Only Snake Curaremimetic Toxins with a Fifth Disulfide Bond Have High Affinity for the Neuronal α7 Nicotinic Receptor*

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Long chain and short chain curaremimetic toxins from snakes possess 66–74 residues with five disulfide bonds and 60–62 residues with four disulfide bonds, respectively. Despite their structural differences all of these toxins bind with high affinity to the peripheral nicotinic acetylcholine receptors (AChR). Binding experiments have now revealed that long chain toxins only, like the neuronal α-bungarotoxin, have a high affinity for a chimeric form of the neuronal α7 receptor, with Kd values ranging from about 1 to 12 nM. In contrast, all other toxins bind to the chimeric α7 receptor with a low affinity, with Kd values ranging between 3 and 22 μM. These results are supported by electrophysiological recordings on both the wild-type and chimeric α7 receptors. Amino acid sequence analyses have suggested that high affinities for the neuronal receptor are associated with the presence of the fifth disulfide at the tip of the toxin second loop. In agreement with this conclusion, we show that a long chain toxin whose fifth disulfide is di-sulfiyldiated has a low affinity (Kd = 12 μM) for the neuronal α7 receptor, whereas it retains a high affinity (Kd = 0.35 nM) for the peripheral AChR. Thus, a long chain curaremimetic toxin having a reduced fifth disulfide bond behaves like a short chain toxin toward both the peripheral and neuronal AChR. Therefore, functional classification of toxins that bind to AChRs should probably be done by considering their activities on both peripheral and neuronal receptors.

Animal venoms produce a wide diversity of toxins that bind to a variety of different receptors, ion channels, and enzymes. Thus, they block some physiological pathways of prey and/or predators that, as a result, are usually rapidly immobilized and often killed. In addition, however, animal toxins are capable of exerting more subtle activities. For example, mamba venoms contain an unexpectedly large number of muscarinic toxins that exert a spectrum of fine activities toward a diversity of muscarinic receptor subtypes, acting on them as agonists or antagonists (1). Certainly, identification of subtle pharmacological activities constitutes a real challenge in the domain of toxin discovery.

In this respect, the well known snake curaremimetic toxins and their current classification as long chain and short chain toxins constitute a most puzzling situation. These monomeric toxins possess 66–74 residues with five disulfide bonds and 60–62 residues with four disulfide bonds, respectively.-form known prototypes of long chain and short chain toxins are, respectively, α-bungarotoxin (Bgtx) from venom of the krait Bungarus multicinctus (3) and toxin α from the African cobra Naja nigricollis (4–6). The amino acid sequences of long chain and short chain toxins can be aligned readily, using four invariant half-cystines (2). Not surprisingly, therefore, x-ray crystallographic studies and NMR analyses (7–12) have demonstrated that the polypeptide chains of both types of toxins adopt the same overall fold, which consists of three adjacent loops, rich in β-pleated sheet, which are connected to a globular fold where the four invariant disulfide bonds are located. However, a closer inspection of both amino acid sequences (see Table I) and spatial structures (2) reveals that toxins from both categories display quite precise structural differences. Thus, virtually all long chain toxins have a longer COOH-terminal tail, an additional small loop cyclized by the fifth disulfide at the tip of the central loop, and a slightly shorter first loop. Notwithstanding these clear structural differences, toxins from both categories bind to similar sites and with comparable high affinities on peripheral nicotinic acetylcholine receptors, such as AChR from Torpedo marmorata (13). It has been claimed that long chain toxins may bind more irreversibly than short chain toxins to AChR (14, 15). Therefore, one may wonder why a large number of Elapidae and Hydrophiidae produce simultaneously two categories of toxins (2) which apparently exert the same function.

In the present study, binding experiments have revealed that long chain and short chain toxins present marked biological differences. Thus, long chain toxins recognize a chimeric construction of the neuronal α7 nicotinic acetylcholine receptor with an affinity that is nearly 4 orders of magnitude higher than that of short chain toxins. Electrophysiological experiments performed with wild-type (WT) and chimeric (α7-V201-5HT3) α7 receptors support these observations. Finally, we demonstrate that this discriminating property is associated with a specific structural feature of long chain toxins, i.e. the presence of an additional cyclized loop at the tip of their central loop. Our findings suggest that toxins should be regarded with more scrutiny concerning the expression of their possible biological activities.

EXPERIMENTAL PROCEDURES

Materials—The cDNA of the chimeric α7-V201-5HT3 receptor was kindly provided by Prof. J. P. Changeux (Institut Pasteur, Paris).

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† The abbreviations used are: Bgtx, bungarotoxin; AChR, acetylcholine receptor; Cbtx, cobratoxin; Eb and Ea, erabutoxin a and b, respectively; HEK, human embryonal kidney; PBS, phosphate-buffered saline.
Neuronal Receptor Discriminates Short from Long Toxins

α-Bgtx, β-conotoxins MI and SI, Naja naja oaxiana I, t-tubocurarine, and (–)-nicotine were purchased from Sigma. Laticauda semifasciata III and methyllycaconitine were obtained from Latoxan (Rosans, France). α-Bgtx was obtained from Biotopo Incorporated (St. Cloud, FL). Cobratoxin (Cbtx) from Naja siamensis, α-toxin from N. nigricollis, Laticauda semifasciata toxin, and erabutoxin a and b (EA and Eb) from L. semifasciata were purified from their respective venoms in our laboratory according to a procedure not yet published. α-Conotoxin GI was synthesized as described previously (16). 125I-Bgtx (210–250 Ci/mmol) was purchased from Amersham. 2,2'-Dithiopyridine was purchased from Aldrich.

α-Conotoxin in Human Embryonal Kidney (HEK) Cells—Chimeric cDNA (α7-5HTT) was transfected into HEK 293 cells (ATCC CRL 1573) by calcium precipitation with a careful control of the pH (6.95), CO2 level (3%), and cDNA amount (15 μg) as described previously (17). Each mutant was submitted to amino acid analysis and sequencing.

Selective Reduction and Alkylation of Cbtx—1.2 mM Cbtx was incubated with 2.5 mM dithiothreitol in 0.2 mM Tris buffer, pH 8.5, 1 mg/ml EDTA for 90 min at 4 °C. After this selective reduction, 15 mM 2,2'-dithiopyridine was added in methanol for 1 h at room temperature. After purification by gel filtration (Bio-Gel P-2) and high pressure liquid chromatography (Vydac C8) the Cbtx-dithiopyridine obtained was analyzed by mass spectrometry.

Binding Assays—The affinity of 125I-Bgtx was tested by incubating the labeled toxin (final concentration, 5 nM) with 350 μl of cells in suspension in PBS. The nonspecific binding was determined in the presence of 1 mM (–)-nicotine. After a 40-min incubation, the sample was diluted in 3 ml of cold PBS, filtered through a GF/C filter (Whatman) previously dipped in non-fat dry milk (1%), and rinsed with 3 ml of cold PBS. The radioactivity of the filter was determined on a Rackbeta counter.

RESULTS

Production and Characterization of the Chimeric α7 AChR—The extracellular domain of the α7 nicotinic AChR has been fused previously to the membrane region of the 5HTT receptor (α7-5HTT) (18) displaying thus all of the pharmacological properties of the natural α7 receptor and allowing an efficient expression in HEK 293 cells, which failed in the case of the entire receptor (18, 23). In agreement with previous results obtained from pharmacological or electrophysiological experiments with the entire α7 receptor (24, 25), we found that 125I-Bgtx binds to the chimeric receptors with an equilibrium dissociation constant (Kd) equal to 0.96 ± 0.12 nM, as deduced from both the ratio of the dissociation and association rate constants and competition experiments (data not shown). The yield of receptor production was approximately 0.3 pmol of 125I-Bgtx binding site/106 cells. Methyllycaconitine, a well known α7-specific ligand (26, 27), completely inhibited the binding of radioactive α-Bgtx with a binding affinity characterized by a Kd value of 1 nM, as deduced from the binding inhibition curve (data not shown). This value is in agreement with those reported in previous studies describing the interaction of methyllycaconitine with entire α7 receptors produced in other types of cells (26–28).

Binding of Snake Toxins to Chimeric α7 and Torpedo AChRs—The family of curaremimetic snake toxins includes a large number of toxin members (2). However, only α-Bgtx has been shown to bind with high affinity to the α7 receptor (29). We now investigated the capacity of other snake toxins to inhibit the binding of 125I-Bgtx to the recombinant chimeric α7 receptor. Thus, we examined the inhibitory activity of (a) various long chain curaremimetic toxins from snakes, including Cbtx, the α-cobratoxin from Naja kaouthia; L. semifasciata III, N. naja oaxiana I, a new toxin from L. colubrina, and 2 α-Bgtx a neuronal toxin from B. multicinctus; and (b) some short chain curaremimetic toxins from snakes, including N. nigricollis toxin α and erabutoxins a and b (EA and Eb) from L. semifasciata. The amino acid sequences of these toxins are shown in Table I. In general, short chain toxins possess 60–62 residues and four disulfide bonds, whereas long chain toxins possess 66–74 residues and five disulfide bonds (2). L. colubrina toxin appears therefore as an exception since, although it possesses 69 residues, it lacks the 2 cysteines 29 and 33 that usually form

2 D. Servent, V. Winckler-Dietrich, H.-Y. Hu, P. Kessler, P. Drevet, D. Bertrand, and A. Ménez, unpublished data.
Amino acid sequences of short chain and long chain snake toxins

| Short chain toxins | Loop I | Loop II | Loop III |
|--------------------|--------|---------|----------|
| (1): RICF---NHQSSQPQPTKTCPQESSTSCYKKMNSD-F---RTTIERGC---CCPVKPGIKLS---CESEVCCN| | | |
| (2): RICF---NHQSSQPQPTKTCPQESSTSCYKKMNSD-F---RTTIERGC---CCPVKPGIKLS---CESEVCCN| | | |
| (3): LECH---NQSSQPQPTKTCPGETNCYKVKWR--H---RTTIERGC---GGPTVRPGIKLN---CTTDKCCN| | | |

Long chain toxins

| | 10 | 20 | 30 | 40 | 50 | 60 | 70 |
|---|---|---|---|---|---|---|---|
| (4): IVCHTTASPS-AV----CPPGKLICYRKNDCDCCSIRGGKVVEIAGCAATCPSSKFPYEYVCTCCSTDCNPFPKRP| | | | | | | |
| (5): IRCF---ITPDITSKD--CPNH---VCYKTVMDACFSCIRGKKVDELGAACTCPVTVKTVGTSICCCSTDCNPFPTRKRP| | | | | | | |
| (6): ITCY---KTPPIPTSET---CPQONILTYKTVMDACGSRGGKVEIAGCAATCPYVESQDI1K---CCSTDCNPFPKRP| | | | | | | |
| (7): RECY---LNPHQDTQT---CCPGEIYKSVKNWACSCGBKVLEAGCAATCPVNNTGTEIXC---CCADKCNTP| | | | | | | |
| (8): RACY---LAPPOTXI---CCPGEIYKSVNDSGSSGSSGRKLEAGCAATCPVTVPDGDIK---CCSTDCNPFPKIA| | | | | | | |
| (9): RTCL---ISPSSTPQ---CCPSQDPICFPLAQCDKFCSTIRGPVEIAGCVATCPQFRSNYPLLCCCTDDCN| | | | | | | |

Neuronal receptor discriminates short from long toxins

(1), erabutoxin a from L. semifasciata (62 amino acids); (2), erabutoxin b from N. nigricollis (61 amino acids); (3), toxin a from N. nigricollis (61 amino acids); (4), α-bungarotoxin from B. multicinctus (74 amino acids); (5), α-cobrotoxin from N. kaouthia (71 amino acids); (6), toxin I from N. naja oissana (79 amino acids); (7), LcIII from L. semifasciata (66 amino acids); (8), Lc from L. colubrina (69 amino acids); (9), k-bungarotoxin from L. semifasciata (66 amino acids). Bold residues and disulfide bonds showed in (1) are conserved in all toxins; amino acids underlined are discussed in the text.

Neuronal toxin

A disulfide bridge in other long chain toxins. Therefore, L. colubrina toxin is an unusual long chain toxin with four disulfide bonds, like short chain toxins.

Fig. 1 shows the capacity of varying concentrations of different toxins to inhibit the binding of a constant amount of 125I-Bgtx to the chimeric α7 receptor. In all cases, a complete dose-dependent inhibition was observed, with IC50 values ranging between 10-10 and 2 × 10-5 M for Ea and Eb. Clearly, two groups of competitors emerged. The most potent group is constituted exclusively of long chain toxins, whereas the weak competitors include all short chain toxins and L. colubrina toxin, whose chemical characteristics are hybrid between long chain and short chain toxins. To quantify these data better, we calculated the Hill coefficients and the protection constants that were then assimilated to Kd values, as described previously (6). Results from these calculations are summarized in Table II. Thus, α-Bgtx, Cbtx, N. naja oissana I, and L. semifasciata III display a high affinity for the α7 chimeric receptor with Kd values equal to 1, 4.5, 12, and 5 nM, respectively. Similarly, the neuronal k-Bgtx is characterized by a Kd value equal to 5 nM. In sharp contrast, the other toxins are much weaker ligands for the chimeric receptor, the Kd values of toxin a, Ea, Eb, and L. colubrina toxin being equal to 3, 21, 22, and 7 μM, respectively.

Snake curaremimetic toxins have been initially recognized for their capacity to bind to peripheral AChR from Torpedo electric organs with high affinities, irrespective as to whether they are long chain or short chain toxins (13). In agreement with this general view, competition experiments made between 3H-α-toxin or 125I-Bgtx and various snake toxins toward AChR from Torpedo (6,30–33, this study) revealed that both short chain and long chain curaremimetic toxins, including L. colubrina toxin, had high affinities for Torpedo receptor, with Kd values ranging between 10-9 and 10-11 M. Only the neuronal k-Bgtx had a lower affinity for this receptor. To appreciate better the differential affinities of the toxins for the α7 receptor over Torpedo receptor, we calculated the ratios of their respective affinities. As shown in Table II, the ratios vary between 2 and 35 for long chain curaremimetic toxins, except L. colubrina toxin, whereas they are close to 3 × 105 for both short toxins and L. colubrina toxin. Therefore, long chain curaremimetic toxins exert similar binding affinities for both α7 and Torpedo AChRs, whereas short chain toxins and L. colubrina toxin have a marked preference for Torpedo AChR. Not unexpectedly, the ratio of affinities of α-Bgtx is unique, with an affinity approximately 100-fold higher for the α7 chimeric receptor compared with Torpedo, further indicating the particular character of the α7 receptor, which is capable of binding both long chain curaremimetic and neuronal toxins with high affinities. Recently, it was observed that α-Bgtx and two new short chain toxins from the Australian snake Oxyuranus scutellatus bind to homogenates from chick optic lobes, a tissue that is likely to contain various neuronal receptors, including the α7 receptor. How-

**TABLE I**

| Short chain toxins | Loop I | Loop II | Loop III |
|--------------------|--------|---------|----------|
| (1): RICF---NHQSSQPQPTKTCPQESSTSCYKKMNSD-F---RTTIERGC---CCPVKPGIKLS---CESEVCCN| | | |
| (2): RICF---NHQSSQPQPTKTCPQESSTSCYKKMNSD-F---RTTIERGC---CCPVKPGIKLS---CESEVCCN| | | |
| (3): LECH---NQSSQPQPTKTCPGETNCYKVKWR--H---RTTIERGC---GGPTVRPGIKLN---CTTDKCCN| | | |

Long chain toxins

| | 10 | 20 | 30 | 40 | 50 | 60 | 70 |
|---|---|---|---|---|---|---|---|
| (4): IVCHTTASPS-AV----CPPGKLICYRKNDCDCCSIRGGKVVEIAGCAATCPSSKFPYEYVCTCCSTDCNPFPKRP| | | | | | | |
| (5): IRCF---ITPDITSKD--CPNH---VCYKTVMDACFSCIRGKKVDELGAACTCPVTVKTVGTSICCCSTDCNPFPTRKRP| | | | | | | |
| (6): ITCY---KTPPIPTSET---CPQONILTYKTVMDACGSRGGKVEIAGCAATCPYVESQDI1K---CCSTDCNPFPKRP| | | | | | | |
| (7): RECY---LNPHQDTQT---CCPGEIYKSVKNWACSCGBKVLEAGCAATCPVNNTGTEIXC---CCADKCNTP| | | | | | | |
| (8): RACY---LAPPOTXI---CCPGEIYKSVNDSGSSGSSGRKLEAGCAATCPVTVPDGDIK---CCSTDCNPFPKIA| | | | | | | |
| (9): RTCL---ISPSSTPQ---CCPSQDPICFPLAQCDKFCSTIRGPVEIAGCVATCPQFRSNYPLLCCCTDDCN| | | | | | | |

**FIG. 1.** Competitions between 125I-labeled Bgtx and various snake toxins for binding to the chimeric α7 receptor (α7-V201-5HT) (18). The recombinant receptor was produced at the surface of HEK 293 cells. Experiments were carried out by preincubating varying concentrations of the competitors for 45 min with cell suspension and by filtering through GF/C filters 6 min after the addition of 125I-Bgtx at a final concentration of 5 nM. The continuous lines correspond to theoretical dose responses fitted through the data points using the nonlinear Hill equation. The error bars are shown on two competition curves representative of the two toxin families. LsIII, L. semifasciata III; NnoI, N. naja oissana I; Lc, L. colubrina.
Neuronal Receptor Discriminates Short from Long Toxins

TABLE II
Affinity constants of snake toxins on Torpedo and chimeric α7-5HT3 receptors

| toxin                  | Torpedo AChr | α7/Torpedo |
|------------------------|--------------|-------------|
|                        | Kp (nM)      | nH          | Kd (nM) | Ref. |
| Short chain toxins     |              |             |         |      |
| α-Bgtx                 | 0.95 ± 0.12  | 1.5         | 0.4     | (31) |
| Cbtx                   | 4.5 ± 0.35   | 1.3         | 0.2     | (32) |
| Cbtx-TP                | 12,000 ± 1,200 | 1.2       | 0.35    | (this study) |
| Nosil                  | 12 ± 2       | 1.1         | 0.38    | (this study) |
| LSIII                  | 3 ± 0.3      | 1.2         | 1.6     | (15) |
| Lc                     | 7,000 ± 1,500 | 0.8         | 0.02    | (this study) |
| Neuronal toxin         |              |             |         |      |
| α-Bgtx                 | 5 ± 0.8      | 0.8         | 130–1000 | (41, 42) | 0.035–0.005 |

ever, only the long chain toxin, α-Bgtx, binds to the homog enate with high affinity (34).

**Functional Inhibition of the WT and Chimeric α7 Receptors by Long and Short Chain Toxins**—In view of the differences in apparent binding coefficients exhibited by the short chain and long chain toxins, one obvious related question is how do these values compare with functional inhibition? To address this question, reconstitution experiments using Xenopus oocytes were performed, and the functional properties of the receptors were examined by electrophysiological recordings. Oocytes expressing the chick α7 WT receptor were challenged with short pulses of acetylcholine at a concentration near the EC50 (120 μM, 2 s). Cells expressing the receptor were then incubated in the presence of a long chain (α-Cbtx) or a short chain (Eb) toxin at a given concentration (see “Experimental Procedures”), and their responses to acetylcholine were determined again. A plot of the fraction of the current remaining after toxin incubation yielded typical dose-response inhibition curves that can be fitted by the empirical Hill equation.

Data obtained with this experimental procedure for the α-Cbtx yielded an IC50 of about 0.3 nM (see Fig. 2A), a value in good agreement with the binding experiments. It is important to notice that, as for α-Bgtx, little or no recovery from the blockade could be observed within 1 h of superfusion with the control medium (data not shown). When the same experiments were performed with Eb, an inhibition of the acetylcholine-evoked current was observed only when the toxin concentration was raised to the micromolar range (Fig. 2A). Comparison of the inhibition curves measured with α-Cbtx and Eb reveals a comparable apparent cooperativity for both toxins. In addition, as for the α-Cbtx, no significant recovery of the acetylcholine-evoked current could be measured over long washing periods with the control solution (data not shown).

Previous experiments done with the chimeric α7-5HT3 subunit have shown that the receptor reconstituted with this fusion protein displays a pharmacological profile resembling that of the chick α7 WT receptor (18). Because this fusion protein comprises only the receptor NH2-terminal domain, which is thought to contain the acetylcholine binding site, it was important to examine whether the blockade by α-Cbtx or Eb could be modified in the hybrid construct. Inhibition dose-response curves shown in Fig. 2B were made as for the WT receptor. Comparison of the α-Cbtx IC50 values for the α7 WT and chimera reveals a reduction of the apparent affinity for the chimera, as deduced from the shift of the inhibition curve from 0.3 to 10 nM. Similarly, the chimera displays also a slightly lower affinity for the Eb. However, despite this shift, experiments made on WT (Fig. 2A) and chimeric (Fig. 2B) α7 receptors show the same difference in the affinities of α-Cbtx and Eb, confirming the similar pharmacological profile of these two receptors.

A further illustration of the difference in apparent affinity of the short chain and long chain toxins is given by the recordings obtained in a single cell exposed first to 300 nM Eb and then to 3 nM α-Cbtx (Fig. 2C). The data clearly illustrate that a 30-minute incubation with 300 nM Eb induces only a small reduction of the acetylcholine-evoked current, whereas incubation with a 100-fold lower α-Cbtx concentration practically abolishes any further agonist-evoked current.

**On the Epitopes by Which Snake Toxins Recognize the Chimeric α7 and Torpedo Receptors**—That short chain toxins have a much lower affinity for the α7 receptor than for the Torpedo receptor raised the possibility that the sites by which they bind to these two receptors are different. To address this question, we examined whether residues critical in the affinity change of a short chain toxin for the Torpedo receptor (20, 34) also altered its affinity for the chimeric receptor. Thus, we selected (a) three mutants of Ea (S8T, R33E, and K47E) having a substantially lower affinity for the Torpedo receptor compared with the WT toxin (20, 33) and (b) a mutant (I36R) which uniquely exhibited a higher affinity to Torpedo receptor. Fig. 3 shows the capacity of the four mutants to inhibit the binding of radioactive α-Bgtx to the α7 chimeric receptor. The WT Ea (open symbols) completely inhibits the binding of the radioactive tracer, at a concentration of approximately 3 × 10−4 M. In contrast, a concentration of 2.5 × 10−4 M of either EaR33E or EaK47E had practically no effect on the binding of radioactive α-Bgtx (Fig. 3A), indicating that the two residues Arg-33 and Lys-47 are important for Ea to bind to α7 receptor. EaS8T inhibits the binding of radioactive α-Bgtx with a lower potency compared with the WT toxin, the IC50 value of the mutant being in the millimolar range (Fig. 3B). This finding also suggests that Ser-8 is involved in the binding of Ea to α7 receptor. Fig. 3C shows that the mutant EaI36R displays an approximately 5-fold higher binding affinity for the receptor compared with the WT Ea. In the aggregate, the mutants globally behave similarly toward both the α7 chimeric receptor and Torpedo receptors (20, 33), indicating that the epitopes by which Ea recognizes the two receptors do overlap. The observation that Ea recognizes them with so different affinities (see Table I) suggests that the α7 receptor possesses specific mutations that make some functional residues of Ea unable to establish some of the stabilizing contacts, which probably occur with the Tor-
**FIG. 2. Functional inhibition by toxins of the α7 and chimeric receptor.**

Panel A, functional inhibition of the α7 WT receptor by varying concentrations of a long chain (α-Cbtx) and a short chain (Eb) toxin. Mean values obtained with one to three cells, as indicated in parentheses, are plotted as a function of toxin concentrations. Continuous lines through the data points correspond to the best fit obtained with the empirical Hill equation with respective IC_{50} values and Hill coefficients of 0.3 nM and n = 2 for α-Cbtx and 0.5 μM and n = 2 for Eb. Typical currents evoked by a short acetylcholine (ACh) test pulse before and after application of toxins are illustrated in the right panel. Bars indicate acetylcholine application (120 μM, 2 s). Panel B, inhibition of the function of the chimeric α7-5HT\textsubscript{3} receptor by the long chain and short chain toxins. Lines through data points correspond to Hill equations with IC_{50} values and Hill coefficients of 10 nM and n = 2 for α-Cbtx and 20 μM and n = 2 for Eb. Current traces in the right panel illustrate a typical blockade of acetylcholine responses induced by a 30-min incubation with either toxin. Bars indicated the ACh application (30 μM, 3 s). Panel C, relative potency of α-Cbtx and Eb on a single cell, as shown by acetylcholine-evoked currents recorded after successive incubations with 300 nM Eb (30 min) and 3 nM α-Cbtx (30 min).

pedo receptor.

**How Do Long Chain Toxins Recognize α7 Chimeric Receptor with High Affinity?**—To identify tentatively the feature(s) that may account for the observed functional difference between long chain and short chain toxins for the neuronal receptor, we examined the amino acid sequences of the toxins shown in Table I. We took advantage of the unexpected observation that the L. colubrina toxin has a long COOH-terminal tail, the amino acid sequence of which is similar to that of other long chain toxins. Therefore, it seems unlikely that these 7 residues are responsible for the low affinity of L. colubrina toxin on the neuronal receptor. In contrast, the 5 other residues, located at the tip of loop II (Asp-29, Gly-31, Ser-32, Gly-33, and Lys-36) are replaced by highly conserved residues in all long chain curaremimetic toxins. Especially the 2 cysteines (Cys-29 and Cys-33) which form the fifth disulfide bond and the vicinal aromatic residue (Trp or Phe-32) are conserved in all long chain curaremimetic toxins. With the above conclusions in mind, one can examine, with a bird's eye view, all toxin sequences presented in Table I. A simple glance at this alignment suffices to reveal that the α7 chimeric receptor, which form the fifth disulfide bond and the vicinal aromatic residue (Trp or Phe-32) are conserved in all long chain curaremimetic toxins.

Therefore, it seems unlikely that these 7 residues are responsible for the low affinity of L. colubrina toxin on the neuronal receptor. In contrast, the 5 other residues, located at the tip of loop II (Asp-29, Gly-31, Ser-32, Gly-33, and Lys-36) are replaced by highly conserved residues in all long chain curaremimetic toxins. Especially the 2 cysteines (Cys-29 and Cys-33) which form the fifth disulfide bond and the vicinal aromatic residue (Trp or Phe-32) are conserved in all long chain curaremimetic toxins. Thus, the fifth disulfide bond and the vicinal aromatic residue (Trp or Phe-32) are conserved in all long chain curaremimetic toxins.

Additionally, we looked at the amino acid sequences of other long chain toxins. Therefore, it seems unlikely that these 7 residues are responsible for the low affinity of L. colubrina toxin on the neuronal receptor. In contrast, the 5 other residues, located at the tip of loop II (Asp-29, Gly-31, Ser-32, Gly-33, and Lys-36) are replaced by highly conserved residues in all long chain curaremimetic toxins. Especially the 2 cysteines (Cys-29 and Cys-33) which form the fifth disulfide bond and the vicinal aromatic residue (Trp or Phe-32) are conserved in all long chain curaremimetic toxins. The results of these experiments (Fig. 4) show that Cbtx and Cbtx-dithiopyridine are both potent competitors for the Torpedo and α7 chimeric receptors. The results of these experiments (Fig. 4) show that Cbtx and Cbtx-dithiopyridine are both potent competitors for the Torpedo receptor.
receptor, their $K_d$ values being equal to 0.2 and 0.35 nM, respectively (Fig. 4A). Therefore, the derivatization did not alter the binding capacity of the toxin for the peripheral receptor. In sharp contrast, Cbtx and Cbtx-dithiopyridine display respectively high and low affinities for the $\alpha 7$ chimeric receptor, with $K_d$ values equal to 4.5 and 12,000 nM respectively (Fig. 4B). The selective reduction of the fifth disulfide has therefore altered the toxin binding capacity for the neuronal receptor; only.

**DISCUSSION**

As a result of their similar capacity to bind to the peripheral nicotinic AChR, snake long chain and short chain curaremi-

metric toxins, which all adopt a similar fold, are usually considered as forming a family of functionally homogeneous proteins. This is however surprising because their respective polypeptide chains display clear differences, in terms of both length and number of disulfides. We now show that toxins from these two subgroups exert marked functional differences regarding their capacity to recognize the neuronal $\alpha 7$ receptor; furthermore, we show that this differential functionality is related to a structural feature that is uniquely present in the long chain toxins, i.e. the fifth disulfide bond.

We used a recombinant chimeric receptor that can be produced readily and relatively abundantly into HEK 293 cells (18), making this construction more appropriate than the natural $\alpha 7$ receptor for molecular pharmacology studies. When it is produced at the surface of HEK cells, $^{125}\text{I}$-labeled $\alpha$-Bgtx and methyllycaconitine bind to the chimeric $\alpha 7$ receptor with high affinities, similar to those obtained on the entire $\alpha 7$ receptor. It has been recognized previously that $\alpha$-Bgtx from venom of the krait $B. \text{multicinctus}$ binds with high affinity to the native $\alpha 7$ receptor (25, 29). This toxin contains 74 residues and five disulfide bonds and belongs to the group of long chain toxins that possess the same number of disulfides and 66–74 residues (2). Competition experiments between labeled $\alpha$-Bgtx and other snake curaremimetic toxins revealed that toxins classified as long chain toxins also bind with high affinities to the $\alpha 7$ chimeric receptor, including not only the curaremimetic toxins but also $\kappa$-Bgtx, a neuronal toxin also from venom of $B. \text{multicinctus}$. The binding of this neuronal toxin to the $\alpha 7$ receptor was reported previously in an electrophysiological study in which 100 nM $\kappa$-Bgtx was necessary to abolish the acetylcholine response completely (29). All other snake toxins bind with a low affinity to the $\alpha 7$ receptor. These toxins include the short chain curaremimetic toxins and the $L. \text{colubrina}$ toxin. The $L. \text{colubrina}$ toxin, like two other long chain toxins from $L. \text{colubrina}$ venom (35), is rather unusual in that it has the expected length (69 residues) of long chain toxins but lacks the fifth disulfide. Therefore, the $\alpha 7$ receptor, which offers a single class of binding sites to the curaremimetic toxins, clearly discriminates between the “true” long chain toxins and other toxins. This is in sharp contrast with the $\text{Torpedo}$ receptor, which recognizes with high affinities both short chain and long chain curaremimetic toxins from snake venoms. A differential selectivity between long chain and short chain toxins has been depicted previously toward the chick optic lobe (34), suggesting that our observations, made with a recombinant $\alpha 7$ receptor, may also occur in vivo. However, for this extrapolation to be valid, one needs to demonstrate that the recombinant chimeric receptor behaves just like the natural $\alpha 7$ receptor toward the toxins. This has been made on the basis of electrophysiology experiments.

Electrophysiological experiments made with the reconstituted $\alpha 7$ WT or chimeric receptors showed that both a long chain toxin ($\alpha$-Cbtx) and a short chain toxin (Eb) inhibit the activation of the receptor by acetylcholine. The persistence of blockade observed with either toxin suggests that their dissociation kinetics are too low to allow a significant recovery within the experimental time frame. However, as expected from binding experiments, the inhibition dose-response curves observed with the chimera indicated that Eb is more than a 1,000-fold less potent than $\alpha$-Cbtx. Although slightly more sensitive to both toxins, the $\alpha 7$ WT receptor displays a comparable pharmacological profile. Therefore, although the composition of these receptors differs significantly in their transmembrane domains as well as in their COOH-terminal regions, both receptors are inhibited by similar toxin concentrations, the long chain toxin being always much more potent than the short.
chain toxin. These data not only confirm that the major determinant for toxin binding and selectivity must be contained within the first 201 amino acids of the α7 receptor (18) but also that the binding and electrophysiological experiments are in good agreement, supporting the notion that both types of measurements are assessing the same state of the receptor. Therefore, binding experiments made with the chimeric receptor can safely be considered as indicative of the functional behavior of the α7 WT receptor.

The 104-fold higher binding affinities of short chain toxins for the peripheral receptor from T. marmorata compared with the α7 neuronal receptor may indicate that these toxins recognize the two receptors by different "toxic" sites. To address this question we tentatively localized the site by which Ea, a short chain toxin, binds to the α7 receptor and compared it with the previously identified site by which it binds with high affinity to the Torpedo receptor (20, 33). This was done using four toxin mutants of Ea harboring a single mutation at residues Ser-8, Arg-33, Ile-36, and Lys-47. The reason for this choice was 2-fold. First, mutations at these positions were shown previously to affect markedly the binding affinity of Ea for the Torpedo AChR (20, 33), indicating that these residues are critical for the toxin to bind to the peripheral receptor. Second, three of them (Ser-8, Arg-33, and Lys-47) are spread at the periphery of the Torpedo AChR binding site, whereas Ile-36 is just in its center. Competition experiments made with these mutants revealed that although Ea has a low affinity for the α7 receptor, individual mutations at three positions (Ser-8, Arg-33, and Lys-47) further decreased the affinity for this receptor, whereas mutation at Ile-36 increased it. These observations are similar to those made previously with the Torpedo receptor (20, 33). More precisely, mutations S8T, R33E, and K47E caused a decrease in affinity equal to or higher than 50-fold for the α7 receptor and, respectively, 780-, 310-, and 120-fold for the Torpedo receptor. Also, the mutation I36R caused an approximately 5-fold affinity increase, a value that is similar to what has been observed with the peripheral receptor (20). Therefore, although the binding of Ea to the α7 receptor is weaker than to the Torpedo receptor, it involves identical residues in both cases, indicating that the functional surfaces by which Ea binds to the two types of receptors largely overlap. Because short chain and long chain toxins interact with the Torpedo AChR by similar sites (11, 20, 37), it is likely that they all recognize both the peripheral and neuronal receptors by related though not necessarily identical sites.

How can we explain the differential affinities between the long chain and short chain toxins toward the α7 receptor? A comparison of the amino acid sequences of the different toxins revealed that all high affinity toxins possess a fifth disulfide at the tip of the central loop, suggesting that this region is associated with the remarkable capacity of long chain toxins to recognize the neuronal α7 receptor. A direct demonstration of the validity of this correlation was obtained by reducing the fifth disulfide of α-Cbtx, a long chain toxin. This modification caused virtually no effect on the affinity of α-Cbtx for the peripheral receptor, indicating in agreement with previous data (32, 37) that residues around the fifth disulfide bond of the long chain toxins play little role in the binding to peripheral Torpedo receptor. In sharp contrast, reduction of the fifth disulfide bond caused a nearly 104-fold affinity decrease for the neuronal α7 chimeric receptor. In other words, by reducing the fifth disulfide of the long chain toxin, its behavior became quite similar to that of the short chain toxins and of the unusual L. colubrina toxin. Clearly, therefore, residues in the vicinity of the fifth disulfide bond must be directly related to the capacity of a long chain toxin to recognize the neuronal α7 receptor. This conclusion does not preclude that other unique elements of long chain toxins may also contribute to their discriminating behavior toward the α7 receptor.

Therefore, we have shown that structural differences between long chain and short chain toxins are directly related to distinct functionalities. The precise residues that, in the area of the fifth disulfide, provide the long chain toxins with their remarkable discriminating property remain to be identified. Because the curaremimetic toxins are amenable to synthesis by both recombinant (38–40) and chemical3 approaches, a systematic alanine-scanning experiment of this region can now be envisioned. Also, it would be worth identifying the residues of the α7 receptor which are uniquely recognized by the fifth bond toxin region. Finally, it would be of interest to investigate whether subtle differences in the primary or tertiary structures of toxins forming other large families, such as, for example, potassium channel-blocking toxins from scorpions, are also associated with fine functional selectivities.

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