification results obtained with the V201, V201 I202T, and V201 P207T chimeric receptors. An alternative explanation is that under extended incubation times (1 h at 24 °C versus 5 or 16 h at 4 °C), interaction between the V201 S218C chimeric receptor and toxin is less stable than its counterparts.

Functional Expression of Wild-type α7 nACh and α7–5HT7 Chimeric Receptors—Xenopus oocyte expression of V201 chimeric and α7 nAChRs served as controls for ion-conducting functional screening of chimeric and point mutant subunit cDNAs used in this study (Table V). Of the subunit cDNAs tested, V201 I202T, α7 T202I, P207T, C218S, and V219 C218S mutants formed functional receptors in this expression system. Of the mutant cDNAs that did express functional receptors in Xenopus oocytes, the peak current amplitudes for the α7 T202I,
T207P, and C218S mutants were significantly different from control (Table V). Functional receptors were detected for the V219 C218S mutant, whereas the V219 chimera did not exhibit function (Table V). At the nicotine concentration used, receptor function is not significantly altered by the V201 I202T mutation.

**DISCUSSION**

Cells express many more surface chimeric α7–5HT3 receptors than α7 nAChRs without a concomitant increase in total receptor number. Therefore, changes in surface receptor number are not due to a mass action effect of increased total receptor expression. These observations indicate that the amino acid sequence of receptor subunits determines surface receptor expression level. To test this idea, we constructed a series of α7–5HT3 chimeric receptor subunit cDNAs, expressed them in cells, and quantified receptor expression with an 125I-α-bungarotoxin binding assay. Toxin binding is a quantitative measurement of receptor expression level, since toxin binds to α7 nAChRs and α7–5HT3 chimeric receptors with equivalent affinity (33). For clarity in this discussion, the term “receptor” is defined as an entity that binds α-bungarotoxin, and “expression level” refers to the number of toxin binding sites produced by a cell. Our studies identified amino acid positions within receptor subunits that affect surface receptor expression through a mechanism that redistributes receptors between surface and intracellular pools. We conclude this because, following point mutation of receptor subunits, we observe changes in surface receptor number without observing changes in total receptor number. Receptor number was demonstrated to be equivalent for each class of receptor studied through toxin binding assay and quantitative receptor purification. Furthermore, only assembled receptors bind toxin, and the mutations introduced into these receptors did not affect apparent receptor affinity for toxin binding. These surface receptor expression

**FIG. 6.** Point mutants of the V201 chimera express the same relative number of surface receptors in neuronal and non-neuronal cell lines. A, number of surface toxin binding sites expressed on SK-N-SH human neuroblastoma and COS cells when transfected with point mutants of the V201 chimera. In SK-N-SH cells, the original and the P207T and S218C point mutants of the V201 chimera expressed 41,150 ± 3,775, 30,476 ± 3,076, and 12069 ± 1631, respectively. Expression levels in COS cells is reported in the legend to Fig. 1. B, when the data are normalized to the surface expression level of the V201 chimera in each cell type, the relative expression is not significantly different between the two cell types (p > 0.05; two-tailed Student’s t test).

**TABLE IV**

**V201 chimeras exhibit equivalent toxin binding characteristics**

The average ± S.E. is shown. Two-tailed Student’s t test results in no significant (p > 0.20) difference from values obtained for the V201 chimera.

| Chimera      | $K_D$, apparent (nM) | Hill coefficient |
|--------------|----------------------|------------------|
| V201         | 0.92 ± 0.13          | 1.4 ± 0.11       |
| V201 I202T   | 1.6 ± 0.44           | 1.1 ± 0.18       |
| V201 P207T   | 1.3 ± 0.56           | 1.3 ± 0.16       |
| V201 S218C   | 0.94 ± 0.33          | 1.6 ± 0.46       |

T207P, and C218S mutants were significantly different from control (Table V). Functional receptors were detected for the V219 C218S mutant, whereas the V219 chimera did not exhibit function (Table V). At the nicotine concentration used, receptor function is not significantly altered by the V201 I202T mutation.
in cells produce α7 subunits in different disulfide-bonded conformations that either form a-bungarotoxin binding sites or do not. Certainly, the differences in total toxin binding sites between the V201 chimera, V219 chimera, and α7 nAChR may result from subunit sequence effects on the formation of a toxin binding site. However, such a mechanism cannot account for our observation that point mutation of a receptor subunit changes the number of surface toxin binding sites without a concomitant change in total number of toxin binding sites produced by the cell. Our work measures the influence of subunit amino acid sequence on surface receptor expression downstream of subunit folding and assembly into α-bungarotoxin binding receptors.

The surface expression phenotypes exhibited by the receptors tested in this study are independent of the transfection method and the cell type used for receptor expression. Chimeric receptor subunits expressed in either COS fibroblast or SK-N-SH neuroblastoma cells exhibit the same rank order of surface receptor numbers, although, overall, SK-N-SH surface expression was lower. Surface expression of α7 nAChRs on SK-N-SH cells was below the detection limit (~1,000 toxin binding sites/cell) of our binding assay, probably due to the lower expression level in these cells. The mechanism(s) by which mutations change surface receptor expression involve cellular factors, but there is an intrinsic component dependent on the amino acid sequence of the receptor subunit. We have demonstrated a structure-function relationship between the sequence of ligand-gated ion channel subunits and the level of surface receptor expression.

**Possible Surface Receptor Determinant Mechanisms**—Surface expression determinants act to redistribute receptors between surface and intracellular pools, since the mutations that affect surface receptor expression level do not change the total number of toxin-binding receptors produced by the cell. Surface expression determinants may act through a receptor transport mechanism: receptor transport to the plasma membrane following subunit assembly and receptor maturation in the endoplasmic reticulum and Golgi apparatus or receptor transport (endocytosis) as part of a surface receptor recycling and/or degradation mechanism. One can imagine an intracellular receptor pool positioned either immediately downstream of the Golgi apparatus or endocytosis from the plasma membrane. Functioning as sink and source, an intracellular pool could effect changes in surface receptor numbers without changing the total number of receptors.

Effects on receptor surface expression resulting from point mutation of ligand-gated ion channel subunits indicate that a conformational change has occurred in these receptors. The mutations that increase surface receptor expression (mutations to a 5HT3 amino acid) may act to stabilize a conformation of the receptor that either promotes or retards a molecular interaction with transport and retention machinery, respectively. Those mutations that increase receptor retention (mutations to an α7 amino acid) could function to either decrease a molecular interaction with protein transport components or increase an interaction with protein retention components. Protein transport and retention components can be part of the Golgi and endoplasmic reticulum or function nearer the plasma membrane, i.e., components of the post-Golgi and endocytic pathways. With the possible exception of the threonine-to-proline mutation at position 207, these mutations are conservative in terms of charge and side chain bulk, suggesting that subtle changes in the amino acid sequence of receptor subunits can significantly change surface receptor expression levels. Elucidation of the molecules and the mechanism underlying the effect that these mutations have on receptor surface expression...
Electrophysiology—Although many chimeric and point-mutated subunit cDNAs produce surface toxin binding sites when expressed in COS cells, many of these constructs did not express functional receptors in *Xenopus* oocytes. The functional phenotype observed for these receptors may reflect the existence of receptor subunits that fold, assemble, and are transported properly (hence cell surface toxin binding), yet no longer gate in the presence of agonist or bind nicotine. Further experimentation is needed to demonstrate such a scenario.

Average peak current recordings of mutant constructs revealed significant changes compared with controls. These differences may reflect the number of receptors expressed, channel conductance, channel permeability, or receptor sensitivity to agonist. For example, the larger whole cell currents exhibited by the *α7* mutants could result from more surface receptors, as is the case for COS cell expression, or changes in channel conductance. Effects on Ca**2**⁺ permeability must also be considered, since *α7* nAChRs currents generated in oocytes are boosted by the Ca**2**⁺-induced Cl⁻ conductance (38). Presumably, this is not a concern with recordings of the chimeric receptors, since transmembrane domain II is encoded by 5HT3 sequence, and 5HT3Rs do not conduct Ca**2**⁺ (24).

**Functional Consequence of Surface Expression Determinants in Nicotinic Cholinergic Transmission**—In brain, the number of *α7* nAChRs measured with α-bungarotoxin binding is in discordance with the number of *α7* nAChRs that are measured with electrophysiology techniques (7, 9, 10, 39, 40). In certain brain areas, the density of α-bungarotoxin binding sites suggests that *α7* nAChR activation would produce a signal easily measured with contemporary electrophysiology techniques. Since α-bungarotoxin binding to sections and lysates of brain tissue does not distinguish between intracellular and surface

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**TABLE V**

*Molecular Determinants of Receptor Expression*

**Chimera receptor functional expression summary**

Results are expressed as average peak current in mA ± S.E.; NF, nonfunctional. Two-tailed Student's *t* test results in significant (*p* < 0.05) difference from control currents, i.e., V201 or *α7* receptors. *n* ≥ 3 *Xenopus* oocytes.

| Chimera | Average peak current | Chimera point mutant | Average peak current |
|---------|----------------------|----------------------|----------------------|
| V201    | 1.4 ± 0.9            | V201 I202T           | 0.5 ± 0.4            |
| L208    | NF                   | V201 P207T           | NF                   |
| I212    | NF                   | V201 S218C           | NF                   |
| B216    | NF                   | V219 T202I           | NF                   |
| V219    | NF                   | V219 M203I           | NF                   |
| A223    | NF                   | V219 P207T           | NF                   |
| S235    | NF                   | V219 C218S           | 0.04 ± 0.01          |
| P267    | NF                   |                      |                      |
| *α7*    | 0.9 ± 0.5            |                      |                      |
| *α7* T202I| 3.1 ± 1.5            |                      |                      |
| *α7* M203I| NF                  |                      |                      |
| *α7* T207P| 3.2 ± 0.5            |                      |                      |
| *α7* C218S| 4.0 ± 0.3            |                      |                      |

will provide insight into fundamental aspects of receptor expression.**

Electrophysiology—Although many chimeric and point-mutated subunit cDNAs produce surface toxin binding sites when expressed in COS cells, many of these constructs did not express functional receptors in *Xenopus* oocytes. The functional phenotype observed for these receptors may reflect the existence of receptor subunits that fold, assemble, and are transported properly (hence cell surface toxin binding), yet no longer gate in the presence of agonist or bind nicotine. Further experimentation is needed to demonstrate such a scenario. Average peak current recordings of mutant constructs revealed significant changes compared with controls. These differences may reflect the number of receptors expressed, channel conductance, channel permeability, or receptor sensitivity to agonist. For example, the larger whole cell currents exhibited by the *α7* mutants could result from more surface receptors, as the case for COS cell expression, or changes in channel conductance. Effects on Ca**2**⁺ permeability must also be considered, since *α7* nAChR currents generated in oocytes are boosted by the Ca**2**⁺-induced Cl⁻ conductance (38). Presumably, this is not a concern with recordings of the chimeric receptors, since transmembrane domain II is encoded by 5HT3 sequence, and 5HT3Rs do not conduct Ca**2**⁺ (24).**

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pools of receptor, the α7 nAChRs in the brain that are not functionally measured could represent mostly an intracellular pool. Large intracellular pools of receptor may be a common theme for neuronal nAChRs. Immunohistochemical techniques used to localize a subclass of non-α7 nAChR in brain sections shows the majority of labeled protein is associated with a cytoplasmic compartment, in addition to immunoreactive protein at synaptic and perisynaptic regions (41–46). In transiently transfected cells, the majority of α7 nAChRs are part of an intracellular pool (our results, and Ref. 43). The discrepancy between the density of α-bungarotoxin binding sites in brain and the magnitude of α7 nAChR-generated currents can be resolved if many of them are intracellular.

The idea that α7 nAChR surface expression is a regulated process in neurons is consistent with the previously discussed channel properties. α7 nAChRs in brain conduct Ca2+ upon activation, a powerful and potentially cytotoxic signaling molecule. The greater the number of α7 nAChRs at the synapse, the larger the Ca2+ signal upon receptor activation. The determinants we have defined within the amino acid sequence of nAChR subunits results in an intrinsically low level of α7 nAChR surface expression and is accompanied by a large intracellular pool of receptor. Mobilization of α7 nAChRs, either recently assembled or internalized, to the synapse from this intracellular pool will boost the Ca2+ signal and increase synaptic efficacy to subsequent receptor activation.

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