Molecular Determinants by Which a Long Chain Toxin from Snake Venom Interacts with the Neuronal α7-Nicotinic Acetylcholine Receptor*

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Long chain curarimimetic toxins from snake venom bind with high affinities to both muscular type nicotinic acetylcholine receptors (AChRs) (Kd in the pM range) and neuronal α7-AChRs (Kd in the nM range). To understand the molecular basis of this dual function, we submitted α-cobratoxin (α-Cbtx), a typical long chain curarimimetic toxin, to an extensive mutational analysis. By exploring 36 toxin mutants, we found that Trp-25, Asp-27, Phe-29, Arg-33, Arg-36, and Phe-65 are involved in binding to both neuronal and Torpedo (Antil, S., Servent, D., and Ménez, A. (1999) J. Biol. Chem. 274, 34851–34858) AChRs and that some of them (Trp-25, Asp-27, and Arg-33) have similar binding energy contributions for the two receptors. In contrast, Ala-28, Lys-35, and Cys-26–Cys-30 selectively bind to the α7-AChR, whereas Lys-23 and Lys-49 bind solely to the Torpedo AChR. Therefore, α-Cbtx binds to two AChR subtypes using both common and specific residues. Double mutant cycle analyses suggested that Arg-33 in α-Cbtx is close to Tyr-187 and Pro-193 in the α7 receptor. Since Arg-33 of another curarimimetic toxin is close to the homologous αTyr-190 of the muscular receptor (Ackermann, E. J., Ang, E. T. H., Kanter, J. R., Tsigelný, I., and Taylor, P. (1998) J. Biol. Chem. 273, 10958–10964), toxin binding probably occurs in homologous regions of neuronal and muscular AChRs. However, no coupling was seen between α-Cbtx Arg-33 and α7 receptor Trp-54, Leu-118, and Asp-163, in contrast to what was observed in a homologous situation involving another toxin and a muscular receptor (Osaka, H., Malany, S., Molles, B. E., Sine, S. M., and Taylor, P. (2000) J. Biol. Chem. 275, 5478–5484). Therefore, although occurring in homologous regions, the detailed modes of toxin binding to α7 and muscular receptors are likely to be different. These data offer a molecular basis for the design of toxins with predetermined specificities for various members of the AChR family.

Venoms of elapid and hydrophid snakes contain a family of small toxic proteins called curarimimetic toxins or α-neurotoxins that bind with high affinity to muscular nicotinic acetylcholine receptors (AChRs) and hence affect synaptic transmission (1, 2). All these toxins adopt a leaf-like shape with three adjacent loops rich in β-sheet that emerge from a small globular core where four disulfide bonds are invariably located (3–7). Notwithstanding their common fold and their similar biological function, α-neurotoxins are currently classified as short chain toxins with 60–62 residues and four disulfide bonds and long chain toxins with 66–74 residues and five disulfide bonds. In agreement with this old chemically based classification, we recently showed that the long chain toxins are also and uniquely capable of binding with high affinity to the neuronal α7 receptor (8). These preliminary data also indicated that the neuron-specific binding capacity may be associated with the unique presence in the long chain toxins of a small cyclic loop at the tip of their central loop. The goal of this work was therefore to identify as precisely as possible the determinants by which long chain toxins bind to the neuronal α7-AChR and to compare them with those involved when toxins bind to the muscular AChR. The toxin used in this study is α-cobratoxin (α-Cbtx) (9) from Naja naja siamensis (probably Naja kaouthia (10)). It is a prototype of long chain curarimimetic toxins with a single polypeptide chain of 71 amino acids and five disulfide bonds. α-Cbtx binds with high affinity to the muscular type AChR from Torpedo marmorata (Kd ≈ 58 pM) and the neuronal α7-AChR (Kd ≈ 9 nM). Its three-dimensional structure is known from both NMR (11) and x-ray crystallographic studies (12). We recently submitted this toxin to an extensive site-directed mutagenesis and found that the residues by which it binds to the Torpedo AChR include a number of amino acids that are highly conserved throughout the family of curarimimetic toxins (13). These are Lys-23, Trp-25, Asp-27, Phe-29, and Arg-33, which belong to the concave face of the toxin loop II, and Lys-49, which belongs to the same face of loop III. The same residues of a short chain curarimimetic toxin are involved in binding to the same AChR (14, 15). In addition, however, long and short chain curarimimetic toxins use specific residues for binding to the Torpedo AChR. These specific residues are located in the C-terminal tail and in loop I of long and short chain toxins, respectively (13).

The goal of this study was 3-fold. First, using a set of 36 toxin mutants, we identified the residues by which α-Cbtx most likely binds to the neuronal α7 receptor. Second, we compared these data with those that previously indicated the residues by which the same toxin binds to the Torpedo AChR (13). Third, to identify the regions of the α7 receptor that are recognized by the toxin, we mutated different residues in various functional loops of the α7 receptor and studied the effect of these muta-

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§The abbreviations used are: AChRs, nicotinic acetylcholine receptors; Cbtx, cobratoxin; Bgtx, bungarotoxin; HEK, human embryonic kidney; 5-HT3, 5-hydroxytryptamine type 3.
tions on toxin binding. Using a double mutant cycle approach, we then studied the possible proximity between receptor Tyr-187 and toxin Arg-33. This choice was based on previous findings that the homologous Tyr-190 of the α subunit of the muscular receptor is close to Arg-33 of another curarimimetic toxin (16). We also investigated other possible interactions between toxin Arg-33 and other receptor residues, including Trp-54, Leu-118, and Asp-163, whose homologs in the muscular receptor were observed to be in proximity to Arg-33 of another toxin (17). Together, our data strongly suggest the following. (i) Toxin binding presumably occurs in homologous regions of the muscular type AChR and the neuronal α7-AChR; however, the detailed modes of binding to both receptor subtypes are likely to be different. (ii) The same core of six residues (Trp-25, Asp-27, Phe-29, Arg-33, Arg-36, and Phe-65) is involved in the binding to both subtypes of AChRs. (iii) Three of these common residues (Phe-29, Arg-36, and Phe-65) contribute differently to the binding energy for the two AChRs. (iv) A number of additional residues are associated with the specific recognition of each receptor subtype. These are Ala-28, Lys-35, and Cys-26–Cys-30, which bind selectively to the neuronal AChR, and Lys-23 and Lys-49, which bind solely to the muscular type receptor. These results offer an explanation as to how a toxic protein recognizes two members of a receptor family and provide a molecular basis for the design of new toxins that may be more specific to these and other subtypes of the AChR family.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Characterization of Recombinant α-Cobra toxin—**Wild-type and mutant recombinant α-cobratoxins were obtained as described previously (13). Briefly, cDNA encoding Cbtx was cloned into the pCP vector (18) and expressed as a fusion protein in E. coli strain BL21(DE3). The fused toxin was purified on an IgG-Sepharose column, cleaved by CNBr, refolded with 4 mM GSH and 2 mM GSSG, and purified on a reverse-phase C$_25$ column. The α-Cbtx mutants described here were prepared using the QuickChange™ kit (Stratagene), and the sequence of the entire gene was checked by automatic sequencing (ABI PRISM™ 310 genetic analyses, Perkin-Elmer Applied Biosystems). Biochemical and biophysical characterization of each mutant was assessed by (i) SDS-polyacrylamide gel electrophoresis with silver staining; (ii) analytical reverse-phase high performance liquid chromatography (Vydac C$_5$, 5-μm column, 0.46 × 25 cm), which provides mass spectrometry and circular dichroic analysis as described previously (13). The concentration of purified Cbtx was determined by measuring the absorbance at 278 nm of a given solution of toxin and by amino acid analysis.

**Expression of Wild-type and Mutant α7 Receptors in Human Embryonic Kidney (HEK)-293 Cells—**A chimeric cDNA of a neuronal type nicotinic receptor (α7/5-HT$_3$) was transfected into HEK-293 cells by calcium precipitation as described previously (8, 19, 20). Two days after the transfection, the cells were harvested in phosphate-buffered saline with 5 mM EDTA, washed two times with phosphate-buffered saline, and finally resuspended in 3 ml of this buffer/plate for the binding experiments. The receptor mutants were obtained using the QuickChange™ kit according to the instructions of the manufacturer, and their sequences were checked by sequencing.

**Binding Assays—**The affinities of the toxins for the α7/5-HT$_3$ receptor were determined as described previously (8, 21) with slight modifications. Competition experiments showed the effect of wild-type and mutant recombinant α-cobratoxins on the initial rate of $^{125}$I-Bgtx binding. Different concentrations of α-Cbtx were preincubated for 4.5 h with cells suspended in phosphate-buffered saline and filtered 6 min after the addition of 5 nM $^{125}$I-Bgtx. This preincubation time was checked to be sufficient to reach equilibrium binding between wild-type or mutant toxins and the receptor (no change after overnight preincubation). Furthermore, the association and dissociation kinetics of $^{125}$I-Bgtx for the Y187F receptor mutant were determined and showed that no significant dissociation of the tracer occurred during the 6-min time of the assay and that, after this time, the rate of association of $^{125}$I-Bgtx was still linear. The protection constant ($K_P$) calculated by fitting the competition data to the empirical Hill equation was shown to correspond to the dissociation constants ($K_D$, 20, 22).

**RESULTS**

**Preparation and Characterization of Wild-type and Mutant α-Cobratoxins—**Recombinant α-Cbtx was produced as a fusion protein in E. coli as described previously (13). The final yield of toxin after purification, cleavage, and refolding reached ~1.5 mg/liter of culture.

To identify the residues by which α-Cbtx binds with high affinity to the neuronal α7 receptor, we produced 36 mutants and hence probed the role of 34 of its 71 residues. The explored positions included all the residues of the toxin loop I, from Phe-4 to Asp-13. They also included all residues (Tyr-21, Lys-23, Trp-25, Arg-36, and Asp-38) of the concave face of loop II, the two faces being defined by the large β-sheet that encompasses the three toxin loops. We also explored Lys-35 in loop II, which points toward the other direction, and all residues at the tip of loop II, from Asp-27 to Arg-33, including the disulfide bond Cys-26–Cys-30. Three residues of loop III (Thr-47, Lys-49, and Asp-53) and two residues of the C-terminal tail (Phe-65 and Pro-66) were also investigated. Finally, the role of the C-terminal fragment was further explored by deleting the five last residues (mutant Δ66). The final yield of toxin after purification, cleavage, and refolding varied between 0.5 and 1.5 mg/liter of culture. The far-UV CD spectra of all mutants were virtually superimposable with that of the venom-purified or wild-type recombinant α-Cbtx (data not shown). Also, the mass of each mutant, as determined by electrospray mass analyses, was identical, within experimental error, to the theoretical calculated mass (data not shown). Therefore, the wild-type and mutant recombinant toxins displayed all the expected physicochemical characteristics.

**Affinities of Wild-type α-Cbtx for the Neuronal α7 Receptor—**We investigated the capacity of wild-type and mutant toxins to inhibit the binding of $^{125}$I-Bgtx to a recombinant form of a chimeric version of the chicken α7 receptor. In this version, the extracellular part of the neuronal α7-type receptor was fused to the membrane and cytoplasmic regions of the 5-HT$_3$ receptor (19). This chimeric construction allowed efficient expression in HEK-293 cells and behaved like the wild-type α7 receptor in many respects (19), including its capacity to be selectively blocked by various long chain curarimimetic toxins (8).

A number of typical inhibition binding curves obtained with different α-Cbtx mutants are shown in Fig. 1. Binding affinities...
Table I

Dissociation constants of wild-type and mutant α-cobratoxins for the α7/HT2 α7 receptor

|         | Kp  | Kp/Kc | ΔG    |
|---------|-----|-------|-------|
| Venom Cbtx | 7.5 ± 1.5 | 0.83 | -0.11 |
| Cbtx rec  | 9.0 ± 3  | 1   | 0.00  |
| Loop I   |      |       |       |
| F44     | 19.4 ± 0.4 | 2.1 | 0.43  |
| I5A     | 20.1 ± 2  | 2.2 | 0.46  |
| T6A     | 17.4 ± 3  | 1.9 | 0.38  |
| P7A     | 9.0 ± 1.0 | 1   | 0.00  |
| D8A     | 11.5 ± 0.5 | 1.3 | 0.17  |
| D8R     | 12.2 ± 1.8 | 1.4 | 0.19  |
| I9A     | 18.1 ± 2  | 2   | 0.41  |
| T10A    | 9.5 ± 0.5 | 1.1 | 0.05  |
| S11A    | 14.2 ± 1.1 | 1.6 | 0.27  |
| K12E    | 23.2 ± 9  | 2.5 | 0.53  |
| D13A    | 8.3 ± 0.3 | 0.9 | -0.08 |
| Loop II  |      |       |       |
| Y21F    | 12.5 ± 1.1 | 1.4 | 0.19  |
| K23E    | 24.5 ± 2.5 | 2.7 | 0.57  |
| W25F    | 9.1 ± 1.0 | 1   | 0.00  |
| W25H    | 36 ± 4    | 4   | 0.81  |
| W25A    | 53.5 ± 7  | 6   | 1.1   |
| D27N    | 27.2 ± 2.5 | 3   | 0.64  |
| D27R    | 450 ± 85  | 50  | 2.28  |
| A28G    | 48 ± 7    | 5.4 | 1     |
| A28R    | 9.5 ± 2   | 1.1 | 0.05  |
| F29L    | 24.5 ± 4.5 | 2.7 | 0.57  |
| F29W    | 6.1 ± 0.2 | 0.7 | -0.21 |
| F29A    | 667 ± 128 | 74  | 2.51  |
| C28S/C30S | 125 ± 27 | 13.9 | 1.54 |
| S3A     | 16 ± 6    | 1.8 | 0.34  |
| I32A    | 18 ± 2    | 2   | 0.40  |
| R33E    | 3055 ± 400 | 339 | 3.40  |
| K35A    | 99 ± 6    | 11  | 1.40  |
| R36A    | 145 ± 15  | 16  | 1.62  |
| D381    | 41.5 ± 0.3 | 0.45 | -0.46 |
| Loop III |      |       |       |
| T47A    | 9.5 ± 0.6 | 1.1 | 0.05  |
| K49E    | 29 ± 3    | 3.2 | 0.68  |
| D53S    | 7.2 ± 0.4 | 0.8 | -0.13 |
| C-terminal |     |       |       |
| F65A    | 139 ± 26  | 15.5 | 1.60  |
| P66A    | 24 ± 1    | 2.7 | 0.57  |
| Δ66     | 30 ± 4    | 3.3 | 0.70  |

Protection constants (means ± S.E.) were obtained from at least three different competition experiments in duplicate as described under "Experimental Procedures." ΔG is the difference in free energy of binding between wild-type and mutant α-cobratoxins. ΔG = ΔGWT − ΔGmut = RT ln(Kp/Kc), with R = 1.99 cal/mol/K and T = 293 K. The residues for which mutations caused an affinity decrease >5-fold (ΔG > 1 kcal/mol) are indicated in boldface. Cbtx rec, recombinant wild-type toxin.

Binding Affinities of Toxin Mutants—All residues of loop I were mutated, but none of the introduced mutations caused more than a 3-fold affinity decrease, which corresponds to a change in free energy of binding of 0.5 kcal/mol. Clearly, none of the residues of loop I individually plays a key binding role with the neuronal α7 receptor (Table I).

In contrast, as many as nine residues from loop II (Trp-25, Asp-27, Ala-28, Phe-29, Cys-26-Cys-30, Arg-33, Lys-35, and Arg-36) were sensitive to mutations, with affinity decreases ranging from 5- to 340-fold (Fig. 1 and Table I). Six of these residues (Trp-25, Asp-27, Ala-28, Phe-29, Arg-33, and Arg-36) have their side chain essentially accessible from the concave face of loop II, this face being defined by the large β-sheet displayed by the three fingers (see Fig. 4). Mutation R33E caused the greatest effect, with a decrease of 340-fold in the α-Cbtx binding affinity (Table I). Arg-33 may therefore be the toxin residue that contributes the most to the α-Cbtx-receptor recognition process. Although perhaps less critical, the other arginine of the loop II (Arg-36) may also be functionally important since its mutation to alanine caused a 16-fold affinity decrease. Mutation of Trp-25 to alanine caused a weak affinity decrease, suggesting the moderate functional contribution of the indole ring. However, replacement of Trp-25 by phenylalanine or histidine had virtually no effect on toxin affinity, indicating that an aromatic side chain of variable size can be accommodated at this position (Table I). A 50-fold decrease in binding affinity was observed in mutation D27R, indicating that the presence of the bulky and positively charged arginine is unfavorable at this position. This does not mean that the presence of a negative charge at position 27 is an important element since mutation D27N caused no significant effect. More mutants are needed to understand better how Asp-27 contributes to binding. Mutation of Ala-28 to glycine caused a 5-fold affinity decrease, suggesting that a methyl group at this position may be involved in binding. Surprisingly, however, mutation of Ala-28 to arginine did not affect toxin affinity. This result suggests that position 28 can accommodate any residue whose side chain possesses some hydrophobic character. Of the three mutations introduced at Phe-29, only F29A caused an affinity decrease. This decrease was quite severe since it was associated with a binding energy change of 2.5 kcal/mol (Table I). In contrast, F29W had no effect, indicating that position 29 can accept the bulkier aromatic indole ring. It can also accommodate the hydrophobic, although not aromatic, leucine residue since mutant F29L has only a 3-fold lower affinity. Therefore, a strong hydrophobicity may be an important functional character at position 29, irrespective of whether it is brought by an aromatic or aliphatic side chain.

The other mutation-sensitive residues in loop II (Cys-26-Cys-30 and Lys-35) have their side chain accessible from the convex face of the β-sheet. This result could indicate that mutation at any residue of the toxin loop II is followed by an affinity decrease and that our general approach may not reflect the actual binding contribution of the mutated residues. This is clearly not the case since various residues of loop II could be mutated without affecting toxin affinity. This is the case for the relatively close Asp-38, Ser-31, and Ile-32 and for the more remote Tyr-21. Therefore, the effect observed with mutations at Cys-26-Cys-30 and Lys-35 most likely indicates the involvement of these residues in binding to the receptor. The result obtained upon mutation of the two half-cystines Cys-26 and Cys-30 agrees with previous data based on reduction and chemical modification of this bond (8). However, the affinity decrease observed upon mutation is substantially less than that found upon chemical modification. That the double mutation C26S/C30S caused an affinity decrease can be interpreted in at least two nonexclusive ways. First, the deletion of the bond between residues 26 and 30 modifies the local structural constraints of the small loop, which in turn may affect the spatial positioning and hence the functional contribution of one or more of the important residues (see above) Asp-27, Ala-28, and Phe-29. However, we previously observed that mutation C26S/C30S (13) or reduction and carboxymethylation of the bond (8) caused virtually no effect on toxin binding to the Torpedo.
AChR, although this binding involved various residues in proximity to the disulfide bond, including Trp-25, Asp-27, and Phe-29. Therefore, we suspect that no major structural change occurred around the disulfide bond between residues 26 and 30. Second, the other explanation is that one or both of half-cystines 26 and 30 are involved in the binding to the neuronal receptor. This conclusion implies that the convex face of loop II may be functionally important. In agreement with this view, we found that Lys-35, whose side chain is accessible from the convex face of the toxin, is also functionally important, as judged from the 11-fold affinity decrease associated with its mutation to alanine. Therefore and in contrast to what was found upon binding to muscular type AChRs (13–15, 23, 24), binding to the neuronal AChRs seems to involve residues located on both the concave and convex faces of the curarimimetic toxin.

Previous mutational analyses indicated the functional importance of the highly conserved Lys-23 or Lys-49 in binding to muscular receptors (13). It was not inconceivable that these residues could also be involved in binding to the neuronal receptor. However, this is not the case since inversion of their charge caused no effect on toxin affinity. Also, the two other residues (Thr-47 and Asp-53) of loop III seem to be excluded from the binding surface of the toxin, with mutations T47A and D53K causing no affinity decrease. The hydroxyl function of the invariant Tyr-21 is also unimportant since mutation Y21F had no effect on toxin affinity (Table I). The competition binding curves obtained with the different toxin mutants described above indicated that both mutations are not equal to the sum of the coupling energy of the two mutated residues and was calculated by the equation

\[
\Delta G = -RT \ln \left( \frac{K_{\text{mut}}}{K_{\text{wt}}} \right)
\]

with \(K_{\text{wt}}\) and \(K_{\text{mut}}\) being the dissociation constants of the wild-type and mutant toxins, respectively. The coupling energy represents the interaction of the two mutated residues and was calculated by the equation:

\[
\Delta G = -RT \ln \Omega,
\]

where \(\Omega = K_{\text{wt}}K_{\text{mut}}/K_{\text{mut},\text{wt}}\), and values less than unity were interpreted as indicating some interaction between the two residues. The coupling coefficient \(\Omega\) was calculated from the following equation:

\[
\ln \Omega = \ln \frac{K_{\text{wt}}K_{\text{mut}}}{K_{\text{mut},\text{wt}}} = \ln K_{\text{wt}} + \ln K_{\text{mut}} - \ln K_{\text{mut},\text{wt}}.
\]

We therefore explored the possibility that the homologous region in the neuronal a7 receptor could also interact with a-Cbtx. Toward this goal, we decided to mutate five receptor residues (Tyr-187, Pro-193, Trp-54, Leu-118, and Asp-163) that are in the putative homologous loops of the a7 receptor. Table II and Fig. 2 show that mutations Y187F and D163K caused 21- and 6-fold decreases in the affinity for a-Cbtx, respectively, suggesting that the corresponding residues are involved in toxin binding. In contrast, P193A, W54F, and L118A caused little, if any, affinity change, suggesting that they may not contribute to toxin binding. It could be argued that the observed affinity decreases could result from both a genuine affinity decrease combined with a dissociation effect of 125I-Bgtx during the time of the binding assay (6 min). Using mutant Y187F, we checked that although the dissociation rate of the toxin was significantly affected by the mutation, it did not alter the proportion of bound radioactive toxin during the 6 min of the assay (data not shown). Therefore, our data suggest the importance of the hydroxyl side chain of Tyr-187 in the toxin interaction.

We then performed a double mutant cycle analysis (16, 26, 27) using toxin mutants R33E and F65A and the different a7 receptor mutants previously mentioned. In the resulting thermodynamic analysis, the difference in energy following a mutation was calculated from the following equation:

\[
\Delta G = -RT \ln \Omega,
\]

where \(\Omega = K_{\text{wt}}K_{\text{mut}}/K_{\text{mut},\text{wt}}\), and values less than unity were interpreted as indicating some interaction between the two residues. The competition binding curves obtained with the different

**Table II**

*Binding and coupling energies of wild-type and mutant toxins or the a7 receptor.*

| Mutations     | \(K_d\) | \(K_{\text{int}}(K_{\text{mut}})\) | \(\Delta G\) | \(\Omega\) | \(\Delta G_{\text{int}}\) | \(\text{mut} / \text{wt}\) |
|---------------|-------|-----------------------------|-------------|--------|-----------------|------------------|
| a1 receptor   |       |                             |             |        |                 |                  |
| WT            | 9 ± 3 | 1                          | 3.40        | -3.36  | -0.71           |                  |
| R33E          | 3055 ± 180 | 339                    | 2.70        | 1.78   |                 |                  |
| WT            | 185 ± 17 | 21                    | 1.80        | 1.06   | 0.03            |                  |
| R33E          | 18,680 ± 2030 | 2075               | 4.45        | -3.36  | -0.71           |                  |
| WT            | 1817T/1/R/K | NB                  | 0.15        | 0.11   | 0.03            |                  |
| F65A          | 139 ± 25 | 15.5                   | 1.80        | 1.04   | -0.11           |                  |
| WT            | 3020 ± 380 | 335                  | 3.39        | -4.38  | -0.86           |                  |
| WT            | 19 ± 5 | 2                        | 0.41        | 0.71   |                 |                  |
| R33E          | 1473 ± 250 | 163                  | 2.97        | -0.11  | 0.03            |                  |
| WT            | 54 ± 14 | 6                      | 1.04        | 0.11   |                 |                  |
| R33E          | 15,270 ± 1800 | 1686               | 4.33        | -1.20  | -0.11           |                  |
| WT            | 24 ± 8 | 2.7                    | 0.57        | -0.15  |                 |                  |
| R33E          | 6250 ± 590 | 694                  | 3.76        | -1.30  | -0.15           |                  |
| WT            | 39 ± 19 | 4.4                    | 0.85        | -1.30  | -0.15           |                  |
| R33E          | 10,220 ± 220 | 1136               | 4.10        | -1.30  | -0.15           |                  |
only two coupling values were above the threshold. These are those corresponding to the double mutants Y187F/R33E and P193A/R33E, whose coupling values are equal to -0.71 and -0.86 kcal/mol, respectively. The three other mutant pairs involving R33E in the toxin were associated with much smaller ΔG_int values (ranging from 0.11 to 0.15 kcal/mol), also like the double mutant Y187FF65A, which was characterized by a ΔG_int of 0.03 kcal/mol. Therefore, we suggest that Tyr-187 and Pro-193 in the α7 receptor are in proximity to Arg-33 in α-Cbtx. Sometimes, coupling values may increase when different types of mutations are introduced at a receptor residue position (16, 17, 29). For example, coupling energies of 0.6 and 1.7 kcal/mol were observed between Y190F/R33E and Y190T/R33E, respectively, at the αγ interface of the muscular α subunit (16). Therefore, we explored a possible mutation-type effect using various mutations at receptor position 187. However, no detectable α-Bgtx binding was observed with the different mutants obtained (Y187T, Y187A, Y187R, and Y187K) (Table II), suggesting that the presence of an aromatic residue at this position is crucial either for toxin binding or for correct receptor expression. Finally, no coupling was seen between toxin Arg-33 and α7 receptor Thr-54, Leu-118, and Asp-163, suggesting, at least from these preliminary results, that there is no interaction between Arg-33 and these receptor residues. Similarly, Tyr-187 on the receptor does not seem to interact with Phe-65 on the toxin.

**DISCUSSION**

Previous studies demonstrated that a long chain toxin can bind with high affinity to both the muscular type AChR and the neuronal α7-AChR (8). In this work, we have used a mutational approach to search for residues that are neuron-specific. It is always difficult to establish a cutoff value above which a decrease in binding affinity, as caused by a mutation, reflects involvement of the mutated residue in a binding process. However, from various comparative studies of structural and mutational analyses of protein-protein complexes, it was suggested that a mutation causing a variation in binding energy of >1 kcal/mol may reflect such an involvement (30–32).

**The Site by Which α-Cbtx Binds to the Neuronal α7 Receptor**—Ten residues of α-Cbtx (Trp-25, Cys-26–Cys-30, Asp-27, Ala-28, Phe-29, Arg-33, Lys-35, Arg-36, and Phe-65) are mutation-sensitive and may therefore constitute the surface by which the toxin interacts with the neuronal α7 receptor. This delineation is further supported by the observation that numerous surrounding residues in loop I (from Phe-4 to Asp-13), at the base of loop II (Tyr-21, Lys-23, and Asp-38), and in loop III (Thr-47, Lys-49, and Asp-53) are mutation-insensitive and therefore probably excluded from the binding surface (see Fig. 4B and Table I). With the exception of Phe-65 in the C-terminal tail, all the functionally important residues of the toxin belong to the toxin loop II. Six of these residues (Trp-25, Asp-27, Ala-28, Phe-29, Arg-33, and Arg-36) have their side chain oriented toward the concave face of the large toxin β-sheet, whereas the side chains of Lys-35 and Cys-26–Cys-30 are accessible from the other face. This organization gives the impression that the toxin loop II interacts with receptor residues that form a sort of groove. The additional Phe-65 is located relatively far from the binding region displayed by the central finger and hence may further stabilize the toxin-receptor complex. It is also noticeable that the receptor-binding site includes a cluster of three positively charged residues, Arg-33, Lys-35, and Arg-36, with Arg-33 probably playing the most critical binding role. However, we must note that Arg-33 was mutated to Glu, whereas the two other residues were mutated to alanine, and we cannot exclude the possibility that the charge inversion might have amplified an unfavorable effect on toxin interactions observable in the structure of the complex and occurring between charged and uncharged residues separated by 4–7 Å are characterized by coupling energies ranging between 0.35 and 1.2 kcal/mol. We therefore retained the value of 0.35 kcal/mol as the threshold above which an interaction may occur.
binding. In any case, the positively charged cluster is surrounded by numerous hydrophobic side chains, including three aromatic residues (Trp-25, Phe-29, and Phe-65) and, to a lesser extent, the aliphatic residue Ala-28. Only one negatively charged residue (Asp-27) seems to be important, although its negative character is not functionally determinant since mutation D27N had no effect on the stability of the toxin-receptor complex. Therefore, the determinant that ensures toxin binding to the α7 receptor is compactly located on the toxin central loop, except for Phe-65, with three positively charges surrounded by a hydrophobic ring.

Molecular Basis for the Dual Selectivity of α-Cbtx—A major goal of this study was to understand how α-Cbtx can recognize two subsets of AChRs. To this purpose, we compared the data reported in this paper with those describing the residues through which α-Cbtx also binds to a muscular type (Torpedo) AChR (13). The comparative data are compiled in Fig. 3 and show the effects of the same mutations on the differential binding energy for both neuronal and Torpedo AChRs. Although this comparison should be considered with caution due to the different competition methods used with both receptors, a qualitative comparison revealed two groups of interacting residues: first, residues for which at least one mutation affects toxin binding affinity for both types of AChRs, and second, those for which a mutation affects binding affinity for only one type of receptor. In this comparison, we have considered the mutational effects on one or both of the two physically different toxin-binding sites that are present in the Torpedo receptor. For sake of clarity, a summary of the data from Fig. 3 is shown in Fig. 4, with all excluded residues displayed in green and those that are important for binding to one and/or both receptors in yellow, orange, and red.

The site by which the toxin recognizes the Torpedo AChR covers a surface of ~900 Å² that crosses the concave face of the toxin on loops II and III and part of the C-terminal tail. The neuronal functional site covers a similar surface of 800 Å² spread on both the concave and convex faces of loop II and the C-terminal tail. The two binding sites therefore display similarities and marked differences.

Six toxin residues were mutation-sensitive to both receptors and caused a differential binding energy of at least 1 kcal/mol. These are Trp-25, Asp-27, Arg-33, Arg-36, and Phe-65, which can be divided into two groups. First, mutations at Trp-25, Asp-27, and Arg-33 comparably affect the binding to both receptors (Fig. 3). Thus, mutation R33E always caused the largest effect, with the binding energy differences being ~3.5 kcal/mol for both receptors. Asp-27 may also play a major binding role in both cases since a change in binding energy of ~2 kcal/mol was observed for both receptors in mutation D27R. In both cases, however, the negative charge is not the important binding factor since mutation D27N did not affect binding to either receptor. Although its importance is more moderate, Trp-25 may also play a comparable role in binding to either receptor. The three mutations introduced at this position suggest that the presence of an aromatic side chain at this position could be an important binding element. Therefore, the mutation data suggest that Arg-33, Asp-27, and Trp-25 may play similar binding functions for the Torpedo and neuronal AChRs and hence might recognize similar determinants on both receptors.

Mutations at Phe-29, Arg-36, and Phe-65 also caused affinity decreases for both receptors, but the observed effects were different in terms of binding energy. Mutation F29A caused a large affinity decrease for the neuronal receptor only, whereas F29L caused a strong effect regarding the Torpedo receptor. Also, mutations R36A and F65A caused much larger effects on toxin binding to the neuronal receptor. Therefore, if these three residues are clearly important for the toxin to bind to both receptors, their differential binding contribution suggests that they recognize either distinct receptor determinants or the same determinants, but differently.

Further indicating that the toxin-binding sites are not identical in the two receptors, we found a number of residues whose mutations affect only one of the two AChRs. Thus, Ala-28, Cys-26–Cys-30, and Lys-35 are selectively involved in binding to the α7 receptor, even if the mutations introduced at these positions induce only moderate affinity decreases. We note that Lys-35 and the disulfide bond Cys-26–Cys-30 are on the convex face of the toxin, which is not considered to be important for curarimimetic toxins to bind to the muscular AChR (13–15). These residues may therefore interact with determinants that are present in the α7-AChR, but not in the Torpedo AChR. Interestingly enough, the homologous fifth disulfide bond of the neuronal κ-neurotoxins is also involved in the binding to the neuronal α3β2-AChR (33), suggesting that neuronal AChRs might possess a common determinant recognized by the fifth disulfide bond of long chain three-fingered toxins.

Two mutation-sensitive positions are clearly Torpedo AChR-specific. These are Lys-23 and Lys-49. More precisely, mutations at these positions affect the low affinity binding site of the muscular receptor, but not its high affinity binding site. Therefore, the two highly conserved Lys-23 and Lys-49 residues seem to be important for the curarimimetic toxins to recognize specific determinants at the low affinity binding site of the muscular receptor, perhaps at the interface of the αγ subunits (16, 23).

In summary, α-Cbtx commonly “uses” Trp-25, Asp-27, Phe-29, Arg-33, Arg-36, and Phe-65 to bind to both the neuronal α7 and muscular type AChRs, with some of them (Phe-29, Arg-36, and Phe-65) contributing differentially to the binding to both receptors. The common functional residues are assisted by Ala-28, Cys-26–Cys-30, and Lys-35, which may bind to α7-specific AChR determinants, and by Lys-23 and Lys-49, which may recognize muscular-specific AChR determinants. Thus, this work unambiguously reveals that a toxin recognizes two subtypes of AChRs using a common binding core assisted by additional residues that bind to determinants that are likely to be specific to each receptor subtype. This is a scenario that may be general for most, if not all, toxin-receptor interactions, in-
including those from sea anemones (34) and scorpions (31, 35) that bind to various subtypes of potassium channels.

Whereas both long and short chain curarimimetic toxins bind with high affinities to muscular type AChRs (2), only the long chain toxins also bind with high affinity to the neuronal α7 receptor (8). The data reported in this paper may explain the origin of this differential behavior: α-Cbtx and most long chain curarimimetic toxins, but not short chain toxins (2), possess residues that may bind to α7-specific AChR determinants, including Ala-28, the disulfide bond between residues 26 and 30, and Lys-35.

**Toxin-binding Sites Include Homologous Regions of the Muscular AChR and the α7-AChR**—Since Trp-25, Asp-27, and Arg-33 are comparably important for α-Cbtx and to bind to both neuronal and muscular AChRs, we wondered whether these residues recognize homologous determinants in both receptors. To explore this possibility, we investigated whether at least one of these residues interacts with a homologous residue in both receptors. In this respect, Arg-33 in the toxin was particularly appealing for two reasons. First, its mutation to Glu causes the largest α-Cbtx affinity decrease for both the Torpedo (13) and neuronal (this work) receptors, suggesting that this residue is most crucial for binding to both receptors. Second, Arg-33 of another curarimimetic toxin was shown to interact with Val-188 and Tyr-190 located on the α subunit of the mouse muscular AChR (16). Using double mutant cycle methodology, we found a coupling energy of ~0.71 kcal/mol for the mutant pair R33E/Y187F. This value is clearly above the numerous background values that probably reflect the absence of coupling (0.15 kcal/mol). Also, the value of ~0.71 kcal/mol fits nicely within the range (0.35–1.2 kcal/mol) previously identified to characterize interactions occurring between charged and uncharged residues separated by 4–7 Å (26). Although we are unable to estimate the distance separating Arg-33 in α-Cbtx and Tyr-187 in the α7 receptor, our data suggest that they are close to each other. Since a similar situation was observed between Arg-33 of another curarimimetic toxin and the homologous Tyr-190 of a muscular type receptor (16), it is likely that toxin binding to neuronal and muscular receptors takes place in homologous regions.

To better define the region of the neuronal receptor that is recognized by α-Cbtx, we proceeded to further double mutant cycle analyses. The mutant pair P193A/R33E was characterized by a coupling energy of ~0.86 kcal/mol, which also suggests a proximity between Arg-33 in the toxin and Pro-193 in the receptor. The low coupling values (0.15 kcal/mol) observed for all other mutant pairs suggest that Arg-33 is not close to Trp-54, Leu-118, and Asp-163, in contrast to what has been observed in a homologous situation between another curarimimetic toxin and the mouse muscular AChR (17). Therefore, although occurring in homologous regions of neuronal and muscular receptors, toxin binding does not involve identical determinants. Finally, the low coupling value (ΔΔGΔ = 0.03 kcal/mol) that characterized the pair Y187F/F65A also suggests no proximity between Tyr-187 and Phe-65, a finding that is in agreement with the observation that in α-Cbtx, Arg-33 and Phe-65 are separated by 15 Å, making it unlikely that both residues could interact simultaneously with Tyr-187.

In conclusion, this study shows that a long chain toxin from snake venom binds to homologous, although not identical, regions of two AChR subtypes using a common core of amino acids assisted by a number of additional subtype-specific residues. We are now currently investigating how these data can be exploited to confer new specificity profiles to the toxin.

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