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Molecular Dissection of Subunit Interfaces in the Acetylcholine Receptor: Identification of Determinants of α-Conotoxin M1 Selectivity

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Summary

The acetylcholine receptor from vertebrate skeletal muscle is a pentamer of homologous subunits with composition $\alpha_2\beta\gamma\delta$. Its two ligand binding sites, formed at α–γ and α–δ interfaces, differ in their affinities for agonists and competitive antagonists, owing to different contributions of the γ and δ subunits. To identify portions of the γ and δ subunits that contribute to the binding sites, the experiments described here use γ–δ subunit chimeras and site-specific mutants to determine the basis of the 10,000-fold selectivity of conotoxin M1 for the sites. Three distinct regions of the extracellular domain were found to contribute to conotoxin M1 selectivity, each containing a single residue responsible for the contribution of that region. Residues K34, S111, and F172 of the γ subunit confer low affinity to the α–γ binding site, whereas the corresponding residues of the δ subunit, S36, Y113, and I178, confer high affinity to the α–δ site. Identification of three separate determinants of ligand selectivity suggests a limited model of the folding pattern of the extracellular domain of the subunits.

Introduction

The acetylcholine receptor (AChR) from vertebrate skeletal muscle is an integral membrane protein that binds nerve-released ACh to elicit rapid changes in transmembrane permeability to small cations. The AChR contains two ligand binding sites within a pentamer of homologous subunits with composition $\alpha_2\beta\gamma\delta$. Each subunit contains an amino-terminal extracellular domain of approximately 210 amino acids, followed by four candidate transmembrane domains. Although the two binding sites originally appeared to reside entirely within the extracellular domains of the α subunits, substantial evidence has accumulated to show that the binding sites reside at α–γ and α–δ subunit interfaces. In the native AChR, the ligand binding sites show very different affinities for ACh (Sine et al., 1990) and several types of competitive antagonists (Neubig and Cohen, 1979; Sine and Taylor, 1981; Kreienkamp et al., 1994). The molecular basis for the different affinities is thought to reside in the portions of the γ and δ subunits that contact the two α subunits to form the binding sites (Blount and Merlie, 1989; Pedersen and Cohen, 1990; Sine and Claudio, 1991).

Sine (1993) recently identified three residues in the extracellular domain of the γ and δ subunits responsible for site selectivity of the competitive antagonist dimethyl-d-tubocurarine (DMT). One of these residues, Y117 of the γ subunit, contributes to stabilization of the DMT–receptor complex through quaternary ammonium–aromatic interactions (Fu and Sine, 1994).

α-Conotoxins are a class of peptide neurotoxins found in venom of fish-eating marine snails that potently inhibit neuromuscular transmission (Myers et al., 1993). We recently demonstrated that the α-conotoxin from Conus magnus, conotoxin M1, shows marked site selectivity in binding to the mouse AChR (Kreienkamp et al., 1994). Unlike DMT, which binds more tightly to the site at the α–γ interface, conotoxin M1 binds more tightly to the site at the α–δ interface. Also, whereas the affinities of the two sites differ by approximately 80-fold for DMT, they differ by nearly 10,000-fold for conotoxin M1. Owing to these differences in site selectivity, DMT and conotoxin M1 most likely interact with different residues in the γ and δ subunits at the binding sites.

To gain further insight into the architecture of the ligand binding sites, the work described here examines the structural basis of conotoxin M1 selectivity for the two binding sites. By constructing chimeras composed of segments of the γ and the δ subunits, we identify three residues at equivalent positions of each subunit that govern selectivity of conotoxin M1 for the two binding sites.

Results

α-Conotoxin M1 binding was measured by its competition against the initial rate of ^{125}I-labeled α-bungarotoxin binding to cell-surface receptors composed of α, β, and either γ, δ, or chimeric subunits. Omitting the complementary γ or δ subunit leads to formation of pentamers that contain a second copy of the non-α/β subunit, thus potentially creating two identical binding sites (Sine and Claudio, 1991). Figure 1 shows that AChRs containing only α–γ sites bind conotoxin M1 with low affinity, whereas AChRs containing only α–δ sites bind with high affinity. Moreover, the chimera δ225γ, which contains δ sequence from the amino terminus to the first transmembrane domain M1, produces AChRs with pure δ-like affinity. Thus, the determinants of conotoxin M1 selectivity lie in the major extracellular portion of the subunit.

To localize selectivity determinants within the major extracellular domain, segments of approximately 50 residues of the γ subunit were replaced by homologous segments of δ sequence (Figure 2). Inserting the segment of δ sequence either at the amino terminus or just preceding transmembrane domain M1 increases conotoxin M1 affinity 15- to 30-fold relative to that of the native γ subunit. However, when these two segments of δ sequence are combined, as in the fourth chimera (δ56γ171δ225γ), the

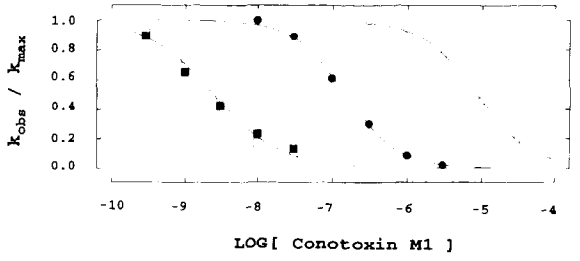

Figure 1. Concentration Dependence of Conotoxin M1 Binding to Cell Surface AChRs

The following combinations of subunits were coexpressed: α, β, and γ (open circles); α, β, and δ (open squares); α, β, and δ225γ (closed squares); and α, β, and δ57γ171δ225γ (closed circles). Conotoxin M1 binding was measured by its competition against the initial rate of α-bungarotoxin binding, as described in Experimental Procedures, and is plotted as the rate of toxin binding in the presence of conotoxin M1 divided by the rate in its absence (k_{obs}/k_{max}). The curves are fits to the Hill equation for these data. For all the data, the fitted dissociation constants had the following means and standard deviations: αβ–γ, Kd = 7.8 ± 1.3 μM; αβ–δ, Kd = 2.8 ± 1.3 nM; αβ–δ225γ, Kd = 2.5 ± 0.5 nM; αβ–δ57γ171δ225γ, Kd = 0.133 ± 0.016 μM.

affinity is only slightly greater than with chimeras containing either segment alone. Also, the absolute affinity conferred by δ56γ171δ225γ is still intermediate to that of the γ and the δ subunits (see Figure 1), suggesting the presence of additional determinants of conotoxin M1 selectivity between segments 1–57 and 171–225. Thus, three distinct regions of the major extracellular domain, labeled I–III, contribute to conotoxin M1 selectivity.

To search for determinants within amino-terminal region I, chimeras were constructed in which the chimera junction was stepped in the two possible amino- to carboxyl-terminal directions (Figure 3): from δ to γ (upper panel) and from γ to δ (lower panel). In both sets of chimeras, the affinity for conotoxin M1 changes when the subunit junction is stepped past position 34, which harbors K in the γ subunit and S in the corresponding position of the δ subunit (unless otherwise specified, position numbers correspond to the γ subunit). When the junction is stepped from δ to γ, the affinity change is about 30-fold, whereas when it is stepped from γ to δ, the change is only 3-fold. As described below, this asymmetry is due to interaction between residue 34 and a determinant of conotoxin M1 selectivity contained in region III. The chimera δ31γ differs from the other members of the series of δ–γ chimeras by the presence of a potential glycosylation site at position 30 (Figure 3). However, the presence of this nearby glycosylation site does not affect conotoxin M1 selectivity.

The intermediate affinity conferred by chimera δ56γ-171δ225γ indicates that a major determinant of conotoxin M1 selectivity lies between residues 56 and 171; this region, labeled region II in Figure 1, contains the disulfide loop found in all AChR subunits. When δ sequence is extended from position 56 to 118, as in δ118γ171δ225γ, conotoxin M1 affinity increases to approach that of the native δ subunit (Figure 4), narrowing the region containing selectivity determinants to between residues 56 and 118. Several additional chimeras were constructed with junctions between positions 56 and 118, but none formed surface receptors when cotransfected with α and β subunits, so it was not possible to further localize determinants using chimeras. Therefore, a portion of this segment, 104–118, was examined by introducing point mutations into the γ subunit at positions where the γ and δ subunits differ (Figure 4). Segment 104–118 is also significant because it contains a major determinant of DMT selectivity, the Y to T difference at position 117 (Sine, 1993). γY117 produces high affinity at the α–γ site by associating with one of two quaternary ammonium groups in DMT (Fu and Sine, 1994). The point mutation γS111Y markedly increases conotoxin M1 affinity, whereas mutations at three other candidate positions have little effect. The increase in affinity produced by γS111Y is nearly 2 orders of magnitude, similar to that caused by the entire segment 56–118. Thus, the S to Y difference at position 111, which precedes the conserved disulfide loop, is likely to be the major selectivity determinant in region II.

Region III begins approximately 30 residues carboxyl-terminal to the conserved disulfide loop. We examined region III by changing the position of the amino terminal γ–δ junction of chimera γ171δ225γ. In this base chimera, the presence of δ sequence between positions 171 and 225 increases conotoxin M1 affinity 16-fold relative to that

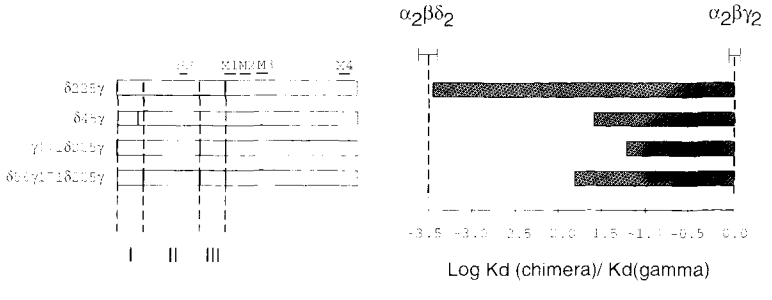

Figure 2. Effect of γ–δ Subunit Chimeras on Conotoxin M1 Binding Affinity

At left are shown schematic drawings of the chimeras. Shaded segments represent δ sequences, and unshaded segments represent γ sequences. Chimeras are designated as follows. The first symbol is the subunit from which amino-terminal sequence is taken, the following number is the position of the chimera junction, and the following symbol is the subunit from which carboxyl-terminal sequence is taken. Marked above the chimeras are positions of the conserved disulfide loop (SS) and transmembrane domains M1–M4.

For data shown at right, the dissociation constant for conotoxin M1 binding was determined for each chimera as in Figure 1 and divided by the dissociation constant determined for the $\alpha_2\beta\gamma_2$ AChR in each experiment. The dashed vertical lines mark the mean ratios of dissociation constants for $\alpha_2\beta\gamma_2$ and $\alpha_2\beta\delta_2$ AChRs, and the error bars indicate ± SD (13 determinations for $\alpha_2\beta\gamma_2$ and 7 determinations for $\alpha_2\beta\delta_2$). The results from these chimeras reveal three distinct regions, labeled I, II, and III, likely to harbor determinants of conotoxin M1 selectivity.

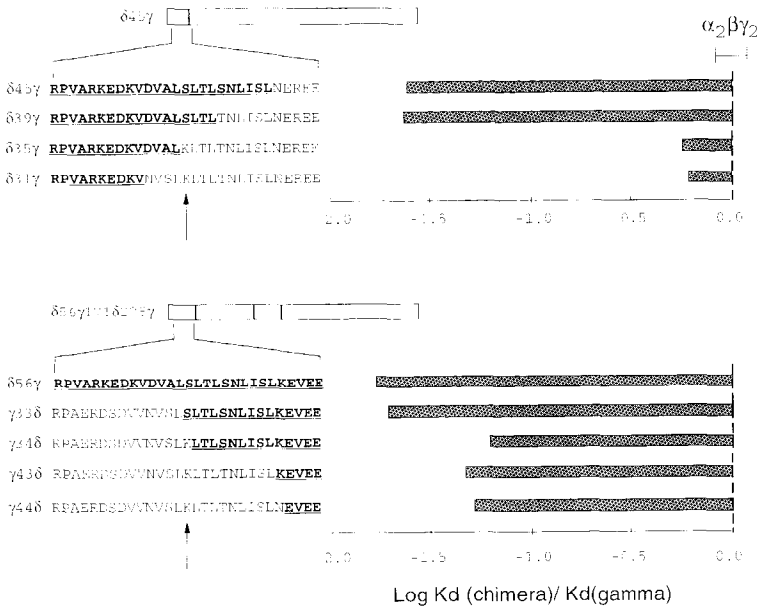

Figure 3. Molecular Dissection of Amino-Terminal Region I

At left are schematic drawings of base chimeras, with sequences preceding and following the chimera junction given below; δ subunit residues are bold and underlined, and γ subunit residues are in plain text. The column to the left gives the position of the amino-terminal chimera junction.

For data shown at right, conotoxin M1 binding for each chimera was determined and plotted as in Figure 2. The upper set of data shows dissection of region I with δ–γ chimeras, whereas the lower set shows dissection with γ–δ chimeras. In both sets of chimeras, conotoxin M1 affinity changes where lysine and serine are exchanged at position 34 of the γ subunit and position 36 of the δ subunit (arrows).

of the γ subunit (see Figure 2). When the subunit junction is stepped past position 172, conotoxin M1 affinity decreases sharply to a value close to that of the native γ subunit (Figure 5). Stepping the junction further toward the carboxyl terminus produces no further change in conotoxin M1 affinity. Thus, position 172, which harbors F in γ and I in the equivalent position of δ, is the major selectivity determinant in postdisulfide region III.

The preceding results identify three residues at equivalent positions of the γ and δ subunits likely to determine conotoxin M1 selectivity. To confirm these assignments, point mutations were introduced into the three key determinant positions of both subunits (Figure 6). For each point mutation, residues at the three positions are shown, with mutant residues bold and underlined. In the γ subunit, simultaneous mutation of all three residues to their δ counterparts, as in γ(SYI), increases conotoxin M1 affinity by 3 orders of magnitude, to a value close to that of the native δ subunit. The converse triple mutant, δ(KSF), decreases conotoxin M1 affinity to that of the native γ subunit. These results confirm that the three residues identified here are the major determinants of conotoxin M1 selectivity for the two AChR binding sites.

The observation that the triple point mutations transfer conotoxin M1 selectivity from one subunit to the other suggests that the α-carbon backbones of the γ and δ subunits each contribute similar scaffolds to the binding site, and that the side chains of the three conotoxin M1 determinants arise from equivalent positions in each scaffold. However, point mutations at single determinant positions produce quantitatively different changes in affinity in the two subunits (Figure 6). In the γ subunit, S111Y increases conotoxin affinity by 60-fold, whereas the homologous mutation in the δ subunit, Y113S, decreases affinity by only

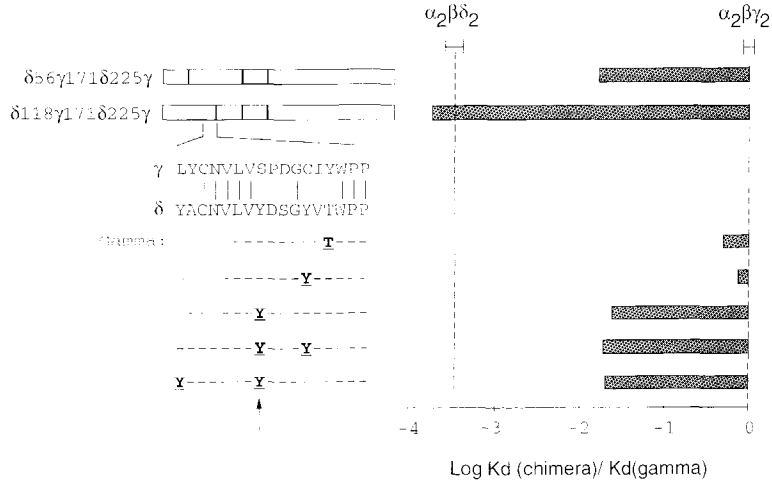

Figure 4. Molecular Dissection of Predisulfide Region II

Upper portion shows two chimeras and their effect on conotoxin M1 binding affinity.

Middle portion is a comparison of γ and δ sequences within part of the region that differs in the above two chimeras.

Lower portion shows point mutations at candidate positions in the γ subunit and their effect on conotoxin M1 affinity. Nonmutant residues are indicated by dashes, and mutant residues are bold and underlined. Note the marked increase in affinity caused by the presence of tyrosine at position 111 (arrow).

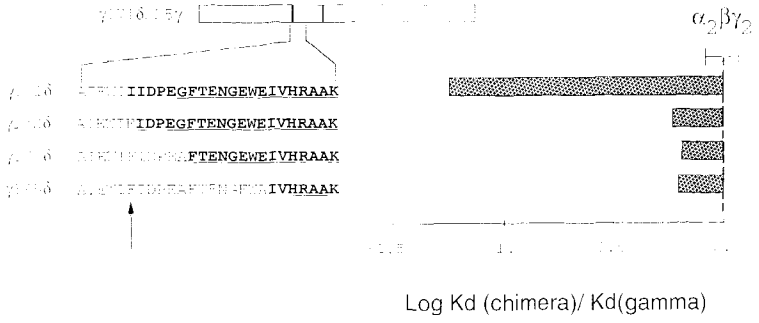

Figure 5. Molecular Dissection of Postdisulfide Region III

At left, the base chimera is shown above (γ171δ225γ), with sequences preceding and following the chimera junction shown below. The column to the left gives the position of the amino-terminal chimera junction.

At right is conotoxin M1 binding affinity for each chimera, as presented in Figure 2. Note the increase in conotoxin M1 affinity where isoleucine and phenylalanine are exchanged at position 172 of the γ subunit (arrow).

10-fold. Similarly, γF172I increases affinity by 16-fold, but the converse mutation, δI178F, has little effect in decreasing affinity.

In addition to subunit-dependent contributions of single determinants, interactions between determinants I and III are observed that again depend on the subunit. Whereas γK34S does not lead to expression of surface AChR, the double mutation γ(K34S + F172I) produces amounts of surface AChR exceeding those of the native γ subunit (186% ± 6% of $\alpha_2\beta\gamma_2$). The converse point mutation, δS36K, produces amounts of surface AChR similar to those of the native δ subunit (92% ± 18% of $\alpha_2\beta\delta_2$), but the double mutation δ(S36K + I178F) shows no further increase in expression (76% ± 22% of $\alpha_2\beta\delta_2$), contrary to the results with the γ subunit. The effect of γK34S on conotoxin M1 affinity could not be determined because of lack of expression, but the double mutation, γ(K34S + F172I), increases conotoxin M1 affinity 3-fold relative to γF172I. The converse point mutation, δS36K, produces a similar change in affinity, a decrease of about 5-fold, but when δS36K is combined with the apparently silent δI178F, conotoxin M1 affinity decreases by nearly 1000-fold. These observations suggest that the three determinants of conotoxin M1 selectivity are not independent, but are in close proximity and interact to confer the affinity of each binding site. Further, a particular matching of residues distant in the linear sequence appears to be required to achieve fidelity of assembly sufficient for cell surface expression.

Discussion

The experiments described here use γ/δ subunit chimeras to identify residues that confer the remarkably large 10,000-fold selectivity of conotoxin M1 for the two binding sites in the AChR. The three selectivity determinants identified lie in the major extracellular domain of the subunit, each separated by about 60 residues. Although our experiments cannot distinguish direct from indirect interactions with conotoxin M1, the proximity of determinants II and III to previously identified points of ligand interaction suggests direct interactions with these residues. The presence at the binding site of three residues distant in the linear sequence would mean at least three distinct regions of the γ and δ subunits converge to the subunit interface. Because exchange of these three residues transfers selectivity from one subunit to the other, the α-carbon backbones of the γ and δ subunits appear to contribute similar scaffolds to the binding sites. Further, because their individual contributions depend on each other and on the subunit in which they reside, the side chains of the determinants appear to interact to form the surface of the non-α subunit interface.

Determinant I, located at position 34 of the γ subunit and position 36 of the δ subunit, is the most amino-terminal residue so far identified to contribute to either ligand selectivity or expression efficiency. It is conserved as lysine in all species of γ subunits, but it is either serine or alanine among δ subunits. In both the α and β subunits, the corresponding residue is glycine, which is conserved across all species of these subunits. Considered together with the overall findings of this study, conservation at position 34 suggests a role for these residues in formation of subunit interfaces. The low affinity of the γ subunit, which

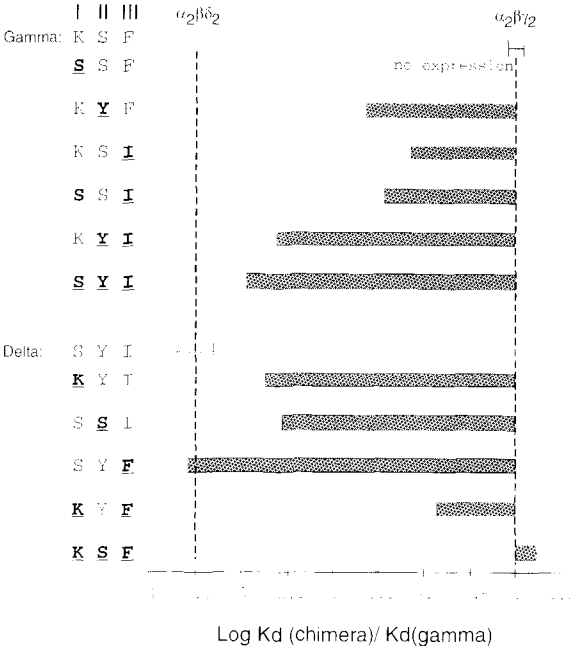

Figure 6. Point Mutations at Positions of the γ and δ Subunits That Determine Conotoxin M1 Selectivity

Mutant subunits are designated according to residues present at the three key conotoxin M1 selectivity positions, I, II, and III, with mutant residues bold and underlined. For each point mutation, conotoxin M1 affinity is presented as in Figure 2.

harbors lysine at position 34, is consistent with repulsion of one or more of three positive charges in conotoxin M1 and thus suggests a direct interaction.

Our results also show that the contribution of determinant I depends on determinant III, which is F172 in γ and I178 in δ. This interdependence is important for expression efficiency where γK34S does not form surface receptors when coexpressed with α and β subunits, but γ(K34S + F172I) produces amounts of surface receptor similar to those of the native γ subunit. Interdependence is also seen in conotoxin M1 selectivity, in which δS36K decreases conotoxin affinity 5-fold and δI178F has no effect, but combining both mutations decreases conotoxin M1 affinity by 1000-fold. This 1000-fold decrease imparted by the δ subunit contrasts with only a 45-fold increase caused by the converse double mutation in the γ subunit. Interdependence is also subunit specific, in that it is most important for expression efficiency in the γ subunit, but it is more important for conotoxin M1 affinity in the δ subunit. Complementarity between determinants I and III suggests that these two residues come into close apposition at the non-α surface of the binding site.

Determinant II, located at position 111 of the γ subunit and position 113 of the δ subunit, lies among a cluster of residues that have been shown to contribute to either ligand selectivity or expression efficiency. Determinant II is not a conserved residue in either γ or δ subunits, being either serine, tyrosine, or arginine in different species, so it is not surprising that it affects ligand selectivity rather than expression efficiency. The high affinity produced by tyrosine suggests stabilization of a positively charged amino group in conotoxin M1. In studies of selectivity of conotoxin M1 and G1 for the Torpedo AChR, Hann et al. (1994) and Utkin et al. (1994) demonstrated a 150- to 200-fold preference of the α–γ site over the α–δ site, a selectivity opposite to that seen in the mouse AChR. Together with our identification of determinant II, this opposite selectivity suggests a tyrosine–cation interaction, because in Torpedo, determinant II is tyrosine in the high affinity γ subunit, whereas it is arginine in the low affinity δ subunit.

Determinant II is near the pair of residues that contribute to selectivity of DMT, I116, and Y117 in the γ subunit and V118 and T119 in equivalent positions of the δ subunit (Sine, 1993). Studies of side chain specificity showed that γY117 stabilizes one of two quaternary ammonium groups in DMT, and that the other quaternary group is stabilized by Y198 of the α subunit (Fu and Sine, 1994). Also, we recently showed that R117 of the β subunit is essential for expression of cell surface pentamers, suggesting a role for this residue in formation of subunit interfaces (Kreienkamp et al., 1995). Similarly, residues 106 and 115 of the ε subunit promote its association with the α subunit, thus affecting efficiency of assembly (Gu et al., 1991), and two groups of residues in this region of the glycine receptor affect subunit stoichiometry (Kuhse et al., 1993). Thus, determinant II is near residues that affect DMT selectivity and subunit assembly, suggesting that it too is at the subunit interface, where it can stabilize positively charged groups in conotoxin M1.

Determinant III is located at position 172 of the γ subunit and position 178 of the δ subunit, again near residues that contribute to ligand binding. Determinant III is not conserved among γ subunits, being either phenylalanine, glutamate, or histidine. The electron-rich character of these residues could allow stabilizing interactions with K34, which might underlie the synergy between these two residues in affecting conotoxin M1 selectivity and expression efficiency. Determinant III is isoleucine in δ subunits from all species, suggesting the need for hydrophobicity at this position. Just amino-terminal to determinant III are residues in corresponding positions of the subunits, γS161 and δK163, which also contribute to DMT selectivity (Sine, 1993), though interaction of these residues with DMT appears to be allosteric (Fu and Sine, 1994). Also nearby is δD180, which reacts with a cross-linking reagent tethered to one of the vicinal cysteines at positions 192 and 193 of the α subunit (Czajkowski and Karlin, 1995) and contributes to agonist affinity (Czajkowski et al., 1993). The weight of the evidence thus suggests that determinant III is also at the subunit interface, where it can contribute to conotoxin M1 selectivity. A novel feature of determinant III is its interaction with determinant I, some 140 residues away in the linear sequence. Matching of these two residues determines their combined contribution to conotoxin M1 selectivity, as well as their effect on expression efficiency, suggesting a role for these residues in determining specificity of subunit association.

The determinants of conotoxin M1 selectivity identified here can be combined with affinity labeling and site-directed mutagenesis studies to map residues to the two faces of the homologous subunits, which we term counterclockwise (+) and clockwise (−) (Figure 7; Table 1; see Czajkowski et al., 1993). On the presumed (+) face of the α subunit, reduction of the bridged cysteines 192 and 193 allows labeling by the site-specific reagent MBTA (N-[4-maleimido]benzyltrimethylammonium iodide; Kao, et al., 1984). Also on the (+) face, tyrosine 190 covalently reacts with the coral toxin lophotoxin (Abramson et al., 1989); tyrosine 93, tryptophan 149, tyrosine 151, tyrosine 190, and cysteines 192–193 react with the photolytic labeling agent DDF (p-N,N-dimethylaminobenzenediazonium; Dennis et al., 1988; Galzi et al., 1990); and tyrosine 190, cysteine 192, and tyrosine 198 react with nicotine following

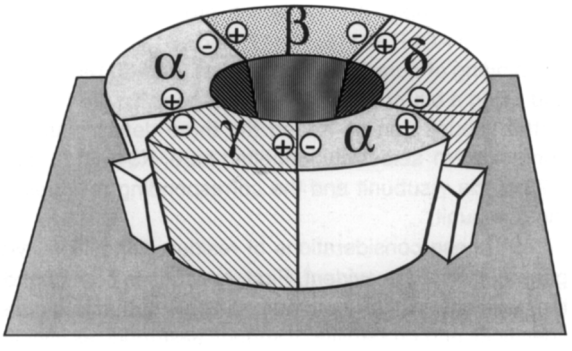

Figure 7. Schematic Diagram of the AChR Depicting the Circular Arrangement of the Subunits and the (+) and (-) Surfaces of the Subunit Interfaces

Table 1. Disposition of Residues in the Major Extracellular Domain on the Two Faces of the Subunit Interface

Subunit	Residues	Basis
(+) Counterclockwise face		
α	Tyr 93	Affinity labeling, agonist affinity
α	Trp 149	Affinity labeling, agonist affinity
α	Tyr 190	Affinity labeling, ligand affinity
α	Cys 192	Affinity labeling
α	Cys 193	Affinity labeling
α	Tyr 198	Affinity labeling, ligand affinity
γ, ε, δ	Ile, Lys 145	Subunit assembly
γ, ε, δ	Thr, Lys 150	Subunit assembly
(−) Clockwise face		
γ, δ	Lys, Ser 34	Ligand selectivity
γ, δ	Trp 55, 57	Affinity labeling
γ, δ	Ser, Tyr 111	Ligand selectivity
γ, δ	Tyr, Thr 117	Ligand selectivity
γ, δ	Ser, Lys 161	Ligand selectivity
γ, δ	Phe, Ile 172	Ligand selectivity
δ	Asp 180	Subunit cross-linking
β	Arg 117	Surface expression
ε	Ser 106, Tyr 115	Subunit assembly

photodecomposition (Middleton and Cohen, 1991). Several of these α subunit residues have been shown to contribute to ligand binding where mutation at these sites produced marked increases in dissociation constants for agonist binding or receptor activation (Galzi, et al., 1991; Tomaselli et al., 1991; O'Leary and White, 1992; Sine et al., 1994). Also, asparagines 187 and 189 of the α subunit can be surmised to be near the (+) face of the α subunit, because glycosylation of these residues prevents binding of peptide α-toxins but not the smaller α-conotoxins (Kreienkamp et al., 1994; Keller et al., 1995). Studies of assembly of the (+) face of the γ subunit with the (−) face of the α subunit show that γ145 and γ150 promote association of these two subunits, whereas the presence of lysines in the corresponding positions of the δ subunit inhibit this association (Kreienkamp et al., 1995).

On the opposing clockwise (−) face of the subunit interface, tryptophans 55 and 57 of the γ and δ subunits, respectively, are labeled by d-tubocurarine following photodecomposition (Chiara and Cohen, 1992). Also on the presumed (−) face are residues that confer ligand selectivity and subunit assembly referred to in context with the three conotoxin selectivity determinants identified here. They include εS106 and εY115, which affect efficiency of subunit assembly, βR117, which promotes surface expression of AChR, γI116, γY117, and γS161, which stabilize binding of curariform antagonists, and δD180, identified using a bifunctional labeling reagent. To the (−) face of the subunit can be added the three determinants of conotoxin M1 selectivity identified here: K34, S111, and I172 of the γ subunit and the corresponding residues of the δ subunit.

From these considerations of residue disposition, two general trends are evident. First, residues in corresponding positions in the homologous AChR subunits dictate specificity and probability of subunit assembly, as well as govern ligand selectivity. Second, in spite of caveats and biases inherent to residue labeling and possible long-range conformational influences of mutagenesis, this analysis shows no glaring inconsistencies where neighboring residues in the linear sequence are found to be projected on opposite faces of a given subunit. These observations shed a ray of optimism on the prospect of developing a limited model for the peptide folding pattern in the extracellular domain of the nicotinic receptor.

Experimental Procedures

Materials

Conotoxin M1 was purchased from the American Peptide Company, Sunnyvale, CA. ^{125}I-labeled α-bungarotoxin was purchased from Dupont NEN. The 293 HEK fibroblast cell line was obtained from the American Type Culture Collection. Mouse AChR subunit cDNAs were generously provided by Drs. John Merlie and Norman Davidson (see Sine, 1993 for references to cDNA sequences).

Construction and Expression of Wild-Type and Mutant AChR

Mouse AChR subunit cDNAs were subcloned into the CMV-based expression vector pRBG4 as described (Sine, 1993). Subunit chimeras and point mutations were constructed by bridging naturally occurring or mutagenically installed restriction sites with synthetic double-stranded oligonucleotides. All constructs were confirmed by restriction mapping and dideoxy sequencing. HEK cells were transfected with mutant or wild-type AChR subunit cDNAs using calcium phosphate precipitation as described (Sine, 1993).

Conotoxin M1 Binding Measurements

At 3 days after transfection, intact HEK cells were harvested by gentle agitation in PBS plus 5 mM EDTA (Sine, 1993). Cells were briefly centrifuged, resuspended in high potassium Ringer's solution, and divided into aliquots for conotoxin M1 binding measurements (Sine, 1993). Specified concentrations of conotoxin M1 were added 30 min prior to addition of ^{125}I-labeled α-bungarotoxin, which was allowed to bind for 30 min to occupy at most half of the surface receptors. Unbound toxin was removed by washing twice with potassium Ringer's solution containing 300 μM d-tubocurarine followed by centrifugation, and radioactivity bound to the cells was measured with a gamma counter. Nonspecific binding was determined in the presence of 300 μM d-tubocurarine. The initial rate of toxin binding was determined to yield fractional occupancy of sites by conotoxin M1 (Sine and Taylor, 1979).

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References

Abramson, S. N., Li, Y., Culver, P., and Taylor, P. (1989). An analog of lophotoxin reacts covalently with tyrosine 190 in the α-subunit of the nicotinic acetylcholine receptor. J. Biol. Chem. *264*, 12666–12672.

Blount, P., and Merlie, J. (1989). Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. Neuron *3*, 349–357.

Chiara, D. C., and Cohen, J. B. (1992). Identification of amino acids contributing to high and low affinity d-tubocurarine sites on the Torpedo nicotinic acetylcholine receptor subunits. Biophys. J. *61*, A106.

Czajkowski, C., and Karlin, A. (1995). Structure of the nicotinic receptor acetylcholine-binding site: identification of acidic residues in the delta subunit within 0.9 nm of the α subunit binding site disulfide. J. Biol. Chem. *270*, 3160–3164.

Czajkowski, C., Kaufmann, G., and Karlin, A. (1993). Negatively charged amino acid residues in the nicotinic receptor δ subunit that contribute to the binding of acetycholine. Proc. Natl. Acad. Sci. USA *90*, 6285–6289.

Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J. Y., Lazure, C., Chretien, M., and Changeux, J. P. (1988). Amino acids of the Torpedo marmorata acetylcholine receptor alpha subunit labeled by a photoaffinity ligand for the acetylcholine binding site. Biochemistry *27*, 2346–2357.

Fu, D.-X., and Sine, S. M. (1994). Competitive antagonists bridge the α–γ subunit interface of the acetylcholine receptor through quaternary ammonium-aromatic interactions. J. Biol. Chem. *269*, 26152–26157.

Galzi, J. L., Revah, F., Black, D., Goeldner, M., Hirth, C., and Changeux, J. P. (1990). Identification of a novel amino acid alpha-tyrosine 93 within the cholinergic ligand-binding sites of the acetylcholine receptor by photoaffinity labeling. J. Biol. Chem. *265*, 10430–10437.

Galzi, J. L., Bertrand, D., Devillers-Theiry, A., Revah, F., Bertrand, S., and Changeux, J. P. (1991). Functional significance of aromatic amino acids from three peptide loops of the alpha 7 neuronal nicotinic receptor site investigated by site-directed mutagenesis. FEBS Lett. *294*, 198–202.

Gu, Y., Forsayeth, J., Verrall, S., Yu, X., and Hall, Z. (1991). Assembly of the mammalian muscle acetylcholine receptor in transfected COS cells. J. Biol. Chem. *114*, 799–807.

Hann, R. M., Pagan, O. R., and Eterovic, V. A. (1994). The α-conotoxins GI and MI distinguish between the nicotinic acetylcholine receptor agonist sites while SI does not. Biochemistry *33*, 14058–14063.

Kao, P. N., Dwork, A. J., Kaldany, R. R., Silver, M. L., Wideman, J., Stein, S., and Karlin, A. (1984). Identification of the alpha subunit half-cystine specifically labeled by an affinity reagent for the acetylcholine receptor binding site. J. Biol. Chem. *259*, 11662–11665.

Keller, S. H., Kreienkamp, H.-J., Kawanishi, C., and Taylor, P. (1995). Molecular determinants conferring α-toxin resistance in recombinant DNA-derived acetylcholine receptors. J. Biol. Chem. *270*, 4165–4171.

Kreienkamp, H.-J., Sine, S. M., Maeda, R. K., and Taylor, P. (1994). Glycosylation sites selectively interfere with α-toxin binding to the nicotinic acetylcholine receptor. J. Biol. Chem. *269*, 8108–8114.

Kreienkamp, H.-J., Meada, R., Sine, S. M., and Taylor, P. (1995). Intersubunit contacts governing assembly of the mammalian nicotinic acetylcholine receptor. Neuron *14*, 635–644.

Kuhse, J., Laube, B., Magalei, D., and Betz, H. (1993). Assembly of the inhibitory glycine receptor: identification of amino acid sequence motifs governing subunit stoichiometry. Neuron *11*, 1049–1056.

Middleton, R. E., and Cohen, J. B. (1991). Mapping of the acetylcholine binding site of the nicotinic acetylcholine receptor: [^{3}H]nicotine as an agonist photoaffinity label. Biochemistry *30*, 6987–6997.

Myers, R. A., Cruz, L. J., Rivier, J. E., and Olivera, B. M. (1993). Conus peptides as chemical probes for receptors and ion channels. Chem. Rev. *93*, 1923–1936.

Neubig, R. R., and Cohen, J. B. (1979). Equilibrium binding of [^{3}H] tubocurarine and [^{3}H] acetylcholine by Torpedo postsynaptic membranes: stoichiometry and ligand interactions. Biochemistry *18*, 5464–5475.

O'Leary, M. E., and White, M. M. (1992). Mutational analysis of ligand-induced activation of the Torpedo acetylcholine receptor. J. Biol. Chem. *267*, 8360–8365.

Pedersen, S. E., and Cohen, J. B. (1990). *d*-tubocurarine binding sites are located at α-γ and α-δ subunit interfaces of the nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA *87*, 2785–2789.

Sine, S. M. (1993). Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of residues that determine curare selectivity. Proc. Natl. Acad. Sci. USA *90*, 9436–9440.

Sine, S. M., and Taylor, P. (1979). Functional consequences of agonist-mediated state transitions in the cholinergic receptor. J. Biol. Chem. *254*, 3315–3325.

Sine, S. M., and Taylor, P. (1981). Relationships between reversible antagonist occupancy and the functional capacity of the acetylcholine receptor. J. Biol. Chem. *256*, 6692–6699.

Sine, S. M., Claudio, T., and Sigworth, F. J. (1990). Activation of Torpedo acetylcholine receptor expressed in mouse fibroblasts: single channel current kinetics reveal distinct agonist binding affinities. J. Gen. Physiol. *96*, 395–437.

Sine, S. M., and Claudio, T. (1991). γ- and δ-subunits regulate the affinity and cooperativity of ligand binding to the acetylcholine receptor. J. Biol. Chem. *266*, 19369–19377.

Sine, S. M., Quiram, P., Papanikolaou, F., Kreienkamp, H.-J., and Taylor, P. (1994). Conserved tyrosines in the alpha subunit of the nicotinic acetylcholine receptor stabilize quaternary ammonium groups of agonists and antagonists. J. Biol. Chem. *269*, 8808–8816.

Tomaselli, G. F., McLaughlin, J. T., Jurman, M. E., Hawrot, E., and Yellen, G. (1991). Mutations affecting agonist sensitivity of the nicotinic acetylcholine receptor. Biophys. J. *60*, 721–727.

Utkin, Y. N., Kobayashi, Y., Hucho, F., and Tsatlin, V. I. (1994). Relationships between the binding sites for an α-conotoxin and snake venom neurotoxins in the nicotinic acetylcholine receptor from Torpedo californica. Toxicon *32*, 1153–1157.