Pairwise Interactions between Neuronal α7 Acetylcholine Receptors and α-Conotoxin PnIB*

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This work uses α-conotoxin PnIB to probe the agonist binding site of neuronal α7 acetylcholine receptors. We mutated the 13 non-cysteine residues in CTx PnIB, expressed α7/5-hydroxytryptamine-3 homeric receptors in 293 HEK cells, and measured binding of each mutant toxin to the expressed receptors by competition against the initial rate of 125I-α-bungarotoxin binding. The results reveal that residues Ser-4, Leu-5, Pro-6, Pro-7, Ala-9, and Leu-10 endow CTx PnIB with affinity for α7/5-hydroxytryptamine-3 receptors, side chains of these residues cluster in a localized region within the three-dimensional structure of CTx PnIB. We next mutated key residues in the seven loops of α7 that converge at subunit interfaces to form the agonist binding site. The results reveal predominant contributions by residues Trp-149 and Tyr-93 in α7 and smaller contributions by Ser-34, Arg-186, Tyr-188, and Tyr-195. To identify pairwise interactions that stabilize the receptor-conotoxin complex, we measured binding of receptor and toxin mutations and analyzed the results by double mutant cycles. The results reveal a single dominant interaction between Leu-10 of CTx PnIB and Trp-149 of α7 that anchors the toxin to the binding site. We also find weaker interactions between Pro-6 of CTx PnIB and Trp-149 and between both Pro-6 and Pro-7 and Tyr-93 of α7. The overall results demonstrate that a localized hydrophobic region in CTx PnIB interacts with conserved aromatic residues on one of the two faces of the α7 binding site.

The α-conotoxins have attracted considerable attention as probes of AChR1 binding sites owing to their remarkable receptor subtype and species specificity (1–5). Previous work probed the binding site of the α7 AChR with α-conotoxin ImI (CTx ImI) and identified pairwise interactions that stabilize the toxin-receptor complex (3). Here we further probe the binding site of the α7 AChR with α-conotoxin PnIB, a neuronal-specific toxin structurally divergent from CTx ImI, with the aim of uncovering novel pairwise interactions at the binding site. We mutate both the receptor and the toxin and use double mutant cycle analysis to identify pairs of residues that stabilize the conotoxin-receptor complex.

α-Conotoxins are small disulfide-rich peptides that competitively antagonize muscle and neuronal nicotinic AChRs (4). All α-conotoxins have a conformationally constrained two-loop structure formed by two disulfide bridges. However, the various α-conotoxins differ by the number and type of residues in each loop, allowing specific targeting to different receptor subtypes. For example, the neuronal-specific conotoxins CTx PnIB and MII contain four residues in the first loop and seven in the second, whereas the muscle-specific conotoxins MI and GI contain three residues in the first loop and five in the second (Fig. 1). Furthermore the α7-specific toxin CTx ImI differs from either of these classes containing four residues in the first loop and three in the second. Residue side chains also range widely from small to polar, hydrophobic, charged, and aromatic. Thus, diversity of backbone structure and residue composition makes α-conotoxins excellent probes of different receptor subtypes.

Mutagenesis and site-directed labeling studies establish that the ligand binding sites of the AChR are formed at interfaces between subunits (6, 7). Whereas binding sites of muscle AChRs are formed at interfaces between α1, δ, ε, or γ subunits, binding sites of α7 neuronal AChRs are formed at interfaces between identical α7 subunits. Residues of the α face of the binding site, termed the (+) face, cluster in three well separated regions of the primary sequence, termed loops A, B, and C (6). Using the numbering system for human α7 receptors, residues key to AChR function in these loops include Tyr-93 in loop A, Trp-149 in loop B, and Tyr-188 and Tyr-195 in loop C. Similarly, residues of the non-α face of the binding site, termed the (−) face, cluster in four separate regions of the primary sequence, termed loops I through IV. Residues key to AChR function in these loops include Ser-34 in loop I, Trp-55 in loop II, Gln-117 in loop III, and Asp-164 in loop IV. The convergence of these seven loops to form a localized binding site has led to a multiloop model of the major extracellular domain of the AChR (7).

**Binding site specificity of α-conotoxins should ultimately be traceable to differences in residues supplied by either the receptor or the conotoxin that form the complex. For example, α-conotoxin MI and GI bind with high affinity to muscle but not neuronal AChRs, presumably owing to differences in residues on both the (+) and (−) faces of the binding sites between the two classes of receptors (8). Furthermore, α-conotoxins MI and GI select between binding sites of the muscle AChR owing to differences in residues supplied by the γ, ε, or δ subunits to the (+) face of the binding sites (2). Recent work mutated both the receptor and conotoxin and identified pairwise interactions that stabilize the complex formed by α7 receptors and CTx ImI (3). The primary anchor of the complex comprises a positively charged arginine supplied by the N-terminal loop of CTx ImI that interacts with a conserved tyrosine at position 195 of the (+) face of the binding site. The present study was motivated by the substantial differences between CTx PnIB and CTx ImI (Fig. 1), with the aims of establishing orientation of CTx PnIB in the α7 binding site and uncovering novel pairwise interactions that stabilize the complex.

By mutating residues in both α7 and CTx PnIB, the present
work delineates pairwise interactions that stabilize the receptor-conotoxin complex. We find that CTx PnIB interacts with the (+) face of the α2 binding site through hydrophobic side chains from both its N- and C-terminal loops. Moreover, the key side chains in CTx PnIB form a localized hydrophobic patch in its three-dimensional structure, which interacts with a cluster of conserved aromatic residues on the (+) face of the α2 binding site.

**EXPERIMENTAL PROCEDURES**

**Materials**—Labeled α-bungarotoxin (α-bgt) was purchased from NEN Life Science Products, d-tubocurarine chloride was from ICN Pharmaceuticals, the 293 human embryonic kidney cell line (293 HEK) was from the American Type Culture Collection, and unlabeled α-bgt was from Sigma.

**Synthesis and Purification of Conotoxin PnIB**—Wild-type and mutants of α-conotoxin PnIA and PnIB were synthesized by the Mayo Protein Core Facility using standard Fmoc- (9-fluorenyl)methoxycarbonyl) chemistry on an Applied Biosystems 431A peptide synthesizer. During synthesis, cysteine (S-triphenylmethyl) protecting groups were incorporated at cysteines 2 and 8. The linear peptide was lyophilized prior to formation of the second disulfide bond. The acetamidomethyl-protecting groups on cysteines 2 and 8 were removed oxidatively by iodine as described (9), except the peptide/iodine reaction was allowed to progress for 16 h prior to carbon tetrachloride extraction. The pure product was separated from residual iodine by high pressure liquid chromatography. Formation of disulfide bonds was confirmed by negative reaction with Ellman’s reagent, and the molecular weight was verified by mass spectrometry, as described previously (10). The conotoxin mutants are named as follows: the first letter and number refer to the wild-type residue and position, and the following letter is the substituted residue at that position.

**Mutagenesis and Expression in HEK Cells**—AChR subunit cDNAs were subcloned into the cytomegalovirus-based expression vector pRBC4 (11). Mutant cDNAs were constructed by bridging naturally occurring or mutagenically installed restriction sites with double-stranded oligonucleotides or by the Quick Change™ site-directed mutagenesis kit (Stratagene). All constructs were confirmed by dideoxy sequencing. To increase expression, the extracellular domain of human α2 was joined to the rat 5-HT3 sequence at the start of the M1 transmembrane domain, and all mutations were constructed in the resulting α2/5-HT3 chimeras (9). The α2 point mutants are named as follows: the first letter and number refer to the wild-type residue and position, and the following letter is the substituted residue at that position. HEK cells were transfected with either wild-type or mutant cDNAs using calcium phosphate precipitation as described (11). For ligand binding measurements, cells two days after transfection were harvested by gentle agitation in phosphate-buffered saline containing 4 mM EDTA.

**Ligand Binding Measurements**—Ligand binding to intact cells was measured by competition for the initial rate of α1/2-bgt binding (11). The cells were briefly centrifuged, resuspended in potassium Ringer’s solution (11). The cells were then allowed to bind for 10 min to occupy approximately half of the surface receptors. Binding was terminated by the addition of 2 ml of potassium Ringer’s solution containing 600 mM d-tubocurarine chloride. All experiments were performed at 24 °C ± 2 °C. Cells were harvested by filtration through Whatman GF-B filters and washed three times with 3 ml of phosphate buffered saline, adjusted to pH of 7.4 with NaOH. Specified concentrations of wild-type or mutant CTx PnIB were added 30 min prior to the addition of 3.75 nM 125I-α-bgt, which was then allowed to bind for 10 min to occupy approximately half of the surface receptors. Binding was measured by incubation with toxin for 120 min. The initial rate of binding was calculated as described (12) to yield the fractional occupancy of competing ligand. Binding measurements were analyzed according to the Hill equation,

\[ 1 - Y = \frac{1}{1 + \left(\frac{\text{ligand}}{K_{app}}\right)^{n_H}} \]

where Y is fractional occupancy of the competing ligand, \( K_{app} \) is the apparent dissociation constant, and \( n_H \) is the Hill coefficient. Fitted parameters and standard errors were obtained using UltraFit (BIO-SOFTWARE). For multiple experiments, means of the individual fitted parameters and standard deviations are presented (Tables I-IV).

**FIG. 1.** Comparison of neuronal-specific α-conotoxins PnIB, PnIA, MII, and Iml with muscle-specific α-conotoxins SI, GI, and MI.
RESULTS

Mutagenesis of CTx PnIB—To identify residues in CTx PnIB essential for binding to $\alpha_7$ receptors, we mutated each non-cysteine residue using standard peptide synthesis methods (see “Experimental Procedures”). We expressed $\alpha_7$/5HT-3 receptors in 293 HEK cells and measured binding of each mutant toxin by competition against the initial rate of $^{125}$I- $\alpha$-bungarotoxin binding. The resulting dissociation constants show that residues in both loops of CTx PnIB are essential for binding to $\alpha_7$/5HT-3 receptors. These key residues comprise Ser-4, Leu-5, Pro-6, Pro-7, Ala-9, and Leu-10 (Fig. 2 and Table I).

These six essential residues localize to one end of the three-dimensional structure of CTx PnIB (13). They run the length of a helix within the compact structure of the toxin, with their side chains radiating outward to create hydrophobic ridges and grooves positioned to contact the $\alpha_7$ binding site (Fig. 3). Thus within the three-dimensional structure of CTx PnIB, bioactive residues coalesce to form an ordered hydrophobic motif that likely complements a portion of the $\alpha_7$ binding site.

The overall mutagenesis results indicate both hydrophobic and steric contributions to CTx PnIB affinity (Fig. 2 and Table I). Within the N-terminal loop (Fig. 1), Pro-6 and Pro-7 make the greatest hydrophobic contributions, as hydroxyl substitutions of either of the prolines decrease affinity by an order of magnitude (Table I). The position of the hydroxyl substitution on the imino ring is not decisive, as substitutions at either position 3 or 4 decrease affinity to the same extent. The adjacent Leu-5 contributes in a primarily steric manner, as the mutations L5A and L5S increase affinity by one order of magnitude, perhaps by allowing deeper penetration of Pro-6 and Pro-7 into the binding site.

Residues in the C-terminal loop of CTx PnIB also make hydrophobic and steric contributions to affinity. Ala-9 makes a nearly pure hydrophobic contribution, as mutation to serine decreases affinity, whereas mutation to leucine increases affinity. Leu-10 also contributes in a hydrophobic manner, as shortening the aliphatic side chain with L10A decreases affinity. On
For WT relative to wild type (WT) CTx PnIA. The affinity of wild type CTx PnIA for the CTx PnIA mutants are expressed as the log ratio whereas CTx PnIB contains leucine at position 10 and serine at position 11, whereas CTx PnIB contains alanine at position 10 and asparagine at position 11. CTx PnIA differs by only two residues from CTx PnIB. CTx PnIA contains alanine at position 10 and asparagine at position 11, whereas CTx PnIB contains leucine at position 10 and serine at position 11 (Fig. 1). As a naturally occurring variant of CTx PnIB, CTx PnIA prompted us to test the hydrophobic contribution of residue 10 and to look further for a possible contribution of residue 11. As expected, CTx PnIA binds less tightly to α7/5HT-3 receptors compared with CTx PnIB, supporting the importance of the hydrophobic contribution of residue 10 (Fig. 4). The CTx PnIA mutation A10L markedly increases affinity by two orders of magnitude, further supporting the hydrophobic contribution of residue 10. The absolute affinity of the A10L mutant is 0.02 nM, which is approximately 10^4 fold higher than that of the wild type (WT) CTx PnIA (Table I).

By contrast to the cluster of essential hydrophobic residues in CTx PnIB, the remaining six non-cysteines do not contribute significantly to affinity for α7/5HT-3 receptors (Fig. 2). Particularly striking are the lack of contributions by Asp-14 and Tyr-15, where neutralizing the negative charge with D14N or removing the aromatic hydroxyl with Y15A are without effect. Collectively, these nonessential residues segregate to one hemisphere of CTx PnIB to form a hydrophilic surface (Fig. 3). The overall results demonstrate the essential hydrophobic nature of the essential residues in CTx PnIB.

Mutagenesis of CTx PnIA—The closely related toxin CTx PnIA differs by only two residues from CTx PnIB. CTx PnIA contains alanine at position 10 and asparagine at position 11, whereas CTx PnIB contains leucine at position 10 and serine at position 11 (Fig. 1). As a naturally occurring variant of CTx PnIB, CTx PnIA prompted us to test the hydrophobic contribution of residue 10 and to look further for a possible contribution of residue 11. As expected, CTx PnIA binds less tightly to α7/5HT-3 receptors compared with CTx PnIB, supporting the importance of the hydrophobic contribution of residue 10 (Fig. 4). The CTx PnIA mutation A10L markedly increases affinity by two orders of magnitude, further supporting the hydrophobic contribution of residue 10. The absolute affinity of the A10L mutant is 0.02 nM, which is approximately 10^4 fold higher than that of the wild type (WT) CTx PnIA (Table I).

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**Table II**

| High affinity α7 and point mutants | K_{app} (mut/K_{app} wt) | n_{H} | N |
|-----------------------------------|-------------------------|------|---|
| High affinity α7/5HT-3            |                         |      | 10|
| (+) Face                          |                         |      | 10|
| Loop A                           |                         |      | 10|
| Y93T                             | 1.73                    | 1.00 | 5.06|
| Y93F                             | 0.91                    | 0.85 | 4.03|
| Loop B                           |                         |      | 10|
| W149T                            | 2.3                     | 0.86 | 4.06|
| W149F                            | 1.0                     | 0.96 | 1.3|
| Y151T                            | 0.36                    | 1.00 | 4.06|
| Y151F                            | 0.12                    | 1.10 | 0.07|
| G152D                            | -0.15                   | 0.99 | 0.03|
| G153S                            | -0.02                   | 0.97 | 0.03|
| W154A                            | -0.02                   | 1.08 | 0.06|
| Loop C                           |                         |      | 10|
| R186V                            | -0.94                   | 1.20 | 0.05|
| R186E                            | -0.95                   | 1.10 | 0.08|
| R186Q                            | -0.91                   | 1.00 | 0.02|
| Y188T                            | NE                      | 1    | 1  |
| Y188W                            | -0.99                   | 1.0  | 0.04|
| Y188H                            | 0.02                    | 1.0  | 0.04|
| Y188F                            | -0.69                   | 1.0  | 0.04|
| Y195F                            | -0.57                   | 1.0  | 0.04|
| Y195T                            | 1.03                    | 1.0  | 0.07|
| Y195R                            | 1.98                    | 1.2  | 0.01|
| Y195K                            | 2.04                    | 0.95 | 0.08|
| Loop I                           |                         |      | 10|
| S34K                             | -1.39                   | 0.94 | 0.04|
| Loop II                          |                         |      | 10|
| W55R                             | 0.02                    | 1.1  | 0.08|
| T77R                             | 0.14                    | 1.1  | 0.07|
| W55R + S59Q                      | 0.09                    | 1.1  | 0.02|
| Loop III                         |                         |      | 10|
| Q117S                            | 0.01                    | 1.0  | 0.07|
| Loop IV                          |                         |      | 10|
| D164A                            | 0.13                    | 1.1  | 0.09|
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Binding parameters for high affinity and mutant α7 receptors and CTX PnIA

Data are least square fits to the Hill equation for experiments in Fig.

FIG. 5. Pairwise coupling between high affinity α7 and CTX PnIB or PnA. The coupling coefficients ℧, calculated according to Equation 2 for each pair of mutations, are also given in Table IV.

FIG. 6. Pairwise binding of mutant and high affinity α7 and CTX PnIB. A, 293 HEK cells were transfected with high affinity α7 or α,W149T subunit cDNAs, and binding of CTX PnIB or the L10A mutant of CTX PnIB was determined as described under “Experimental Procedures.” The curves through the data are fits to the Hill equation (Equation 1) with means and standard errors of the fitted parameters given in Tables II and IV. The drawing at the top is a schematic representation of CTX PnIB with the arrow indicating the site of the L10A mutation. B, 293 HEK cells were transfected with high affinity α7 or α,W149T subunit cDNAs, and binding of CTX PnIB or the S4A mutant of CTX PnIB was determined as described under “Experimental Procedures.” The curves through the data are fits to the Hill equation (Equation 1) with means and standard errors of the fitted parameters given in Tables II and IV. The drawing at the top is a schematic representation of CTX PnIB with the arrow indicating the site of the S4A mutation. WT, wild-type.

We mutated key residues in the seven loops of α7 known to converge to the binding site interface (6, 7). On the (+) face of the binding site, we mutated aromatic residues in loops A, B, and C, as well as local flanking residues. The results reveal that Trp-149 in loop B provides the strongest source of stabilization of CTX PnIB, as shown by the 200-fold loss in affinity produced by W149T (Fig. 5 and Table II). In addition to the overall aromatic contribution by Trp-149, its indole ring provides optimal stabilization, as mutation to phenylalanine decreases affinity by 10-fold. We find that Tyr-93 in loop A provides an additional strong source of stabilization of CTX PnIB, with Y93T decreasing affinity by 10-fold and Y93F decreasing affinity by 10-fold. Additional weaker sources of stabilization include Arg-186, Tyr-188, and Tyr-195 in loop C, with R186V and Y188W producing unusual increases rather than decreases in affinity of CTX PnIB. Thus residues in loops A, B, and C of the (+) face of the α7 binding site contribute to CTX PnIB affinity, with Trp-149 and Tyr-93 making dominant contributions.

On the opposing (−) face of the α7 binding site, we mutated key residues in loops I-IV (Fig. 5 and Table II). We find that Ser-34 in loop I stabilizes CTX PnIB, with the mutation S34K increasing affinity by more than an order of magnitude. No additional contributions were detected for residues on the (−) face of the binding site.

We also examined the effects of binding site mutations on the affinity of CTX PnIA. We mutated key residues in α7 important for CTX PnIB binding and found similar effects on binding of CTX PnIA (Table III). The lone exception to this trend is the mutation W149T, which only slightly decreases affinity of CTX PnIA, in contrast to the 200-fold decrease in affinity observed for CTX PnIB. This observation, together with mutant cycle data presented below, suggests a pairwise interaction between Trp-149 of α7 and Leu-10 of either CTX PnIA or PnIB.

To summarize, we have examined key residues in the seven loops in α7 receptors known to contribute to the ligand binding site. We find that Trp-149 and Tyr-93 are major contributors to CTX PnIB affinity and that several residues on both the (+) and (−) faces of the binding site are minor contributors.

Thermodynamic Mutant Cycle Analysis—We previously used thermodynamic mutant cycles analysis to identify pairwise interactions between α7 receptors and CTX ImII (3). To generate a mutant cycle for pairs of CTX PnIB and α7 receptor mutations, dissociation constants are determined for the four possible combinations of wild-type (W) and mutant (M) receptors (r) and toxins (t): WrWt, MrWt, WrMt, and MrMt. The resulting set of dissociation constants are then used to calculate a coupling coefficient Ω (14).

Ω = \frac{K_r(WrWt) \times K_t(MrMt)}{K_r(WrMt) \times K_t(MrWt)}

(Eq. 2)
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Table IV

| Mutant CTx | High affinity α7 point mutants | Omega (Ω) | Kapp | nH | N |
|-----------|-------------------------------|-----------|------|----|---|
| S4A       | High affinity α7-HT-3          | 0.7 ± 0.09 | 1.07 ± 0.03 |
|           | S34K                          | 1.9 ± 0.1 | 1.05 ± 0.03 |
|           | Y93T                          | 1.9 ± 0.13 | 0.90 ± 0.03 |
|           | W149T                         | 1.1 ± 0.2 | 0.98 ± 0.03 |
|           | Y151T                         | 1.6 ± 0.2 | 0.98 ± 0.03 |
|           | R186V                         | 1.0 ± 0.08 | 1.07 ± 0.03 |
|           | Y188W                         | 1.4 ± 0.01 | 1.07 ± 0.02 |
|           | Y195T                         | 1.5 ± 0.1 | 1.04 ± 0.02 |
| L5A       | High affinity α7-HT-3          | 0.09 ± 0.02 | 0.95 ± 0.02 |
|           | S34K                          | 2.7 ± 0.02 | 1.00 ± 0.02 |
|           | Y93T                          | 1.3 ± 0.3 | 1.1 ± 0.03 |
|           | Q117S                         | 1.7 ± 0.08 | 0.95 ± 0.02 |
|           | W149T                         | 4.4 ± 0.2 | 0.91 ± 0.03 |
|           | Y151T                         | 3.9 ± 0.1 | 0.91 ± 0.04 |
|           | R186V                         | 3.8 ± 0.04 | 1.02 ± 0.02 |
|           | Y188W                         | 2.1 ± 0.02 | 0.97 ± 0.02 |
|           | Y195T                         | 2.2 ± 0.1 | 1.1 ± 0.04 |
| P6HP-3    | High affinity α7-HT-3          | 93.5 ± 2.0 | 1.1 ± 0.3 |
|           | S34K                          | 1.6 ± 0.2 | 1.0 ± 0.04 |
|           | Y93T                          | 19.5 ± 5.4 | 1.0 ± 0.53 |
|           | Q117S                         | 1.7 ± 0.5 | 1.0 ± 0.04 |
|           | W149T                         | 78.6 ± 2.0 | 1.07 ± 0.03 |
|           | Y151T                         | 1.2 ± 0.13 | 1.0 ± 0.06 |
|           | R186V                         | 1.4 ± 0.15 | 1.0 ± 0.04 |
|           | Y188W                         | 2.0 ± 1.4 | 1.1 ± 0.02 |
|           | Y195T                         | 1.2 ± 0.85 | 0.82 ± 0.04 |
| P6HP-4    | High affinity α7-HT-3          | 96.9 ± 2.9 | 1.1 ± 0.63 |
|           | S34K                          | 2.6 ± 0.1 | 1.1 ± 0.02 |
|           | Y93T                          | 9.0 ± 0.6 | 0.9 ± 0.62 |
|           | Q117S                         | 2.0 ± 0.9 | 1.1 ± 0.04 |
|           | W149T                         | 22.0 ± 0.03 | 0.91 ± 0.07 |
|           | Y151T                         | 1.0 ± 0.23 | 1.0 ± 0.04 |
|           | R186V                         | 3.3 ± 0.2 | 1.1 ± 0.03 |
|           | Y188W                         | 1.8 ± 1.7 | 1.0 ± 0.08 |
|           | Y195T                         | 1.6 ± 119.0 | 1.1 ± 0.08 |
| P7HP-3    | High affinity α7-HT-3          | 32 ± 1.0 | 1.0 ± 0.04 |
|           | S34K                          | 2.3 ± 0.09 | 0.94 ± 0.03 |
|           | Y93T                          | 14.5 ± 3.3 | 0.96 ± 0.63 |
|           | Q117S                         | 2.6 ± 0.5 | 1.1 ± 0.02 |
|           | W149T                         | 9.7 ± 3.0 | 1.1 ± 0.04 |
|           | Y151T                         | 1.5 ± 0.9 | 0.78 ± 0.05 |
|           | R186V                         | 1.5 ± 0.5 | 1.1 ± 0.04 |
|           | Y188W                         | 2.2 ± 0.5 | 1.1 ± 0.03 |
|           | Y195T                         | 1.1 ± 3.67 | 1.0 ± 0.03 |
| P7HP-4    | High affinity α7-HT-3          | 14.8 ± 0.6 | 0.86 ± 0.02 |
|           | S34K                          | 2.8 ± 1.7 | 1.1 ± 0.03 |
|           | Y93T                          | 14.6 ± 5.5 | 0.92 ± 0.04 |
|           | Q117S                         | 1.8 ± 7.6 | 0.91 ± 0.07 |
|           | W149T                         | 12.4 ± 2.3 | 1.07 ± 0.03 |
|           | Y151T                         | 1.5 ± 2.2 | 0.89 ± 0.03 |
|           | R186V                         | 2.5 ± 0.3 | 1.1 ± 0.02 |
|           | Y188W                         | 3.3 ± 5.1 | 1.1 ± 0.03 |
|           | Y195T                         | 3.5 ± 84.5 | 0.8 ± 1.0 |
| A9S       | High affinity α7-HT-3          | 27.9 ± 1.6 | 1.1 ± 0.03 |
|           | S34K                          | 2.3 ± 0.07 | 1.0 ± 0.03 |
|           | Y93T                          | 1.480 ± 178 | 1.2 ± 0.06 |
|           | Q117S                         | 1.0 ± 0.3 | 1.1 ± 0.07 |
|           | W149T                         | 4.6 ± 38.8 | 0.9 ± 0.03 |
|           | Y151T                         | 1.2 ± 6.7 | 1.2 ± 0.1 |
|           | R186V                         | 1.9 ± 6.0 | 1.1 ± 0.03 |
|           | Y188W                         | 1.4 ± 4.0 | 1.0 ± 0.05 |
|           | Y195T                         | 1.1 ± 340.1 | 0.9 ± 0.07 |

If a pair of residues does not interact, then Ω equals unity, whereas if a pair interacts, then Ω deviates significantly from unity. The free energy of the interaction is given by the following equation.

\[ \Delta G = \text{RT} \ln \Omega \]  

(Eq. 3)

Pairwise Interactions That Stabilize the α7 Receptor-CTx PnIB Complex—To identify pairwise interactions between α7 and CTx PnIB, we focused on residues in the receptor and the toxin that significantly stabilize the complex. Results for the most strongly interacting pair of residues are shown in Fig. 6A, which displays binding curves for the receptor/conotoxin pair W149T/L10A. The receptor mutation W149T decreases affinity for wild-type CTx PnIB by 190-fold, whereas the CTx PnIB mutation L10A decreases affinity for wild-type α7 by 5-fold. However, when examined together, the two mutations decrease affinity by only 10-fold, contrary to the 950-fold decrease expected if their contributions were additive. Mutant cycles analysis reveals a coupling coefficient of 690 for the W149T/L10A pair, corresponding to an interaction free energy of 3.9 kcal/mol.

On the other hand, results demonstrating a noninteracting pair of residues are shown in Fig. 6B for the receptor-conotoxin pair W149T/S44A. The receptor mutation W149T decreases affinity for wild-type CTx PnIB by 190-fold, whereas the CTx PnIB mutation S44A increases affinity for wild-type α7 by 4.3-fold. The two mutations together decrease affinity by 43-fold, which is purely additive, indicating that W149T and Ser-4 do not interact. Thus mutant cycle analysis readily distinguishes interacting from noninteracting pairs of residues in the α7-CTx PnIB complex.

We next tested all possible combinations of receptor and conotoxin mutations, pairing each receptor mutation against a single conotoxin mutation, and repeating the process for each of the six bioactive residues in CTx PnIB. Among all possible pairs of receptor and conotoxin mutations, the pair α7-TrpL149/α7-Leu-10 shows the greatest coupling coefficient, as described above, indicating that this pair is the primary anchor of the α7-CTx PnIB complex. As suggested by the lack of effect of the α7 mutation W149T on affinity of CTx PnIA, we also find a large coupling coefficient of 100 for the pair α7-W149T/PnIA A10L. Thus the majority of pairwise combinations show small or negligible interactions, in contrast to the large stabilizing interaction revealed by the W149T/L10A pair.
In addition to its primary coupling to L10, α7-Trp-149 couples weakly to Pro-6 and Pro-7 in CTx PnIB (Fig. 7 and Table IV). The pairs W149T/P6HP-3 and W149T/P7HP-3 show coupling coefficients of 78 and 12, respectively, suggesting that Trp-149 also receives hydrophobic stabilization from the imino rings of Pro-6 and Pro-7. Interaction of Trp-149 is restricted to a small region of CTx PnIB, as no significant coupling is detected to other bioactive residues in the toxin, including Ser-4, Leu-5, and Ala-9.

The second major source of stabilization in α7, Tyr-93, couples significantly to Pro-6, Pro-7, and Leu-10 in CTx PnIB (Fig. 7 and Table IV). The coupling coefficients for the pairs Tyr-93/P6HP, Tyr-93/P7HP, and Tyr-93/L10A range from 10 to 20 and are comparable to those observed for the W149T/P7HP pair. Coupling of both Trp-149 and Tyr-93 to the same side chains in CTx PnIB indicates either the close approach of loops A and B in the α7 face of the binding site. Side chains of Pro-6 and Pro-7 protrude one turn above Leu-10 to interact with Trp-149 in loop B of the α7 binding site. The leucine side chain at position 10 of Pro-6 and Pro-7 forms a hydrophobic spiral at one end of the compact three-dimensional structure (Fig. 3). The leucine side chain at position 10 protrudes from one end of the spiral to interact strongly with Trp-149 in loop B of the α7 face of the binding site. Side chains of Pro-6 and Pro-7 protrude one turn above Leu-10 to interact with Tyr-93 in loop A of the α7 face, as well as with Trp-149. The turn above Pro-6 and Pro-7 comprises side chains of the aromatic residues in the binding site. The overall results reveal multiple pairwise interactions between a cluster of hydrophobic residues in CTx PnIB and aromatic residues at the α7 binding site.

**DISCUSSION**

The present work defines the essential hydrophobic nature of the interaction between CTx PnIB and the neuronal α7 receptor and places into close proximity aromatic residues in two of the three loops of the α7 face of the α7 binding site. The key bioactive residues in CTx PnIB run the length of a helix to form a hydrophobic spiral at one end of the compact three-dimensional structure (Fig. 3). The leucine side chain at position 10 protrudes from one end of the spiral to interact strongly with Trp-149 in loop B of the α7 face of the binding site. Side chains of Pro-6 and Pro-7 protrude one turn above Leu-10 to interact with Tyr-93 in loop A of the α7 face, as well as with Trp-149. The turn above Pro-6 and Pro-7 comprises side chains of the aromatic residues in the binding site. The overall results reveal multiple pairwise interactions between a cluster of hydrophobic residues in CTx PnIB and aromatic residues at the α7 binding site.

Both strong and weak hydrophobic interactions stabilize CTx PnIB in the α7 binding site. The hydrophobic spiral in CTx PnIB comprises leucine side chains at positions 5 and 10, together with proline side chains at positions 6 and 7. Three of these four side chains interact with aromatic side chains on the α7 face of the α7 binding site. The stabilization likely owes to inclusion of water from both the hydrophobic and aromatic surfaces, as well as to hand-in-glove complementarity between the surfaces of the toxin and binding site that maximizes van der Waals contacts. The observed coupling coefficients suggest that the indole ring of Trp-149 packs into the groove bounded by Pro-6, Pro-7, and Leu-10, whereas the phenol side chain of Tyr-93 enters the same groove but from the opposite side. Finally, the phenol side chain of Tyr-151 packs on the external side of this groove, bounding the exposed length of Leu-10. The overall results place into close proximity the aromatic side chains Trp-149, Tyr-93, and Tyr-151 on the α7 face of the α7 binding site.

The Ctx PnIB-α7 complex contains with the previously described CtxIm1-α7 complex (3) in both the chemical nature of the interaction and the points of contact in the α7 binding site. The CtxIm1-α7 complex is anchored by an aromatic-quaternary interaction between arginine at position 7 of CtxIm1 and tyrosine at position 196 of the α7 face of CtxIm1. Also, tryptophan at position 10 of CtxIm1 interacts with multiple residues on the α7 face of the α7 binding site. The present results detect no interaction between residues in Ctxs PnIB and Tyr-195 of α7 nor between Ctxs PnIB and the α7 face of the binding site. The very different interactions of Ctx Im1 and Ctx PnIB suggest that the various α-conotoxins evolved to target different microdomains within the AChR binding site.

Although we only detect interaction between Ctx PnIB and the α7 face of the binding site, we cannot exclude possible interactions with residues on the α7 face. We find significant contributions to Ctx PnIB affinity of Ser-34 in loops I of the α7 face of the site, with the mutation S34K increasing affinity by about an order of magnitude. Also, pairwise interactions remain to be discovered for the bioactive residues Ser-4 and Leu-5 in Ctx PnIB. Thus the possibility remains that residues not yet discovered on the α7 face interact with Ser-4 or Leu-5. Because residues at the α7 face of the binding site are conserved across AChR subtypes, selectivity of the various α-conotoxins for different AChR subtypes likely owes to different contributions of the α7 face.

The large coupling coefficient between α7-Trp-149 and Leu-10 of Ctx PnIB does not by itself ensure that the two residues interact directly. However, the absence of other strong pairwise interactions for Leu-10 or Trp-149 strongly suggests that the interaction is direct. For example, if Trp-149 interacted with a second residue in α7, that in turn contacted Leu-10, mutating that second residue should both weaken Ctx PnIB binding and show strong coupling to Leu-10. Our systematic mutagenesis shows no strong coupling of Leu-10 to other key residues of either the α7 or the α7 face of the α7 binding site, even though Ctx PnIB binding is very sensitive to mutation of these key residues.

Similarly without atomic structures of the mutant conotoxins, global conformational changes are difficult to distinguish from local changes at the site of mutation. For example, suppose hydroxyproline mutations at positions 6 or 7 distorted the peptide structure so the primary anchor, leucine at position 10, moved out of alignment with its counterpart in the receptor. Hydroxyproline retains the original restricted rotation about the N-Ca bond, but its hydroxyl group may increase hydration locally. However, we made a chemically similar mutation, A9S, which is even closer to the active residue Leu-10, but again found no coupling to Trp-149, indicating specificity of the hydroxyproline mutations. Furthermore, on the other flank of Leu-10, the mutation S11L did not affect Ctx PnIB affinity, although local hydrophobicity was altered. Thus of the many possible combinations of receptor and conotoxin mutations, only a few interact in a pairwise manner; this high degree of specificity suggests the interactions are direct.

The present observations of hydrophobic contributions of Ctx PnIB to affinity suggest similar interactions for related α-conotoxins. Ctx MII has the same number of residues in the two-loop structure, and contains proline at position 6, valine at position 7, and leucine at position 10 (Fig. 1). Ctx E1 also has the same number of residues in the two-loop structure and contains proline at position 6, threonine at position 7, and methionine at position 10. Our findings suggest that these hydrophobic residues from Ctx MII and E1 interact with Trp-149, Tyr-93, and Tyr-151 of the corresponding α7 subunit target.

The overall results disclose the essential hydrophobic nature of the interaction between CTx PnIB and aromatic residues in the α7 binding site. The specificity of Ctx PnIB for α7 receptors is due to its rigid scaffold that presents a hydrophobic spiral of side chains to the α7 face of the α7 binding site. Therefore the scaffold of Ctx PnIB may be useful as a template to construct toxins selective for neuronal acetylcholine receptors. The pairwise interactions we identify provide spatial constraints to refine our picture of the α7 binding site.
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REFERENCES

1. Ackerman, E. J., Ang, E. H.-T., Kanter, J., Tsigelny, I., and Taylor, P. (1998) J. Biol. Chem. 273, 10958–10964
2. Sine, S. M., Kreienkamp, H.-J., Bren, N., Maeda, R., and Taylor, P. (1995) Neuron 15, 205–211
3. Quiram, P. A., Jones, J. J., and Sine, S. M. (1999) J. Biol. Chem. 274, 19517–19524
4. McIntosh, J. M., Santos, A. D., and Olivera, B. (1999) Annu. Rev. Biochem 68, 59–88
5. Groebe, D. R., Gray, W. R., and Abramson, S. N. (1997) Biochemistry 36, 6469–6474
6. Prince, R. J., and Sine, S. M. (1997) in The Nicotinic Acetylcholine Receptor: Current Views and Future Trends (Barrantes F. J., ed) pp. 31–59, Landes Bioscience, Austin, TX
7. Tsigelny, I., Sugiyama, N., Sine, S. M., and Taylor, P. (1997) Biophys. J. 73, 52–66
8. Quiram, P., and Sine, S. M. (1997a) J. Biol. Chem. 273, 11001–11006
9. Andrue, D., Albericio, F., Sokh, N. A., Munson, M. C., Ferrer, M., and Barany, G. (1994) in Methods in Molecular Biology: Peptide Synthesis Protocols (Pennington, M. W., and Dunn, B. M., eds) Vol. 35, pp. 139–140, Humana Press, Totowa, NJ
10. Quiram, P. A., and Sine, S. M. (1998b) J. Biol. Chem. 273, 11007–11011
11. Sine, S. M. (1993) Proc. Natl. Acad. Sci. 90, 9436–9440
12. Sine, S. M., and Taylor P. (1979) J. Biol. Chem. 254, 3315–3325
13. Hu, S. H., Gehram, J., Alewood, P. F., Craik, D. J., and Martin, J. L. (1997) Biochemistry 36, 11325–11330
14. Horowitz, A., and Persht, A. R. (1996) J. Mol. Biol. 214, 613–617
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