Identification of Pairwise Interactions in the α-Neurotoxin-Nicotinic Acetylcholine Receptor Complex through Double Mutant Cycles*

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α-Neurotoxins are potent inhibitors of the nicotinic acetylcholine receptor (nAChR), binding with high affinity to the two agonist sites located on the extracellular domain. Previous site-directed mutagenesis had identified three residues on the α-neurotoxin from Naja mossambica mossambica (Lys27, Arg33, and Lys47) and four residues on the mouse muscle nAChR α-subunit (Val188, Tyr190, Pro197, and Asp200) as contributing to binding. In this study, thermodynamic mutant cycle analysis was applied to these sets of residues to identify specific pairwise interactions. Amino acid variants of α-neurotoxin were expressed as fusion proteins in Escherichia coli, and the free toxins were purified as described (36). Because several of the mutant toxins produced rather low affinity complexes with mutant receptors, relatively large amounts of Nmml α-neurotoxins were required for this study. Typically, 0.5–1.5 mg of toxin could be purified to homogeneity from 1 liter of cells.

The critical tool utilized in the initial identification of the receptor and in subsequent structural analyses is the family of three-fingered snake α-neurotoxins (for review, see Refs. 28 and 29), which form high affinity complexes with the receptor; for example, the α-bungarotoxin-receptor complex has a KD of $10^{-12}$. The structure of the α-neurotoxins has been solved through nuclear magnetic resonance (30–32) and x-ray crystallographic studies (33–35). These polypeptides (~7 kDa) are characterized by three large loops which extend from a rigid globular domain held together by 4 or 5 conserved disulfide bonds. Even though α-neurotoxin structures have been solved, little is known about the structure of the toxin-receptor complex, and interacting residues have not been identified.

The structure of the α-neurotoxin receptor (nAChR) is a member of the large family of neurotransmitter-gated ion channels (for review, see Refs. 1–4). It is composed of five subunits that are arranged in the circular order of αβγδβ'. The two ligand binding sites reside in the extracellular domain at the αγ and αδ subunit interfaces (5, 6). A high resolution structure is not available for any member of the family of neurotransmitter-gated ion channels. However, within this family the structure of the nAChR has been most extensively characterized through site-directed labeling (7–12), site-specific mutagenesis (13–23), electron microscopy reconstruction analysis (24, 25), and homology modeling (26). In current models, three discontinuous regions or domains on the α-subunit (encompassing residues around 93, 149–154, and 180–200) and four regions on the γδ subunits are thought to participate in the formation of the binding sites (2, 26). Because of sequence differences in the δ and γ subunits, the binding sites on the receptor are not identical; consequently, many ligands bind to each site with different affinities (15, 22, 27).

The experimentally determined binding affinities (15, 22, 27).

EXPERIMENTAL PROCEDURES

Materials—[125I]-α-Bungarotoxin (specific activity ~16 μCi/μg) was obtained from NEN Life Science Products. α-Conotoxin M1 was purchased from American Peptide Company.

Nmml Expression and Purification—Recombinant wild-type and mutant Nmml were expressed as fusion proteins in Escherichia coli, and the free toxins were purified as described (36). Because several of the mutant toxins produced rather low affinity complexes with mutant receptors, relatively large amounts of Nmml α-neurotoxins were required for this study. Typically, 0.5–1.5 mg of toxin could be purified to homogeneity from 1 liter of cells.

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The abbreviations used are: nAChR, nicotinic acetylcholine receptor; Nmml, α-neurotoxin I from Naja mossambica mossambica.

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Receptor Mutagenesis—All receptor mutants were made as described previously (20, 23, 27, 36, 40).

Expression of Wild-type and Mutant nAChR—CDNAs encoding the mouse muscle nAChR subunits (α, β, γ, and δ) in a cytomegalovirus-based expression vector pRBG4 were co-transfected in a ratio of 2:1:1:1 into HEK 293 cells (at densities of 300–500,000 cells/ml) and expression was measured 3–4 days after transfection (20).

Nmr Binding Measurements—Binding assays were carried out on assembled pentameric nAChRs expressed on the surface of intact cells. The cells were harvested by gentle agitation in phosphate-buffered saline plus 5 mM EDTA, centrifuged briefly, and resuspended in high potassium Ringer’s solution. The cells were divided into aliquots for binding measurements (assay volume 200 μl). Specified concentrations of NnmI were added to each tube containing receptor and allowed to bind for 5 h. NnmI dissociation constants were measured by competition against initial rates of [125I]bungarotoxin binding in the presence and absence of NmmI.

Homology Modeling—Using erabutoxin b as a template (an α-neurotoxin of known crystal structure (33) and possessing 60% residue identity with NnmI), segments of NnmI were modeled on the basis of conserved regions and the common disulfide linkages. The final conformation of the α-carbon backbone was adjusted to account for the two unique prolines in erabutoxin b and the single proline in NnmI. The modeled structure was then relaxed by unrestrained steepest descent minimization of 5000 iterations with the program Discover (MSI, 1997).

RESULTS

nAChR Binding Site—Of the three segments of linear sequence in the α-subunit, the region encompassing residues 180–200 appears to be the most critical for NnmI recognition. Substitutions at residues 188, 190, 197, and 200 resulted in substantial decreases in toxin binding affinity (36), indicating major roles of these residues in α-neurotoxin recognition. Determinants in this region include a conserved aromatic residue (Tyr190), two positions where neuronal and muscle receptors differ (Val188 and Pro197), and a negatively charged residue (Asp200). The mutations Y190F and Y190T both resulted in a loss of affinity of ~2 kcal/mol at the αδ site and almost 4 kcal/mol at the αγ site (Table I), suggesting the importance of the aromatic hydroxy group. Introducing a positive charge at position 188 (V188K) was also unfavorable and destabilized the toxin-receptor complex by 1.8 kcal/mol (at the αδ site) and 3.5 kcal/mol (at the αγ site), whereas introduction of a negative charge (V188D) at this position had either no effect or far less of an influence (~0.09 kcal/mol at the αδ site and 1.8 kcal/mol at the αγ site). The selective loss in binding affinity observed

### Table I

| Mutations | Binding energy | Coupling energy |
|-----------|----------------|-----------------|
|            | $K_d^{\alpha\delta}$ | $\Delta G_0^{\alpha\delta}$ | $\alpha\gamma$ | $\Delta G_{INT}^{\alpha\gamma}$ |
| WT        | 0.14 ± 0.04     | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 1.8 ± 0.1       | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 95 ± 1          | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 1.5 ± 0.1       | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 0.12 ± 0.01     | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 2.8 ± 1.0       | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 3.5 ± 1         | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 6.8 ± 2.5       | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 0.47 ± 0.05     | 35 ± 1.5        | 2.1 ± 0.2      |
| WT        | 0.33 ± 0.02     | 35 ± 1.5        | 2.1 ± 0.2      |
| V188D     | 54 ± 3          | 35 ± 1.5        | 2.1 ± 0.2      |
| V188D     | 6,200 ± 700     | 35 ± 1.5        | 2.1 ± 0.2      |
| V188D     | 2.1 ± 1.1       | 35 ± 1.5        | 2.1 ± 0.2      |
| V188K     | 110 ± 20        | 35 ± 1.5        | 2.1 ± 0.2      |
| V188K     | 1,300 ± 100     | 35 ± 1.5        | 2.1 ± 0.2      |
| V188K     | 27 ± 4          | 35 ± 1.5        | 2.1 ± 0.2      |
| V188F     | 520 ± 70        | 35 ± 1.5        | 2.1 ± 0.2      |
| V188F     | 4,500 ± 1,000   | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 71 ± 20         | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 1,100 ± 200     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 9,800 ± 2,600   | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 120 ± 40        | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 150 ± 300       | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 9,000 ± 2,000   | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 1,000 ± 400     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 2,000 ± 200     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 9,000 ± 2,000   | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 1,000 ± 400     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 2,000 ± 200     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 9,000 ± 2,000   | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 1,000 ± 400     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 2,000 ± 200     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 9,000 ± 2,000   | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 1,000 ± 400     | 35 ± 1.5        | 2.1 ± 0.2      |
| V190F     | 2,000 ± 200     | 35 ± 1.5        | 2.1 ± 0.2      |

* a Dissociation constant (μM) from two-site analysis of binding curves with equal population of sites. Mean ± S.E. or standard errors are shown from measurements made from cells in which two or more separate transfections were analyzed.
* b Difference in free energy of binding WT and mutant toxin-receptor pairs, $\Delta G_0 = \Delta G_{mut} - \Delta G_{INT}$ (units kcal/mol); $\Delta G = -RT\ln (K_d)$.
* c Coupling coefficient calculated as shown in Equation 2; values less than unity were inverted and indicated with a negative sign.
* d Coupling energy of each mutant toxin-mutant receptor pair, $\Delta G_{INT} = RT\ln (\Gamma)$ (units kcal/mol); $\Gamma = 1.987$ cal/mol-K, $T = 296$ K. Errors associated with $\Delta G_{INT}$ range from 30 to 50%.
* Values taken from Ref. 36. WT, wild type.
with the introduction of a positive charge but not a negative charge suggested destabilization resulted from coulombic repulsion of the highly cationic α-toxins. Elimination of the negative charge at position 200 (D200Q) decreased binding by 66-fold (2.5 kcal/mol) selectively at the ad site.

**α-Toxin Structure and Binding Site—**Sequence comparisons between members of the α-toxin family have identified ~12 residues that are highly conserved, three of which are positively charged (Lys27, Arg33, Lys47; NmmI numbering). Extensive mutagenesis studies carried out by Menez and co-workers (42–44) using a homologous α-neurotoxin with 60% residue identity to NmmI (erabutoxin a) defined the toxin structure obtained through an energy minimization with the solved structure of erabutoxin Loops I, II, and III of the toxin are labeled. This structure was obtained with side chains of mutated residues darkened. From pairwise interactions. This method is based on simple additivity or non-additivity of mutations. If two residues are interacting, then the sum of the free energy change of the single mutations will usually not equal the free energy change measured with both mutations (45–47). This is shown by Equation 1:

\[
\Delta G_{XY} = \Delta G_X + \Delta G_Y + \Delta G_{INT} \tag{Eq. 1}
\]

where \(\Delta G_X\) represents the change in free energy caused by a mutation at site \(X\) on one interacting species relative to its wild type, \(\Delta G_Y\) represents the change in free energy caused by a mutation at site \(Y\) on the other species relative to its wild type, \(\Delta G_{INT}\) represents the change in free energy caused by both mutations when present together, and \(\Delta G_{INT}\) (coupling energy) is the measure of the interaction of the two components that are mutated. If the two residues are not linked or interacting, \(\Delta G_{INT}\) will equal 0, and if the two residues are interacting then the value of \(\Delta G_{INT}\) may be either positive or negative depending on whether the interaction between the mutated residues reduces or enhances affinity (47). \(\Delta G_{INT}\) can also be described in terms of the equilibrium constants (39):

\[
\Delta G_{INT} = RT \ln (\Omega) \tag{Eq. 2}
\]

where

\[
\Omega = \frac{K_{X\text{WT}}K_{Y\text{WT}}}{K_{X\text{mut}}K_{Y\text{mut}}} \tag{Eq. 3}
\]

**Mutant Pairs and Site Selectivity—**Dissociation constants and changes in free energy for each of the mutant pairs analyzed are shown in Table I. Fig. 2 (A and B), shows typical binding curves obtained with the α-subunit mutations Y190T and P197I, respectively, when assayed with each of the toxin subunit interactions; the wild-type toxin/wild-type receptor curve is also shown for comparison. As seen in Table I and Fig. 2, many of the mutant pairs result in large reductions in the overall affinity of the toxin-receptor complex. The dissociation constant for the R33E/V188D pair at the αγ binding site was too large to measure precisely but corresponded to a \(K_d\) of more than 0.5 mM. The R33E/P197I mutant pair resulted in a loss of affinity of 8.0 kcal/mol (αγ site) or a loss in \(K_d\) of 6 orders of magnitude compared with the wild-type/wild-type interaction. Mutant pairs involving the toxin mutation K27E also resulted in large destabilizations ranging from 3.5 to 7.5 kcal/mol.

As expected by the demonstration of site selectivity conferred by mutations on the α-neurotoxin or receptor when analyzed separately, all of the mutant pairs showed two distinct binding affinities presumably arising from the αδ and αγ sites. Analysis of the binding curves yielded Hill coefficients ranging from 0.3 to 0.8, indicating the presence of two classes of binding sites. When these curves were fit to a two-site analysis of equal affinities presumably arising from the αδ and αγ sites. Analysis of the binding curves yielded Hill coefficients ranging from 0.3 to 0.8, indicating the presence of two classes of binding sites. When these curves were fit to a two-site analysis of equal population, differences in affinity between the two sites ranged from 9- to 85-fold.

To confirm that these binding curves reflected disparate affinities at the αδ and αγ subunit interfaces and to ascertain which site possessed the high affinity binding, assays were carried out in the presence of α-conotoxin M1. α-Conotoxin M1 has a more than 10,000-fold selectivity for the αδ binding site over the αγ site on the wild-type mouse receptor (\(K_{d,\alpha\gamma} = 0.45 \mu M\) (22, 27). α-Conotoxin M1 site selectivity is maintained with each of the receptor mutations studied here despite some changes in the absolute \(K_d\) values. Thus, when
a-conotoxin M1 is included in the α-neurotoxin-receptor binding assays, it protects the αδ site nearly completely, with the residual α-neurotoxin binding observed only at the αγ site. Fig. 2C shows binding curves of K27E/V188D assayed in the presence and absence of 300 nM a-conotoxin M1. In the absence of a-conotoxin M1 the curve gives a Hill coefficient of 0.6 with Kd values of 57 nM and 1.8 μM. In the presence of a-conotoxin M1, the number of sites decreased by ~50% and the resulting Hill coefficient increased to 0.9, consistent with residual α-neurotoxin binding to a single class of sites. Because a-conotoxin M1 will preferentially protect the αδ site, the observed Kd of 1.3 μM reflects lower affinity binding of K27E at the αγ site. Accordingly, identical experiments were carried out with each of the toxin-receptor pairs presented in Table I. In all cases, higher affinity α-neurotoxin binding was found to correspond to the αδ site and lower affinity to the αγ site, as indicated in Table I.

Coupling Analysis—The coupling coefficient ω and coupling energy ΔΔGINT for each toxin-receptor pair (at both the αδ and αγ binding sites) were determined using Equations 1 and 2 (Table I and Fig. 3). Two of the mutant pairs studied gave strong coupling energies above 2.0 kcal/mol and seven pairs gave coupling energies of 1.5 kcal/mol or above. Despite very large shifts in the overall affinities of the mutant pairs relative to that of the wild-type toxin-wild-type receptor complex, the majority of the pairs showed simple additivity of free energy within experimental error (coupling energies approaching 0, 0.1–0.5 kcal/mol). Most strikingly, all of the mutant pairs involving K47A gave values very close to ΔΔGINT = 0, indicating that the introduced receptor mutations and the toxin mutation K47A do not grossly alter the respective protein structures. A few mutant pairs gave values between 1.0 and 1.3 kcal/mol; intermediate energy values in this range are difficult to interpret because of cumulative errors of addition of free energies. As can be seen in Table I, the strengths of the observed linkages are not identical at the αδ and αγ binding sites (Fig. 3, A and B). Because the energy contributions of the individual residues alone were found to differ at the two sites, a difference in the coupling energy at the two sites might also be expected.
Interactions of Receptor Residue Val388 with Toxin Residues Arg33 and Lys27—The two strongest interactions were found between the mutant pairs R33E/V188D and K27E/V188D at the αβ interface (coupling energies of 2.6 and 2.1 kcal/mol, respectively). The R33E/V188D pair also appeared to have a strong coupling at the αγ interface (>1.5 kcal/mol), but a precise number could not be obtained. The very large overall loss in the binding affinity required an unachievable production level of mutant toxin. On the other hand, no significant coupling was observed at the αγ interface with the K27E/V188D mutant pair containing the other charge substitution studied in loop 2.

Analysis of the direction of the free energy changes observed in the double mutant cycles involving V188D or V188K and the toxin mutations R33E and K27E are consistent with the involvement of coulombic attractions and repulsions between the introduced charged mutations. For example, the single toxin mutation R33E and single receptor mutation V188D resulted in changes in free energy from the wild-type counterpart of 5.7 and 1.8 kcal/mol, respectively (at the αγ interface). The summations of the individual changes in free energy is then 7.5 kcal/mol, which corresponds to a 

The linkages between these mutant pairs were further explored by examining a network of mutant cycles proceeding among positive, neutral, and negative substitutions shown in Fig. 4. Here additional cycles involving R33E with K188D and K27E with K188D are also analyzed. The mutant cycle for R33E/K188D results in a very strong coupling coefficient of more than 100 at both the αβ and αγ binding interfaces (2.8 and >3.0 kcal/mol, respectively). This result reveals a previously unobserved strong linkage at the αγ interface. The same analysis carried out with the K27E/K188D mutant pairs also gives roughly equal linkages at the two sites but 10-fold smaller than those observed with the R33E with the same receptor mutations.

Other Interactions—The toxin-receptor mutant pairs K27E/Y190F, K27E/Y190T, K27E/P197I, and K27E/D200Q all showed coupling energies of 1.5 kcal/mol or higher at the αβ binding site with no significant couplings observed at the αγ binding site. Approximately equal coupling values were found with either Y190T or Y190F and the K27E substitution (Table I). Apart from its coupling to position 188 on the receptor, the R33E substitution showed coupling to the Y190T mutation. This was only observed at the αγ binding site.

**DISCUSSION**

Using site-directed mutagenesis, we previously identified residues involved in the high affinity interaction between the α-neurotoxin Nmml and the mouse muscle nAChR. The goal of this study was to identify specific pairs of interacting residues between the toxin and the receptor utilizing double mutant cycles (39, 47). This method has been successfully developed to identify pairwise interactions between the scorpion toxins and the potassium channel (37–39). In those studies, the known structure of the toxin was utilized to establish spatial locations of residues on the potassium channel of unknown structure.

In theory, if two residues are coupled either directly or through another residue then the effect of the double mutation will not be equal to the sum of the effects of the two single mutations (47, 48). Therefore, if 

ΔΔGINT values may exist between residues separated by great distances (49). Conversely, small ΔΔGINT values may exist between residues separated by short distances between the two residues under study exhibit an interaction. In practice, small ΔΔGINT values may exist between residues separated by great distances (49). Conversely, small ΔΔGINT values may exist between residues separated by short distances between the two residues under study (46). In our case, the errors associated with binding measurements of two nonequivalent sites located on one receptor molecule ranged from 10 to 20%. Because of the cumulative errors when summing single mutations, the errors associated with our linkage values ranged from 30 to 50%. Therefore, we have only considered coupling energies above 1.5 kcal/mol for our analysis.
maintained. In addition, failure to detect an interaction does not exclude the close proximity of the two residues. This may be due to either weak interactions between the two residues or to interactions that are compensatory yielding a minimal net change. This is especially true in the case of mutant cycles in which the reference side chain, typically the naturally occurring amino acid, exhibits a dominant influence (50).

For example, when the naturally occurring side chain Val^{188} on the common α-subunit of two binding sites is converted to both cationic and anionic side chains therein creating two parallel cycles, different coupling energies are achieved at the αδ and αγ interfaces (Fig. 4). However, if we coalesce the two cycles by considering a direct substitution from a cationic to an anionic side chain, then differences between the two sites virtually vanish. This suggests the naturally occurring valine common to both small cycles imparts the asymmetry, and the influence of an inserted charge on α-neurotoxin binding is similar at both sites. Interactions intrinsic to the reference residue or steric constraints could influence the αδ and αγ sites in a differential manner (50). A more appropriate frame of reference might come from a neutral side chain isosteric with substituted residues or a side chain with minimal steric perturbation (alanine).

Of the 36 residue pairs studied here (18 at each of the αδ and αγ binding sites), 25 gave ΔΔG values below 1.0 kcal/mol, indicating simple additivity. These results suggest that the gross structural changes do not occur in the interacting molecules with the introduced mutations. Rather, the relatively few but large coupling energies that were observed support the specific interactions between these residues.

The strongest linkages observed are with the R33/V188 and K27/V188 toxin-receptor pairs. The strength of the coupling observed between these pairs varied at the αδ and αγ binding sites and also with the different amino acid substitutions examined. The toxin residue 33 and receptor residue 188 appear to be interacting at both the αδ and αγ binding sites with coupling energies as high as 2.8 and >3.0 kcal/mol observed within the network of cycles. Toxin residue 27 and receptor residue 188 also appear to be interacting at both sites but to a lesser extent, as the coupling energies observed between this pair were generally lower than with the 33/188 pair. In contrast, the toxin residue Lys^{27} did not show any interaction with the receptor residue Val^{188}.

Coupling energies ranging from 1.5 to 1.9 kcal/mol were also found with the toxin residue Lys^{27} and the three receptor residues (Tyr^{190}, Pro^{197}, and Asp^{200}), all at the αδ interface. These results suggest that Tyr^{190}, Pro^{197}, and Asp^{200} are close enough in the receptor structure each to be interacting with Lys^{27}. Another possibility is that some or all of these observed linkages are mediated through a third residue. The lack of coupling observed between these paired residues at the αγ binding site does not preclude their interaction or their close proximity. However, it does demonstrate that the energetic contributions of Tyr^{190}, Pro^{197}, and Asp^{200} to toxin binding differ at the two sites. These data also suggest that the toxin is binding with different orientations to the two ligand sites, where interactions with Tyr^{190}, Pro^{197}, and Asp^{200} are less critical for the αγ site, but further experiments will be necessary to address this point.

An initial model of binding is proposed from these data. The two conserved toxin cationic residues Arg^{33} and Lys^{27}, located on loop II of the toxin structure, are complexing with key receptor residues located on the α-subunit region between 180 and 200. More specifically, we suggest that the toxin residue Arg^{33} is adjacent to the receptor residue Val^{188} and is probably stabilized by adjoining negative or aromatic residues located on the receptor structure. One such candidate may be Tyr^{190}, which did show a linkage with Arg^{33} at the αγ binding site. Other possibilities include residues located on the δ/γ subunits (see below). Lys^{27} also appears to be positioned in the vicinity of Val^{188} but closer to the residues Tyr^{190}, Pro^{197}, and Asp^{200}.

In this case, the lysine cation on the toxin may be directly stabilized through electrostatic interactions with Asp^{200} and cation/π interactions with Tyr^{190}.

The involvement of cationic residues near the tip of loop 2 on the toxin and the receptor sequence between residues 180 and 200 has been implicated from single residue mutations, chemical labeling, and binding of toxin to receptor peptide fragments (50–55), but previous studies have not pinpointed specific residue interactions nor have they distinguished differences in α-neurotoxin binding between the two binding sites. Homology modeling (26) and labeling experiments (12) have indicated that residues 180–200 on the α-subunit are located at the interface formed at the αδ and αγ ligand binding sites. Therefore, besides the α-subunit residues studied here, it is likely that the toxin loop II residues are interacting with δ/γ subunit residues. On the other hand, K47A, which is located on loop III of the toxin structure, does not appear to be interacting with this area of the α-subunit. Further studies aimed at identifying linkages between toxin residues and receptor residues on the δ and γ subunits should provide the additional constraints necessary to describe the toxin orientation and positioning at the two receptor binding sites.

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REFERENCES

1. Unwin, N. (1993) Cell 72, 31–41
2. Galzi, J.-L., and Changeux, J.-P. (1994) Curr. Opin. Struct. Biol. 4, 554–565
3. Karlin, A., and Akabas, M. H. (1995) Neuron 15, 1231–1244
4. Hucho, F., Tsetlin, V. I., and Machold, J. (1996) Eur. J. Biochem. 239, 539–557
5. Blount, P., and Merlie, J. P. (1989) Neuron 4, 349–357
6. Sire, S. M., and Claudio, T. (1991) J. Biol. Chem. 266, 19369–19377
7. Rao, P. N., Dwork, A. J., Kaldany, R. J., Silver, M. L., Wideman, J., Stein, S., and Karlin, A. (1984) J. Biol. Chem. 259, 11662–11665
8. Dennis, M., Giraudat, J., Kotsya-Hibert, F., Goeldner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chretien, M., and Changeux, J.-P. (1988) Biochemistry 27, 2546–2557
9. Abrams, S. N., Li, Y., Culver, P., and Taylor, P. (1989) J. Biol. Chem. 264, 12666–12672
10. Galzi, J.-L., Revah, F., Black, D., Goeldner, M., Hirth, C., and Changeux, J.-P. (1990) J. Biol. Chem. 265, 10430–10437
11. Middleton, R. E., and Cohen, J. B. (1991) Biochemistry 30, 6987–6997
12. Czajkowski, C., and Karlin, A. (1995) J. Biol. Chem. 270, 3160–3164
13. Tomasselli, G. F., McLaughlin, J. T., Jurman, M. E., Hawrot, E., and Yellen, G. (1991) Biophys. J. 60, 721–727
14. O’Leary, M. E., and White, M. M. (1992) J. Biol. Chem. 267, 8360–8365
15. Sire, S. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9436–9440
16. Aylin, M. L., and White, M. M. (1994) Mol. Pharmacol. 46, 1149–1155
17. Aylin, M. L., and White, M. M. (1994) FEBS Lett. 349, 99–103
18. Fu, D.-X., and Sine, S. M. (1994) J. Biol. Chem. 269, 26152–26157
19. O’Leary, M. E., Filatov, G. N., and White, M. M. (1994) Am. J. Physiol. 266, C648–C653
20. Sine, S. M., Quiram, P., Papanikolaou, F., Kreienkamp, H.-J., and Taylor, P. (1994) J. Biol. Chem. 269, 8808–8816
21. Yoshizaki, M., Hisa, H., and Chang, J.-P. (1994) J. Biol. Chem. 269, 52–66
22. Sine, S. M., Kreienkamp, H.-J., Bren, N., Maeda, R., and Taylor, P. (1995) Nature 376, 370–371
23. Sugiyama, N., Boyd, A. E., and Taylor, P. (1996) J. Biol. Chem. 271, 26575–26581
24. Unwin, N. (1993) J. Mol. Biol. 229, 1101–1124
25. Unwin, N. (1996) J. Mol. Biol. 257, 586–596
26. Tsigelny, I., Sugiyama, N., Sine, S. M., and Taylor, P. (1997) Biophys. J. 73, 52–66
27. Kreienkamp, H.-J., Sine, S. M., Maeda, R. K., and Taylor, P. (1994) J. Biol. Chem. 269, 8108–8114
28. Endo, T., and Tamiya, N. (1987) Pharmacol. Ther. 34, 403–451
29. Menez, A. (1991) in Snake Toxins (Harvey, A. L., ed.) pp. 35–90, Pergamon Press, New York
30. Basas, V. J., Bilellet, M., Love, R. A., Stroud, R. M., and Kuntz, I. D. (1988) Biochemistry 27, 2763–2771
31. Hatanaka, H., Oka, M., Kohda, D., Tate, S.-I., Suda, A., Tamiya, N., and Inagaki, F. (1994) J. Mol. Biol. 240, 155–166
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32. Peng, S.-S., Kumar, T. K. S., Jayaraman, G., Chang, C.-C., and Yu, C. (1997) *J. Biol. Chem.* 272, 7817–7823
33. Law, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D., and Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 2991–2994
34. Agard, D. A., and Stroud, R. M. (1982) *Acta Crystallogr. A* 38, 186–194
35. Arnoux, B., Menez, R., Drevet, P., Boulain, J.-C., Ducruix, A., and Menez, A. (1994) *FEBS Lett.* 342, 12–14
36. Ackermann, E. J., and Taylor, P. (1997) *Biochemistry* 36, 12836–12844
37. Ranganathan, R., Lewis, J. H., and MacKinnon, R. (1996) *Neuron* 16, 131–139
38. Naranjo, D., and MacKinnon, R. (1995) *Science* 268, 307–310
39. Sugiyama, N., Marchot, P., Kawanishi, C., Osaka, H., Mollas, B., Sine, S. M., and Taylor, P. (1998) *J. Biol. Chem.* 273, 3315–3325
40. Sine, S., and Taylor, P. (1979) *J. Biol. Chem.* 254, 3315–3325
41. Pillet, L., Tremeau, O., Ducancel, F., Drevet, P., Zinn-Justin, S., Pinkasfeld, S., Boulain, J.-C., and Menez, A. (1993) *J. Biol. Chem.* 268, 909–916
42. Tremeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.-C., and Menez, A. (1995) *J. Biol. Chem.* 270, 9362–9369
43. Drevet, P., Zinn-Justin, S., Boulain, J.-C., and Menez, A. (1996) *J. Biol. Chem.* 271, 31345–31353
44. Carter, P. J., Winter, G., Wilkinson, A. J., and Fersht, A. R. (1984) *Cell* 38, 835–840
45. Schreiber, G., and Fersht, A. R. (1995) *J. Mol. Biol.* 248, 478–486
46. Wells, J. (1990) *Biochemistry* 29, 8509–8517
47. Wells, J. (1990) *Biochemistry* 29, 8509–8517
48. Ranganathan, R., Lewis, J. H., and MacKinnon, R. (1996) *Neuron* 16, 131–139
49. Hidalgo, P., and MacKinnon, R. (1995) *Science* 268, 307–310
50. Sine, S., and Taylor, P. (1979) *J. Biol. Chem.* 254, 3315–3325
51. Barchan, D., Ovadia, M., Kochva, E., and Fuchs, S. (1995) *Biochemistry* 34, 9172–9176