Identification of Residues in the Neuronal α7 Acetylcholine Receptor That Confer Selectivity for Conotoxin ImI

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To identify residues in the neuronal α7 acetylcholine subunit that confer high affinity for the neuronal-specific toxin conotoxin ImI (CTx ImI), we constructed α7-α1 chimeras containing segments of the muscle α1 subunit inserted into equivalent positions of the neuronal α7 subunit. To achieve high expression in 293 human embryonic kidney cells and formation of homo-oligomers, we joined the extracellular domains of each chimera to the M1 junction of the 5-hydroxytryptamine-3 (5HT-3) subunit. Measurements of CTx ImI binding to the chimeric receptors reveal three pairs of residues in equivalent positions of the primary sequence that confer high affinity of CTx ImI for α7-5HT-3 over α1-5HT-3 homo-oligomers. Two of these pairs, α7Trp55/α1Arg55 and α7Ser69/α1Gln59, are within one of the four loops that contribute to the traditional non-α subunit face of the muscle receptor binding site. The third pair, α7Thr77/α1Lys77, is not within previously described loops of either the α or non-α faces and may represent a new loop or an allosterically coupled loop. Exchanging these residues between α7 and α1 subunits exchanges the affinities of the binding sites for CTx ImI, suggesting that the α7 and α1 subunits, despite sequence identity of only 38%, share similar protein scaffolds.

The two neurotransmitter binding sites of muscle nicotinic acetylcholine receptors (AChR)1 are generated by apposition of pairs of nonequivalent subunits, α1β, α2γ, and α3ε. By contrast, the binding sites of α7 neuronal nicotinic receptors are generated by apposition of pairs of identical subunits, α7/α7 (1). Because only the α7 subunit contributes to both faces of the ligand binding site, one can study the traditional α and non-α faces by mutagenesis of a single α7 cDNA.

Ligand affinities of α7 neuronal and muscle AChRs differ owing to the different subunits that form their binding site interfaces. For example, the muscle-specific α-conotoxins M1, GI, and SI bind with high affinity to muscle receptors, whereas they bind with low affinity to α7 neuronal receptors (2). On the other hand, α-conotoxin IM1 (CTx ImI) binds with high affinity to α7 receptors but binds with low affinity to muscle receptors (3). As the only known α7-specific α-conotoxin, CTx ImI is a valuable probe of the homo-oligomeric α7 binding site.

Understanding of the α7 binding site has been limited by low expression of α7 receptors in mammalian cell lines (4, 5). Part of the problem appears due to cell type, as neuronal cell lines promote expression of α7 receptors more efficiently than non-neuronal cell lines (6). The sequence of the subunit also affects expression, as chimeras derived from α7 and 5HT-3 subunits express high levels of functional homo-oligomers in non-neuronal cells (7). Joining the α7 extracellular domain to the M1 junction of 5HT-3 permits expression in 293 HEK cells and preserves the pharmacology of the α7 binding site (7). Thus, inserting portions of 5HT-3 sequence is a powerful tool to express receptors with an intact α7 binding site.

Studies of the muscle AChR have led to a basic scaffold hypothesis to account for observations that residues in equivalent positions of the homologous γ, ε, and δ subunits contribute similarly to ligand affinity (8–10). The hypothesis postulates that owing to their high degree of homology, these subunits fold into similar peptide scaffolds such that residues equivalent in the linear sequence occupy equivalent positions in three-dimensional space. Thus, ligand affinity for a particular site is dictated by differences in primary structure rather than differences in secondary or tertiary structures.

The primary sequence of the α7 subunit reveals conserved residues that contribute to both the α and non-α faces of the ligand binding site. Within the α face of the binding site, α7 and α3 share conserved aromatic residues that stabilize agonists, including α7Tyr92, α3Trp148, α7Tyr187, and α3Tyr195 (11–13). On the other hand, α7 and non-α muscle subunits (γ, ε, and δ) share the conserved α7Trp55, which contributes to binding of agonists and antagonists (14, 15). Thus, despite only 31–38% sequence homology with muscle subunits, α7 subunits maintain conserved residues that contribute to both faces of the ligand binding site.

The experiments described herein identify residues of the α7 binding site that determine selectivity for the competitive antagonist CTx ImI and examine the question of whether α7 neuronal and α7 muscle subunits form similar protein scaffolds. By constructing chimeras composed of α7 and α1 subunits, we identified three pairs of residues in equivalent positions of the subunits that confer selectivity of CTx ImI for binding sites formed from α7 versus α1 subunits. Moreover, exchanging these three selectivity determinants between α7 and α1 subunits exchanges the affinity conferred by the subunit, indicating that α7 and α1 subunits share similar protein scaffolds.

EXPERIMENTAL PROCEDURES

Materials—[125I]-Labeled α-bungarotoxin (α-bgt) was purchased from NEN Life Science Products, d-tubocurarine chloride from ICN Pharmaceuticals, (+)-epibatidine and methylycaminol from Research Biochemicals, 293 human embryonic kidney cell line (293 HEK) from the American Type Culture Collection, and unlabeled α-bgt from Sigma. Human α7 and rat 5HT-3 subunit cDNAs were generously provided by Drs. John Lindstrom and William Green. Sources of the human acetyl-
choline receptor subunit cDNAs were as described previously (16).

Synthesis and Purification of Conotoxin ImI—Conotoxin ImI was synthesized by standard Fmoc-(9-fluorenylmethoxycarbonyl) chemistry on an Applied Biosystems 431A peptide synthesizer. During synthesis, cysteine (S-triphenylmethyl)-protecting groups were incorporated at cysteines 2 and 12, and acetamidomethyl-protecting groups were incorporated at cysteines 3 and 8. The linear peptide was purified by reversed phase high performance liquid chromatography using a Vydac C18 preparative column with trifluoroacetic acid/acetonitrile buffers. Two intramolecular disulfide bridges were formed as follows: the cysteine S-triphenylmethyl-protecting groups of cysteines 3 and 12 were cleaved, then trifluoroacetic acid cleavage of the linear peptide from the support resin, and the peptide was oxidized by molecular oxygen to form the 3-12 disulfide by stirring in 50 mM ammonium bicarbonate buffer, pH 8.5, at 25 °C for 24 h. The peptide was lyophilized prior to the formation of the second bridge. The acetamidomethyl-protecting groups on cysteine 2 and 8 were removed oxidatively by iodine as described (17) except that the peptide/iodine reaction was allowed to progress 16 h prior to carbon tetrachloride extraction. Residual iodine was separated from the pure product by high performance liquid chromatography, and CTx ImI was characterized by mass spectrometry.

**Mutagenesis and Expression in HEK Cells**—Acetylcholine receptor subunit cDNAs were subcloned into the cytomegalovirus-based expression vector pRBG4 (18). Mutant cDNAs were constructed by bridging naturally occurring or mutagenically installed restriction sites with double-stranded oligonucleotides. The chimeras are named as follows: the first subunit is the amino-terminal sequence of the chimera, the number following gives the position of the chimeric junction, and the final subunit gives the subunit from which the carboxyl-terminal sequence of the extracellular domain is taken. The extracellular domains of all chimeras are joined at M1 to the rat 5HT-3 sequence. Chimera a7/5HT-3 (a7/200/5HT-3) was constructed by bridging a 58-bp synthetic oligonucleotide from a TfiI site in a7 to an EcoRI site in rat 5HT-3. Chimera a2/5HT-3 (a2/205/5HT-3) was constructed by bridging a 69-bp synthetic oligonucleotide from a DraIII site in a2 to a StuI site in rat 5HT-3. All constructs were confirmed by dideoxy sequencing. HEK cells were transfected with wild type or mutant cDNAs using calcium phosphate precipitation as described (18). Two days after transfection, intact cells were harvested by gentle agitation in phosphate-buffered saline with 5 mM EDTA.

**Ligand Binding Measurements**—Ligand binding to intact cells was measured by competition against the initial rate of 125I-labeled a-bgt binding (18). The cells were briefly centrifuged, resuspended in potassium Ringer’s solution, and divided into aliquots for ligand binding. Potassium Ringer’s solution contains 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl2, 1.7 mM MgCl2, 25 mM HEPES, and 30 mg/ml bovine serum albumin, adjusted to a pH of 7.4 with 10 mM NaOH. Specified concentrations of ligand were added 30 min prior to addition of 3.75 nM 125I-labeled a-bgt, which was allowed to bind for 15 min to occupy approximately half of the surface receptors. Binding was terminated by addition of 2 ml of potassium Ringer’s solution containing 600 µM of d-tubocurarine chloride. All experiments were performed at 24 ± 2 °C. Cells were harvested by filtration through Whatman GF-B filters using a Brandel cell harvester and washed three times with 3 ml of potassium Ringer’s solution. Prior to use, filters were soaked in potassium Ringer’s solution containing 4% skim milk. Nonspecific binding was determined in the presence of 10 nM a-bgt and was typically 1% of the total number of binding sites. The total number of binding sites was determined by incubation with toxin for 120 min. The initial rate of toxin binding was calculated as described previously (19) to yield the fractional occupancy of ligand. Binding measurements were analyzed according to the Hill equation: 1 − Y = 1/(1 + ([ligand]/Kapp)nH), where Y is fractional occupancy of ligand, Kapp is the apparent dissociation constant and nH is the Hill coefficient. Parameter estimates and standard errors were obtained using UltraFit (BIOSOFT). For multiple experiments, means and S.E. of the fitted parameters are given in Table I. Expression of wild type a2 and a2/5HT-3 surface receptors was typically 7 fmol and 6 pmol per 10-cm plate, respectively.

Ringer’s solution. Following labeling with 3.75 nM 125I-labeled a-bgt for 2 h, cells were washed free of unbound radioactivity. Samples for sucrose gradient centrifugation were solubilized in 1 ml of Triton X-100 buffer (0.6% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 35 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 1 µg/ml pepstatin A, pH 7.5). Extracts were layered on sucrose gradients (3–30%) and centrifuged for 22 h at 40,000 rpm, and fractions were collected and counted with a γ counter. Radioactivity in each fraction was normalized to that of the fraction containing the maximum radioactivity in each gradient.

**RESULTS**

Characterization of a2/5HT-3 and a2/5HT-3 Chimeric Receptors—Previous studies described construction of a chimera containing the extracellular domain of chick a7 joined to the M1 junction of the rat 5HT-3 subunit (a7/201/5HT-3) (7). The studies further showed that addition of 5HT-3 sequence maintained ligand recognition properties of the native a2 binding site. We constructed a similar chimera by joining the extracellular domain of human a7 to the rat 5HT-3 subunit with the chimera junction formed at position 200 (a7/200/5HT-3) (Fig. 1A). To determine whether our human a7/5HT-3 receptor has similar ligand recognition properties to wild type a7, we expressed the constructs in 293 HEK cells and measured binding of agonists and antagonists by competition against the initial rate of 125I-
labeled α-bgt binding. Although expression of α7/5HT-3 receptors exceeds that of wild type α7 receptors by 1000-fold (see Fig. 1 legend), the competitive antagonists CTx ImI and methyllycaconitine and the agonist (+)-epibatidine bind with identical affinities to the two types of receptors (Fig. 1, B–D; Table I). Thus, the α7 ligand binding domain is preserved in α7/5HT-3 receptors, and expression is greatly enhanced by addition of 5HT-3 sequence.

To investigate the basis of the specificity of CTx ImI for α7 receptors, we needed a subunit homologous to α7 and with low affinity for CTx ImI to serve as a frame of reference. We therefore constructed an analogous α5/5HT-3 chimera by joining the α5 subunit extracellular domain to the M1 junction of the 5HT-3 receptor. When transfected into 293 HEK cells, the α5/5HT-3 cDNA leads to expression of α-bgt binding sites on the cell surface. Moreover, CTx ImI binds 50-fold less tightly to α5/5HT-3 than to α7/5HT-3 receptors (Fig. 2A). Similarly, CTx ImI binds 50-fold less tightly to adult human muscle receptors, further demonstrating neuronal specificity of CTx ImI (Fig. 2A). Thus, the α5/5HT-3 receptor provides a homologous muscle-like frame of reference for investigating specificity of CTx ImI for the α7/5HT-3 receptor.

To determine whether surface receptors generated by α7/5HT-3 and α5/5HT-3 are homo-oligomers containing five subunits, we labeled them with 125I-labeled α-bgt and centrifuged the solubilized receptors on sucrose density gradients. We ran a parallel gradient containing the α2βδ human muscle receptor to provide a 9S pentamer standard. The α5/5HT-3 receptor migrates with a sedimentation coefficient just greater than our 9S muscle receptor standard, as described previously (20), and the profile was noticeably broader (Fig. 2B). The increased molecular weight and broad profile suggest either increased glycosylation or an additional 40 kDa due to binding of α-bgt to the five potential binding sites. The α5/5HT-3 chimera co-migrates with our 9S muscle receptor standard. Both the α7/5HT-3 and human muscle receptors show significant 13S peaks due to free 125I-labeled α-bgt, indicating dissociation of some of the α7-receptor complexes during centrifugation. These results show that both α5/5HT-3 and α5/5HT-3 subunits form pentameric homo-oligomers on the cell surface.

To further investigate differences in stability of the α-bgt-receptor complexes suggested by sedimentation analysis, we compared time courses of 125I-labeled α-bgt dissociation from α7/5HT-3, α5/5HT-3, adult mouse muscle, and adult human muscle receptors. 125I-labeled α-bgt dissociates from α7/5HT-3 and adult mouse muscle receptors with a single slow rate constant ($t_{1/2} = 20$ h), whereas adult human muscle receptors show a more rapid single rate of dissociation ($t_{1/2} = 2.5$ h). However, 125I-labeled α-bgt dissociates from α5/5HT-3 receptors in a biphasic manner, with a rapid component having a $t_{1/2}$ of 13 min and a slow component having a $t_{1/2}$ of 13.7 h. The amplitudes of the two components are approximately equal, indicating similar numbers of two classes of sites in the α7/5HT-3 receptor. Thus, the kinetics of 125I-labeled α-bgt dissociation reveal quantitative differences in binding sites of α7/5HT-3, α5/5HT-3, and muscle α2βδ subunits. Data are from single representative experiments; overall mean parameters and S.D. are given in Table II. Panel B, sucrose density sedimentation profiles of surface receptors formed by expressing α7/5HT-3, α5/5HT-3, and muscle α2βδ cDNAs in 293 HEK cells as described under “Experimental Procedures.” Radioactivity in each fraction is normalized to the peak value in each gradient. Arrows and S values indicate α-bgt (1.3 S) and α2βδ pentamer (9 S) peaks. Panel C, time courses of 125I-labeled α-bgt dissociation from α7/5HT-3, α5/5HT-3, human muscle α2βδ, and mouse muscle α2βδ receptors. For each receptor, data are normalized to the binding at zero time. For α7/5HT-3, human muscle α2βδ, and mouse muscle α2βδ receptors, the smooth curves are fits to a single exponential decay, with $t_{1/2}$ given in the text; for α5/5HT-3 receptors, the curve is a fit to a double exponential decay, with $t_{1/2}$ values given in the text.

**Table I**

| Receptor Binding Determinants for Conotoxin ImI |
|-----------------------------------------------|
| K<sub>app</sub> | n<sub>H</sub> | n |
| Conotoxin ImI |
| Wild type α7 | 2.45 ± 0.10 μM | 1.0 ± 0.05 | 2 |
| α5/5HT-3 | 2.38 ± 0.05 μM | 0.85 ± 0.01 | 10 |
| Methyllycaconitine |
| Wild type α7 | 12.5 ± 0.10 nM | 0.82 ± 0.04 | 1 |
| α5/5HT-3 | 11.9 ± 0.05 nM | 0.85 ± 0.08 | 1 |
| (+)-Epibatidine |
| Wild type α7 | 194 ± 11 nM | 1.89 ± 0.23 | 1 |
| α5/5HT-3 | 198 ± 9 nM | 1.72 ± 0.11 | 1 |

$K_{app}$ is the apparent dissociation constant, $n_H$ is the Hill coefficient, and $n$ is the number of independent experiments.

Fig. 2. Characterization of cell surface receptors formed by α5/5HT-3 and α7/5HT-3 chimeras. Panel A, comparison of CTx ImI binding to surface receptors composed of wild type α7, α5/5HT-3, α7/5HT-3 and muscle α2βδ subunits. Data are from single representative experiments; overall mean parameters and S.D. are given in Table II. Panel B, sucrose density sedimentation profiles of surface receptors formed by expressing α5/5HT-3, α7/5HT-3, and muscle α2βδ cDNAs in 293 HEK cells as described under “Experimental Procedures.” Radioactivity in each fraction is normalized to the peak value in each gradient. Arrows and S values indicate α-bgt (1.3 S) and α2βδ pentamer (9 S) peaks. Panel C, time courses of 125I-labeled α-bgt dissociation from α5/5HT-3, α7/5HT-3, human muscle α2βδ, and mouse muscle α2βδ receptors. For each receptor, data are normalized to the binding at zero time. For α5/5HT-3, human muscle α2βδ, and mouse muscle α2βδ receptors, the smooth curves are fits to a single exponential decay, with $t_{1/2}$ given in the text; for α5/5HT-3 receptors, the curve is a fit to a double exponential decay, with $t_{1/2}$ values given in the text.

Determination of CTx ImI Selectivity Identified Using α5-α7 Chimeras—We constructed a series of α5-α7 chimeras to identify residues of the α7 receptor that confer the 50-fold higher affinity of CTx ImI for α5/5HT-3 over α7/5HT-3 receptors. For all chimeras, we joined the extracellular domains to the M1 junction of the rat 5HT-3 subunit. Our first informative chi-
a7 Receptor Binding Determinants for Conotoxin ImI

Table II
Conotoxin ImI binding parameters for receptors containing wild type, chimeric, or point mutant subunits

| receptors              | K_{app} (μM) | n | n_H |
|------------------------|--------------|---|-----|
| Wild type a7           | 2.45 ± 0.1   | 1.0 ± 0.05 | 2   |
| a7/5HT-3               | 2.38 ± 0.05  | 0.85 ± 0.01 | 10  |
| a7/5HT-3 (human)       | 117 ± 4.8    | 1.1 ± 0.05  | 8   |
| a7/55a5/77α7 (human)   | 119 ± 4.0    | 1.1 ± 0.06  | 3   |
| a7/55a5/77α7 (human)   | 6.9 ± 0.2    | 0.91 ± 0.06 | 3   |
| a7/55a5/77α7 (human)   | 23.7 ± 6.0   | 0.92 ± 0.06 | 3   |
| a7/55a5/77α7 (human)   | 25.7 ± 2.7   | 0.9 ± 0.04  | 3   |
| a7/55a5/77α7 (human)   | 22.8 ± 1.6   | 0.92 ± 0.05 | 3   |
| a7/55a5/77α7 (human)   | 23.2 ± 1.6   | 0.95 ± 0.05 | 3   |
| a7/55a5/77α7 (human)   | 119 ± 8.3    | 1.08 ± 0.08 | 3   |
| a7/55a5/77α7 (human)   | 104 ± 5.6    | 1.0 ± 0.05  | 1   |
| a7/55a5/77α7 (human)   | 22.8 ± 3.1   | 0.9 ± 0.05  | 1   |
| a7/55a5/77α7 (human)   | 2.3 ± 0.88   | 0.88 ± 0.04 | 1   |
| a7/111α1/116α1        | 3.6 ± 0.21   | 0.82 ± 0.03 | 3   |
| a7/133α1/153α1        | NE           | 1   |
| a7/164α1/200          | NE           | 1   |
| a7/184α1/200          | NE           | 1   |
| Point mutants a7      |              |     |     |
| a7/W55R               | 7.2 ± 0.26   | 0.92 ± 0.02 | 3   |
| a7/S59Q               | 6.9 ± 0.32   | 0.88 ± 0.03 | 3   |
| a7/T77K               | 23.9 ± 0.37  | 0.89 ± 0.02 | 3   |
| a7/W55R/S59Q          | 20.5 ± 2.0   | 0.84 ± 0.04 | 3   |
| a7/W55R/T77K          | 46 ± 2.2     | 1.0 ± 0.06  | 2   |
| a7/S59Q/T77K          | 41.8 ± 6.9   | 1.0 ± 0.06  | 2   |
| a7/W55R/S59Q/T77K     | 11 ± 5       | 0.99 ± 0.05 | 3   |
| a7/R55W               | NE           | 1   |
| a7/Q59S               | 59.8 ± 2.9   | 1.0 ± 0.08  | 3   |
| a7/K77T               | 29.5 ± 1.15  | 0.95 ± 0.03 | 3   |
| a7/Q59S/K77T          | 9.41 ± 0.78  | 0.88 ± 0.06 | 2   |
| a7/R55W/Q59S/K77T     | NE           | 1   |

Data are the least squares fits to the Hill equation from the series of experiments shown in Figs. 2–5. K_{app} is the apparent dissociation constant, n_H is the number of independent experiments, and NE is no expression.

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Fig. 3. CTX ImI binding to receptors formed by a7–a7 chimeras. Panel A is a schematic drawing of the a755a77α7 chimera, which contains a7 sequence from residues 55 to 77. The black portion represents a7 sequence, the unshaded portion represents a7 sequence, and the shaded portion represents SHT-3 sequence. Comparison of a7 and a7 sequence in this segment indicates candidate residues that confer CTX ImI selectivity. Panel B, CTX ImI binding to intact cells expressing a7/5HT-3, a7/5HT-3, and a755a77α7 receptors. The chimera contains a small insertion of a7 sequence between residues 55 and 77 of the a7 subunit (Fig. 3A). This short segment of a7 sequence fully decreases CTX ImI affinity to that of the pure a7/5HT-3 chimera (Fig. 3B), suggesting that the 55–77 segment is the entire source of high affinity in a7.

We examined additional a7–a7 chimeras to determine whether the 55–77 segment is the sole source of high affinity for CTX ImI (Table II). The chimera a755a77α7 confers pure a7-like affinity, indicating that residues between positions 78 and 103 do not contribute to selectivity. We also constructed chimera to target two of the three regions that confer selectivity of a7-conotoxin MI in muscle receptors (8): a7/28α731α7, a7/27α754α7, and a7/110α116α7. Each of these chimeras confers pure a7-like affinity, indicating no contributions of segments 28–31, 37–54, and 111–116. We also constructed chimera targeting the carboxyl-terminal extracellular domain, including a7/133α1/153α1, a7/164α1/200, and a7/184α1/200, but these did not form functional receptors. Thus, the 55–77 segment in a7 appears to be the sole source of high affinity for CTX ImI.

Dissection of the 55–77 Segment—We constructed a series of stepwise chimera to further localize selectivity determinants within the 55–77 segment. Starting with our reference chimera a755a77α7, we maintained the a7–a7 junction at position 55 but shifted the carboxyl-terminal junction from position 77 to position 57 (Fig. 4). Surpassing only one mismatched residue, the chimera a755a76α7 increases CTX ImI affinity toward that of a7, suggesting that the pair a7/Thr77/Lys77 contributes to selectivity. Shifting the junction from position 76 to 59 produces no further change in affinity, but shifting from position 59 to 57 reveals an additional increase in affinity toward that of a7. The last chimera in this series, a755a57α7, falls 3-fold short of pure a7-like affinity, indicating that residues 55–57 contribute the remaining increment of selectivity. Thus, the stepwise chimera reveal at least three subsets of selectivity determinants within the 55–77 segment; one is between positions 55 and 57, the second is between positions 57 and 59, and the third is the pair a7/Thr77/Lys77.

**Point Mutants of Selectivity Determinants**—We further local-
mutating within the 57–59 segment, α7-S59Q decreases affinity by the same amount observed in the stepwise chimeras. Finally, the mutation α7-T77K decreases affinity as observed in the chimeras. Thus, three residues, α7-Trp55, α7-Ser59, and α7-Thr77, contribute to CTx ImI selectivity for α7 (Fig. 5).

We next combined mutations of two or three determinants into one receptor to look for interactions between the determinants and to ask whether the set of three determinants fully accounts for selectivity of CTx ImI. The three possible double mutations, α7-W55R/S59Q, α7-W55R/T77K, and α7-S59Q/T77K, decreases affinity in an additive manner (Table II), indicating that these pairs of residues contribute independently. Moreover, the triple mutation α7-W55R/S59Q/T77K fully decreases affinity to that observed for both the α7-S55Q,77T/α7-W55R/5HT-3 chimeras (Fig. 5). Thus, α7-Trp55, α7-Ser59, and α7-Thr77 confer CTx ImI selectivity for α7 receptors. The overall results support the basic scaffold hypothesis because exchange of the selectivity determinants from α1 to α7 exchanges the affinity for CTx ImI.

Exchange of Selectivity Determinants between the α7 and α1 Subunits—To further confirm that α7-W55R, α7-S59Q, and α7-T77K confer CTx ImI selectivity, we sought to convert α1 to α7 affinity by constructing the converse mutations in the α1/5HT-3 subunit (Fig. 5). Two of the three point mutants, α1-Q59S and α1-K77T, maintain good levels of expression and increase affinity for CTx ImI, as expected from the chimeras. Combining these two mutations into a single receptor with α1-Q59S/K77T] increases CTx ImI affinity in an additive manner, again showing that these determinants contribute independently. However, the third point mutation, α1-R55W, does not express alone nor when present in the triple mutant α1(R55W/Q59S/K77T). The residual affinity between the double point mutation α1(Q59S/K77T) and α1/5HT-3 equals that conferred by the point mutant α1-W55R, further suggesting that basic scaffold of the α1 subunit is similar to that of the α7 subunit.

DISCUSSION

We probed the binding site of the α7 receptor using the neuronal-specific toxin CTx ImI together with chimeras containing portions of α1 sequence substituted into the extracellular domain of α7. The results reveal three pairs of residues in equivalent positions of the subunits that confer selectivity of CTx ImI for α7 over muscle-like AChRs. Because exchange of these residues between α1/5HT-3 and α7/5HT-3 exchanges affinity for CTx ImI, the extracellular domains of α1 and α7 subunits appear to fold into similar basic scaffolds. This basic scaffold hypothesis was originally developed to explain ligand selectivity conferred by subunits with high homology, such as γ and δ subunits (49% identity), ε and δ subunits (47%), and γ and δ subunits (53%) (8–10, 18, 21). We find that the hypothesis extends to the less homologous α7 and α1 subunits, which are only 38% identical.

To investigate the basis for neuronal specificity of CTx ImI, we needed to achieve high levels of expression of receptors with α7 binding sites and a homologous, muscle-like frame of reference. As described by others (7), we find that substituting 5HT-3 sequence from the M1 domain to the carboxyl terminus markedly increases expression, yet preserves the ligand recognition properties of the α7 binding site. The increased expression was initially surprising because the extracellular domain was widely known for its importance in receptor assembly (22–24). However, studies of α7–α2 chimeras show that formation of homo-oligomers requires matching of particular residues in the M1 and M2 transmembrane domains (25). Thus, our results further confirm that the region carboxyl-terminal to M1 contributes to assembly of homo-oligomers.

Another surprise is that our α7/5HT-3 construct forms homooligomers on the cell surface. This observation contrasts with expression of the α1 subunit alone, which remains monomeric and retained within the cell. Thus, 5HT-3 sequence between M1 and the carboxyl terminus promotes homo-oligomer formation. We found differences, however, between α7/5HT-3 and α1/5HT-3 homo-oligomers in their kinetics of α-bgt dissociation. α-bgt dissociates from α1/5HT-3 homo-oligomers with a single slow rate constant, whereas the toxin dissociates from α7/5HT-3 homo-oligomers with one rapid and one slow rate constant. Thus, despite the presence of only one type of subunit, binding sites in α7/5HT-3 homo-oligomers appear to be non-equivalent. The origin of nonequivalent sites is not known, but they may arise during the course of subunit folding and oligomerization that produces a fully assembled pentamer with high affinity for α-bgt (26). Because acquisition of the toxin binding site is associated with a protein folding event, and folding requires interaction with specific subunits, successive addition of α1/5HT-3 subunits may produce unusual interactions, leading to nonequivalence of the binding sites.

In addition, we find that α7/5HT-3 and α2β6ε muscle receptors bind CTx ImI with similar low affinities. Because the muscle receptor contains the stabilizing residues ε-Trp55/δ-Trp57, and the α1/5HT-3 homo-oligomer contains the destabilizing residue α1-Arg55, one might expect higher affinity of the muscle receptor compared with that of α1/5HT-3. Resolution of the apparent paradox likely lies in the different contributions of the (−) face in the two types of receptors, because they contain identical (+) faces. Potentially, residues flanking the three determinants identified here, but unique to the ε and δ subunits, may decrease affinity, despite the presence of ε-Trp55/δ-Trp57 in the muscle receptor. The unique residue differences may cause small changes in the protein scaffold and prevent interaction between CTx ImI and ε-Trp55/δ-Trp57 as well as other determinants of affinity. In addition, the low affinity may result from reorientation of CTx ImI at the binding site, and the low affinity may be due to stabilization by residues common to the α1, ε, and δ subunits.

We chose CTx ImI to probe the α7 binding site because it is a constrained two-loop structure owing to its two disulfide bridges, similar to the muscle-specific α-conotoxins (Fig. 6). CTx ImI contains four amino acids in its first loop and three in its second, whereas muscle-specific α-conotoxins contain three
and five amino acids in its first and second loops, respectively. In addition, CTx ImI contains basic residues in both loops (Arg\(^{2}\) and Arg\(^{4}\)) and an acidic residue in the first loop (Asp\(^{3}\)), unlike muscle-specific \(\alpha\)-conotoxins; these unique residues may further contribute to neuronal specificity of CTx ImI.

Photoaffinity labeling and mutagenesis studies establish that the ligand binding sites of the muscle AChR contain contributions of both \(\alpha\) and non-\(\alpha\) subunits. Residues of the \(\alpha\) or (+) face of the binding site cluster into three linearly separate regions, leading to a three-loop model of the \(\alpha\) subunit contribution to the binding site. Similarly, residues of the non-\(\alpha\) or (−) face of the binding site cluster into four separate regions of the linear sequence, leading to a four-loop model of the non-\(\alpha\) contribution to the site (reviewed in Refs. 27 and 28). Studies of the highly homologous δ, ε, and γ subunits show that residues in equivalent positions of the subunit make equivalent contributions to ligand affinity (8–10, 18, 21). Thus, the (−) face contributed by these subunits harbors virtually superimposable peptide scaffolds. Here, we extend the basic scaffold hypothesis to the less homologous \(\alpha\) and \(\alpha\) subunits by showing that exchanging selectivity determinants between \(\alpha\) and \(\alpha\) subunits exchanges affinity for CTx ImI.

Two of the three pairs of \(\alpha\) selectivity determinants, \(\alpha\)Trp\(^{54}\)/\(\alpha\)Arg\(^{55}\) and \(\alpha\)Ser\(^{59}\)/\(\alpha\)Gln\(^{59}\), are within one of the four loops that contribute to the (−) face of the binding site. The contribution of \(\alpha\)Trp\(^{54}\) was first demonstrated by photoaffinity labeling with \(\beta\)-[\(\mathrm{TH}]\)tubocurarine, which labeled the equivalent residue Trp\(^{57}\) of Torpedo receptors (29). Subsequent mutagenesis of \(\alpha\)Arg\(^{55}\)/5HT-3 homo-oligomers revealed contributions of the equivalent residue in chick \(\alpha\)Trp\(^{54}\) to agonist and antagonist affinity (15). The second pair in this segment, \(\alpha\)Ser\(^{59}\)/\(\alpha\)Gln\(^{59}\), is equivalent to the residues in non-\(\alpha\) subunits in muscle receptors, eAsp\(^{59}\)/6Ala\(^{59}\), which contribute to dimethyl-d-tubocurarine selectivity of the adult receptor (9). Thus, approximately half of the neuronal specificity of CTx ImI is due to stabilization by one of the four loops at the (−) face of the subunit.

The third pair of selectivity determinants, \(\alpha\)Thr\(^{77}\)/\(\alpha\)Lys\(^{77}\), is not contained within previously described loops of either the \(\alpha\) or non-\(\alpha\) faces of the binding site. One member of this pair, Lys\(^{77}\), is immediately carboxyl-terminal to the main immunogenic region, which extends from the tip of the extracellular lobe of the AChR (30). Lys\(^{77}\) may be far enough from the main immunogenic region that it can fold back to the binding site. We cannot say whether \(\alpha\)Thr\(^{77}\)/\(\alpha\)Lys\(^{77}\) contributes directly or allosterically to the binding site. However, the positively charged Lys\(^{77}\) may repel one of the arginine side chains in CTx ImI, whereas Thr\(^{77}\) may be neutral or stabilize CTx ImI through hydrogen bonding. We observed equal and opposite changes in free energy of binding with \(\alpha\)Thr\(^{77}\)K and \(\alpha\)Lys\(^{77}\)T, suggesting direct contributions to affinity. A long range interaction would not be expected to show such equal and opposite free energy changes because it would have to propagate through intervening residues.

The overall results reveal three pairs of equivalent residues in the \(\alpha\) and \(\alpha\) subunits that confer selectivity of CTx ImI and show that the extracellular domains of \(\alpha\) and \(\alpha\) subunits fold into similar basic scaffolds. The precise contacts between CTx ImI and \(\alpha\) await experiments that mutate residues in both the toxin and the receptor.

REFERENCES
1. Couturier, S., Bertrand, D., Matter, J. M., Hernandez, M. C., Bertrand, S., Millar, N., Valera, S., Barkas, T., and Baliviet, M. (1999) Neuron 31, 477–486.
2. Olivera, B. M., Rivier, J., Scott, J. K., Hillyard, D. R., and Cruz, L. J. (1999) J. Biol. Chem. 274, 22067–22070.
3. McLennan, J. M., Yoshikami, D., Mahe, E., Nielsen, D. B., Rivier, J. E., Gray, W. R., and Olivera, B. M. (1999) J. Biol. Chem. 274, 16733–16739.
4. Puchacz, E., Buisson, B., Bertrand, D., and Lukas, R. J. (1994) FEBS Lett. 354, 185–199.
5. Gopalakrishnan, M., Buisson, B., Touma, E., Giordano, T., Campbell, J. E., Hu, I. C., Donnelly-Roberts, D., Arneric, S. P., Bertrand, D., and Sullivan, J. P. (1995) Eur. J. Pharmacol. 279, 237–246.
6. Cooper, S. T., and Millar, N. S. (1999) J. Neurochem. 68, 2140–2151.
7. Eisele, J. L., Bertrand, S., Galzi, J. L., Devillers-Thiry, A., Changeux, J. P., and Bertrand, D. (1993) Nature 366, 479–483.
8. Sine, S. M., Kreienkamp, H.-J., Bren, N., Maeda, R., and Taylor, P. (1995) Neuron 15, 205–211.
9. Bren, N., and Sine, S. M. (1997) J. Biol. Chem. 272, 30793–30798.
10. Prince, R. J., and Sine, S. M. (1996) J. Biol. Chem. 271, 25770–25777.
11. Sine, S. M., Quiram, P., Papanikolaou, F., Kreienkamp, H.-J., and Taylor, P. (1994) J. Biol. Chem. 269, 8808–8816.
12. Tomasselli, G. F., McLaughlin, J. T., Furman, E. M., Hawrot, E., and Yellen, G. (1991) Biophys. J. 60, 721–722.
13. Galzi, J. L., Bertrand, D., Devillers-Thiry, A., Revah, F., Bertrand, S., and Changeux, J. P. (1991) FEBS Lett. 294, 198–202.
14. O’Leary, M. E., Filatov, G. N., and White, M. M. (1994) Am. J. Physiol. 266, C648–C653.
15. Corringer, P.-J., Galzi, J.-L., Eisele, J.-L., Bertrand, S., Changeux, J.-P., and Bertrand, D. (1995) J. Biol. Chem. 270, 11749–11752.
16. Ohno, K., Wang, H.-L., Milone, M., Bren, N., Brengman, J. M., Nakano, S., Quiram, A., Pruitt, J. N., Sine, S. M., and Engel, A. G. (1996) Neuron 17, 157–170.
17. Andrade, D., Albericio, F., Sökt, N. A., Munson, C. M., Ferrer, M., and Barany, G. (1994) in Methods in Molecular Biology (Pennington, W. M., and Dunn, B. R., eds.) Vol. 35, pp. 139–140, Humana Press, Prinston, NJ.
18. Sine, S. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9436–9440.
19. Sine, S. M., and Taylor, P. (1979) J. Biol. Chem. 254, 3135–3132.
20. Anand, R., Peng, X., and Lindstrom, J. (1993) FEBS Lett. 327, 241–246.
21. Sine, S. M. (1997) J. Biol. Chem. 272, 25521–25527.
22. Kreienkamp, H.-J., Maeda, R., Sine, S. M., and Taylor, P. (1995) Neuron 14, 635–644.
23. Gu, Y., Forsayeth, J. R., Verrall, S., Yu, X. M., and Hall, Z. W. (1991) Neuron 6, 879–887.
24. Kühne, J., Laube, B., Magaile, D., and Betz, H. (1993) Neuron 11, 1049–1056.
25. Vincenzo, Aquilino, F., Rovira, J. C., Campos-Caro, A., Rodríguez-Ferrer, C., Ball, J. A., Saha, S., Saha, F., and Criado, M. (1996) FEBS Lett. 399, 83–86.
26. Green, W. N., and Claudio, T. (1993) Cell 74, 57–69.
27. Prince, R. J., and Sine, S. M., in The Nicotinic Acetylcholine Receptor: Current Views and Future Trends. (Barrantes, F. J., ed) pp. 31–59, Landes Bioscience, Austin, TX.
28. Tsukiyama, K., Sugiyama, N., Sine, S. M., and Taylor, P. (1997) Biophys. J. 73, 52–66.
29. Chiara, D. C., and Cohen, J. B. (1992) Biophys. J. 61, A160.14.
30. Tzartos, S. J., Kokka, A., Walgrave, S. L., and Contis-Tronconi, B. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2898–2903.