Two Novel α-Neurotoxins Isolated from the Taipan Snake, *Oxyuranus scutellatus*, Exhibit Reduced Affinity for Nicotinic Acetylcholine Receptors in Brain and Skeletal Muscle†

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ABSTRACT: Three novel toxic peptides were purified to homogeneity from the venom of the Australian taipan snake, *Oxyuranus scutellatus scutellatus*. On the basis of complete amino acid sequence analyses, two of these toxins belong to the family of short-chain α-neurotoxins found in elapid and hydrophid snake venoms and are the first postsynaptic neurotoxins identified in taipan venom. Radioligand binding studies confirm that taipan toxins 1 and 2 inhibit the binding of [^{125}I]-α-bungarotoxin to nicotinic acetylcholine receptors in skeletal muscle with IC_{50} values of 2.4−2.5 nM but are 5-fold less potent in this assay than α-bungarotoxin or the two short-chain α-neurotoxins erabutoxin a and erabutoxin b. Taipan toxins 1 and 2 do not antagonize [^{125}I]-α-bungarotoxin binding to central neuronal nicotinic receptors at concentrations up to 3 μM. We find that erabutoxin a and erabutoxin b do inhibit the binding of [^{125}I]-α-bungarotoxin to central neuronal nicotinic receptors but are over 350-fold less potent than long-chain α-neurotoxins at these receptors. The novel α-neurotoxins from taipan venom do not inhibit the binding of [^{3}H]nicotine to high-affinity nicotine receptors in brain, a property they share with α-bungarotoxin and the erabutoxins. The results demonstrate that at least two neuromuscular junction-blocking peptides are present in taipan venom. Nonconservative substitutions at position 32 in both taipan toxin 1 and 2 may be responsible for the observed decreases in affinities of the toxins of 5-fold for muscle receptors (compared to α-bungarotoxin) and over 10-fold for α-bungarotoxin-sensitive nicotinic receptors in brain (compared to the structurally similar short-chain α-neurotoxins erabutoxin a and erabutoxin b).

The postsynaptic neurotoxins are a family of structurally related proteins isolated from the venoms of elapid and hydrophid snakes [reviewed in Endo and Tamiya (1991)]. These toxins bind with high affinity to certain nicotinic acetylcholine receptors (AChRs)[1] in skeletal muscle and nervous tissue, where they competitively antagonize the actions of acetylcholine [reviewed in Stroud et al. (1990)]. The AChRs are ligand-gated, cation-selective ion channels that span the cell membrane and consist of various pentameric assemblies of structurally related subunits that include the agonist-binding α subunits (α1−α9 are known) and other subunits termed β, γ, δ, and ϵ. Of the two known subfamilies of postsynaptic neurotoxins, the κ-neurotoxins bind most avidly to a subset of neuronal AChRs made up in part of α3 or α4 nicotinic receptor subunits [Boulter et al., 1987; McLane et al., 1993; reviewed in Chiappinelli (1993)]. In contrast, the α-neurotoxins bind most potently to skeletal muscle AChRs, all of which contain α1 subunits. A further subdivision can be made within the α-neurotoxins, on the basis of the sizes and structures of the toxins. Long-chain α-neurotoxins have 70−74 amino acids and five internal disulfide bonds, while short-chain α-neurotoxins have 60−62 amino acids and four disulfide bonds (see Figure 5).

Although the α-neurotoxins are best known as high-affinity ligands for α1-containing muscle AChRs, they are also valuable probes for characterizing certain neuronal AChRs containing α7, α8, or α9 subunits (Couturier et al., 1990; Séguéla et al., 1993; Elgoyhen et al., 1994). These neuronal receptors have a high affinity (K_d = 0.7−1.7 nM) for the long-chain α-neurotoxins α-bungarotoxin and α-cobratoxin (Tindall et al., 1978; Schneider et al., 1985; Wolf et al., 1988; Alkondon & Albuquerque, 1990). α-Bungarotoxin has been an important antagonist in establishing the functional properties of these neuronal AChRs (Alkondon & Albuquerque, 1993; Zhang et al., 1994). The α-bungarotoxin-sensitive neuronal receptors have certain properties unlike those of other AChRs. The most characteristic of these are (1) the ability to form functional homomeric channels in oocyte expression studies, (2) an extremely rapid desensitization (within milliseconds) in the presence of agonist (Alkondon & Albuquerque, 1993; Zhang et al., 1994), and (3) high permeability to Ca^{2+} ions (Séguéla et al., 1993).

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; LD_{50}, lethal dose for 50% of the population tested; HPLC, high-performance liquid chromatography; RC-toxin, reduced and carboxymethylated toxin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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There are two known stoichiometries of skeletal muscle AChRs: $(\alpha1)_2\beta\gamma\delta$ and $(\alpha1)_2\beta\epsilon\delta$ (Stroud et al., 1990). These muscle receptor subtypes exhibit similar pharmacological properties, including affinities for α-bungarotoxin. At least two subtypes of α-bungarotoxin-sensitive AChRs have been described in the chick optic lobe, with the predominant form containing α7 but not α8 subunits and a minor form containing both α7 and α8 subunits (Schoepfer et al., 1990; Gotti et al., 1994). Both subtypes have similar affinities for α-bungarotoxin and other antagonists but have different affinities for certain agonists. Several studies have provided evidence for at least one as yet unidentified subunit in both of these neuronal receptor subtypes, on the basis of the protein bands present after gel electrophoresis of solubilized receptors (Gotti et al., 1994) and on the basis of the observation that the pharmacology of native α7-containing receptors is quite similar but not identical to that of heterologously expressed α7-homomeric receptors (brain receptors exhibit 50-fold less sensitivity to the nicotinic agonist cytisine; Anand et al., 1993). The binding of short-chain α-neurotoxins to α-bungarotoxin-sensitive neuronal AChRs has not previously been examined.

The venom of the Australian taipan snake (*Oxyuranus scutellatus*) is known to be among the most lethal to man in the world and to have a very complex mixture of components (Fohlman et al., 1976; Lind & Eaker, 1982). While several groups have identified toxic principles in this venom, including taipoxin and taicatoxin (Fohlman et al., 1976; Possani et al., 1992a), there have been no reports of postsynaptic neurotoxins existing in taipan venom.

We now report the isolation, biochemical characterization, and complete amino acid sequences of two novel α-neurotoxins from the venom of the Australian taipan snake. The amino acid sequences of these toxins indicate that they are members of the short-chain α-neurotoxin family but that they have several novel nonconservative substitutions. We find that the new taipan toxins exhibit reduced potency at muscle AChRs and fail to bind to neuronal α-bungarotoxin-sensitive receptors at concentrations at which other short-chain α-neurotoxins do bind to these receptors.

MATERIALS AND METHODS

Isolation of Known Postsynaptic Neurotoxins. α-Bungarotoxin was purified from the crude venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, FL) as previously described (Chiappinelli, 1983). Identification and purity of α-bungarotoxin were confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), isoelectric focusing, quantitative amino acid analyses, and N-terminal amino acid sequencing. Erabutoxin a and erabutoxin b were purified on a CM-cellulose column using a published method (Maeda et al., 1974) from the crude venom of *Laticauda semifasciata* (Sigma, St. Louis, MO). The purity of the toxins was confirmed by SDS−PAGE, where both toxins ran as single bands with molecular weights of approximately 7500, and isoelectric focusing in a polyacrylamide gel, where both toxins ran as single bands in positions consistent with their known, identical isoelectric points of 9.7 (Ui et al., 1982).

Purification of Postsynaptic Neurotoxins from Taipan Venom. Lyophilized venom from the Australian snake *O. scutellatus scutellatus* was obtained from Venom Supplies (18 Creber Street, Whyalla Playford 5600, South Australia). Venom was solubilized in 0.01 M Tris-HCl buffer (pH 8.0) containing 0.01 M NaCl and then centrifuged at 10 000 rpm in a Sorvall centrifuge at 4 °C for 15 min using an SS34 rotor. Soluble venom was applied initially to a (diethylaminoethyl)cellulose resin (DE-cellulose, no. DE-32), prepared according to the specifications of the manufacturer (Whatman, Clifton, NJ). The material unbound to this resin (Possani et al., 1992a) was further separated by a cation exchange resin of (carboxymethyl)cellulose (CM-cellulose, no. CM-32), from the same company. Chromatographic columns were pre-equilibrated with appropriate buffers before loading. For the first column, the DE-cellulose resin was buffered with the solution used for solubilization of the venom. In the second column, the initial buffer was 0.02 M ammonium acetate (pH 4.7), and bound protein was eluted with a linear gradient of 0 to 0.6 M NaCl, in the same buffer. Prior to the unbound material from the first column being loaded, this material was dialyzed against 1 L of the corresponding buffer for 30 min with four to five changes. Spectrapor Type 3 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA) with a molecular weight cutoff of approximately 3500 was used for dialysis. The third step of separation consisted of high-performance liquid chromatography (HPLC) using an analytical C18 (reverse-phase) column (Vydac, Hysperia, CA) in a Waters 625 LC System, equipped with a Photodiode Array Detector (Waters 996). A linear gradient from 0.12% trifluoroacetic acid in water (A) to 60% B (0.12% trifluoroacetic acid in acetonitrile) was applied during 60 min for elution of the peptides from the column.

Chemical Characterization. Purified toxins were sequenced using a ProSequencer Model 6600 apparatus from MilliGen/Biosearch (Division of Millipore), which automatically performs the Edman degradation of samples either adsorbed into Immobilon-P or covalently attached to Sequelon-DITC or Sequelon-AA membranes, following protocols described by the company. Two types of samples were used: (i) native toxin as well as reduced and carboxymethylated toxin (RC-toxin) loaded with approximately 1 nmol each for direct sequencing and (ii) isolated peptides (also 1 nmol each) obtained by HPLC fractionation of RC-toxins after cleavage with enzymes. Trypsin, chymotrypsin, and *Staphylococcus aureus* protease V8 digestions were performed as previously described (Lebreton et al., 1994), using about 100 μg of sample each time. Arg-C and Lys-C endopeptidase (Boehringer Mannheim, Germany) digestion was conducted with 100 μg of RC-toxin each time. For Arg-C, the sample was dissolved in 0.09 M Tris-HCl buffer at pH 7.6, containing 0.0085 M $CaCl_2$ and 0.005 M dithiothreitol (Aldrich Chem Co., Milwaukee, WI), and hydrolyzed during 4 h at 37 °C, using 1:200 dilution (enzyme:peptide). The digestion with Lys-C was performed similarly using 0.025 M Tris-HCl buffer at pH 8.5, in the presence of 0.001 M EDTA. Separation of resulting peptides was obtained through a C18 reverse-phase column (Vydac, Hysperia, CA) with a linear gradient of acetonitrile (0 to 60%) in 0.1% aqueous trifluoroacetic acid.

Amino acid analyses of samples were conducted in a Beckman 6300E analyzer, after hydrolysis for 20 and 40 h at 110 °C in tubes sealed under vacuum, containing 6 N HCl and 0.05% phenol.

Mass spectrometry data were obtained in a Finnegan-MAT electrospray apparatus.

Recovery of protein content during chromatographic procedures was calculated assuming that 1 unit of absorbance at 280 nm with a 1 cm cuvette pathway equals 1 mg/mL protein concentration. However, for final determination of LD_{50} values and binding and displacement experiments, the toxin concentration was determined on the basis of amino acid analysis.

Protein Concentrations. Concentrations of neurotoxin solutions were determined by absorbance at 280 nm. The extinction coefficient ($E^{280}_{0.1\%}$) for both taipan toxins 1 and 2 was 1.73, as determined by quantitative amino acid analyses of samples with known absorption. Published extinction coefficients were used for the following neurotoxins: α-bungarotoxin, 1.32 (Hanley et al., 1977); and erabutoxins a and b, 1.26 (Hori & Tamiya, 1976). For taipan toxin 3, an estimated value of 1.0 was used.

Lethality Tests. Lethality tests and LD_{50} values (lethal dose for 50% of the population) were performed with adult 20−25 g albino mice (strain CD1), according to approved protocols of the Animal Welfare Committee of the Biotechnology Institute, National Autonomous University of Mexico. The effects of the various protein fractions were observed after intraperitoneal injection of different amounts of protein in 0.1−0.2 mL of isotonic solution containing a maximum of 100 μg of protein. Three designations were proposed to define the toxicity of the various chromatographic components. "Lethal" means that the toxic component, at the dose injected, killed the test mouse within 20 h of injection. "Toxic" means that the animal survived after showing one or more symptoms, such as excitability followed by periods of immobilization, sporadic convulsions, and paralysis of limbs (mainly rear limbs). "Nontoxic" means normal behavior similar to that observed following injection of 0.9% NaCl or injection of the buffers used during purification. HPLC fractions were always dried before use in mouse assays, in order to eliminate trifluoroacetic acid and acetonitrile. For LD_{50} determination, eight distinct toxin concentrations were used with six mice each. The value was graphically obtained by plotting percentage of survival as a logarithmic function of toxin concentration, after 24 h of injection.

Source of Chemicals. All chemicals and solvents used were analytical reagents from companies indicated. Water was deionized and double distilled over quartz.

Inhibition of Binding of [^{125}I]-α-Bungarotoxin to Chick Skeletal Muscle and Brain Homogenates. The ability of α-neurotoxins to inhibit the binding of radiolabeled α-bungarotoxin to homogenates of skeletal muscle (obtained from thigh muscle after 14 days of incubation of chick embryos) and optic lobe (from 20−21 days of incubation of chick embryos) was determined using published procedures (Wolf et al., 1988). α-Bungarotoxin was radioiodinated (Amersham Na^{125}I) using a published protocol (Wolf et al., 1988), and two separate iodinations yielded initial specific activities of 470 and 990 Ci/mmol. For inhibition studies, a final concentration of 10 nM [^{125}I]-α-bungarotoxin was used while the concentration of competing ligand was varied. Preincubations were for 2 h with inhibitors or with 1 μM α-bungarotoxin (nonspecific binding), and tubes were always prepared in duplicate. Data were analyzed using Prism (GraphPad Software).

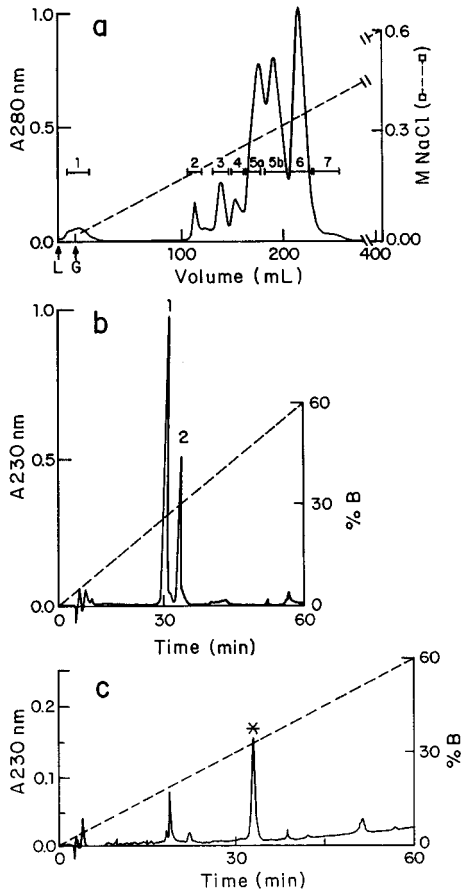

FIGURE 1: Chromatographic separation of taipan toxins 1−3. (a) Fraction 1 from DE-cellulose column purification of crude venom was applied to a CM-cellulose column (0.9 × 30 cm) and separated in ammonium acetate buffer (pH 4.7) using a linear gradient from 0 to 0.6 M NaCl (200 mL each). Fractions of 2 mL were collected and pooled as indicated by the horizontal bars. Fractions 2 and 3 were further separated by HPLC. (b) Fraction 3 from part a (1.6 mg of protein) was applied to a C18 reverse-phase preparative column and separated in a Waters HPLC system, as described in Materials and Methods, using solution A (0.12% TFA in water) to 60% solution B (0.1% TFA in acetonitrile) for 60 min. Components labeled 1 and 2 are respectively toxins 1 and 2. (c) Fraction 2 (680 μg of protein) from part a was separated in a C18 reverse-phase analytical column in the same conditions as for part b. The component labeled with an asterisk is toxin 3.

Inhibition of [^{3}H]Nicotine Binding to Brain Homogenates. Procedures for determining inhibition of [^{3}H]nicotine binding to optic lobe homogenates have previously been published (Wolf et al., 1988). [*N-methyl*-^{3}H]-L-Nicotine (64 Ci/mmol; NEN/DuPont) was used at a final concentration of 10 nM. Preincubation was for 2 h with snake toxins or with 1 mM carbachol (nonspecific binding), and duplicate tubes were used.

RESULTS

Purification Procedures. The profile of the chromatographic purification of postsynaptic neurotoxins from *O. scutellatus* is shown in Figure 1. Soluble whole venom applied to a (diethylaminoethyl)cellulose column separated essentially the same components as described earlier (Possani et al., 1992a), for which reason this chromatogram is not included in Figure 1. The unbound material eluted from this first chromatography step (about 84% of the soluble venom) was loaded onto a cation exchange resin of (carboxymethyl)-cellulose and gave the separation profile shown in Figure

Table 1: Recovery from the Ion Exchange Column at pH 4.7

fraction	protein (mg) by absorbance	percentage (%)	lethality (100 μg)
loaded	59.0	100	lethal
F 1.2	1.6	2.7	lethal
F 1.3	2.9	4.9	lethal
F 1.4	2.3	3.9	nontoxic
F 1.5a	14.9	25.2	lethal
F 1.5b	16.8	28.5	lethal
F 1.6	20.1	34.0	lethal
side tubes[a]	ND[b]		ND
recovered	58.6	99.2	

[a] Side tubes are F 1.1 and F 1.7. [b] ND, not determined. Lethality tests were performed with 100 μg of protein per mouse with a 20 g body weight.

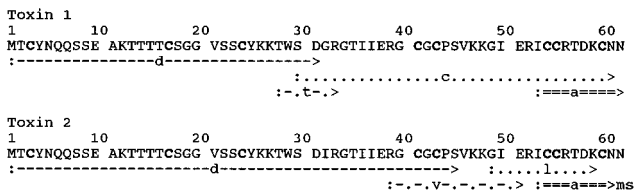

FIGURE 2: Amino acid sequences of toxins 1 and 2. Overlapping amino acid sequences were obtained by direct sequencing or from HPLC-purified fragments of toxins after various enzymatic treatments, as indicated under each sequence by direct sequencing (-d-), chymotrypsin (..c..), trypsin (-.t-.), Arg-C endopeptidase (=a=), Lys-C endopeptidase (..l..), and V8 protease (-.v-.) digestions, and mass spectrometry (ms).

1a. Lethal components 2 and 3 were further separated by HPLC, as described in Materials and Methods. The third fraction of Figure 1a (component labeled with 3) was subfractionated into two peptides (Figure 1b) that were homogeneous on the basis of SDS−PAGE (data not shown) and which were named toxin 1 and 2 respectively because they were lethal to mice (toxin 1, $LD_{50} = 0.063$ mg/kg; toxin 2, $LD_{50} = 0.063$ mg/kg). Similarly toxic was the major subfraction obtained by HPLC from component 2 of Figure 1a, as shown in Figure 1c (labeled with an asterisk). This toxin was named toxin 3. The amount of protein recovered is shown in Table 1. Toxin 1 and 2 (Figure 1b) corresponded respectively to 52 and 26% of the material recovered from the C18 reverse-phase column, while toxin 3 represented 66% of the material recovered (Figure 1c). Thus, toxins 1−3 correspond respectively to 2.1, 1.1, and 1.5% of the initial soluble venom. Additional criteria for sample purity were obtained from the amino acid analysis and sequencing data, as discussed below.

Chemical Characterization. Toxins 1 and 2 were fully sequenced as shown in Figure 2. Amino acid sequences obtained with the native toxins confirmed the homogeneity of these samples, since only one residue was found in each cycle of the microsequencer, except for the half-cystine residues, which were identified by analysis of RC-toxins. Direct sequence data for toxin 1 (labeled d in Figure 2) went unequivocally from Met at position 1 (Met-1) to Asp at position 31, and that of toxin 2 went from Met-1 to Ser-45. Fragments of RC-toxin were obtained by enzymatic digestion as described in Materials and Methods and produced a large number of peptides that resulted in the unique overlapping sequences shown in Figure 2. A chymotryptic petide of toxin 1 corresponds to the sequence from residue Ser-30 to Asn-61 (labeled c in Figure 2), and two additional segments of this sequence were obtained from a tryptic peptide (labeled t) and an Arg-C endopeptidase (labeled a) corresponding respectively to the sequence from Thr-28 to Arg-33 and Ile-53 to Asn-62. The C-terminal peptide of toxin 1 (Ile-53 to Asn-62) was also confirmed by amino acid analysis (data not shown).

Concerning toxin 2, three additional HPLC peptides were enough to complete the sequence: a V8 peptidase fragment corresponding to residues from Arg-39 to Glu-51 (labeled v in Figure 2) and a Lys-C endopeptidase fragment (labeled l) covering the C-terminal part of this toxin from residue Gly-49 to Lys-59. The last segment Ile-53 to Asn-61 was obtained by sequencing a peptide from the hydrolysis with Arg-C (labeled a, in toxin 2, Figure 2).

The molecular weight of toxin 1 was determined by electrospray mass spectroscopy to be 6726, compared with the molecular weight of 6726.6 calculated for the assigned amino acid sequence in Figure 2. The molecular weight of toxin 2 obtained with mass spectrometry was 6781, or 55 mass units greater than that of toxin 1. This difference in experimentally determined molecular weights between the toxins is consistent with a single substitution of Ile for Gly (at position 32) in toxin 2 and supports the presence of two Asn residues at the C-terminus of both toxins, a conclusion that is also consistent with amino acid analyses of the toxins. The amino acid composition of toxin 1 was as follows [calculated nanomoles of amino acid (found by sequence)]: Asp 5.7 (5), Thr 4.9 (8), Ser 6.0 (7), Glu 5.1 (5), Pro 2.6 (1), Gly 7.0 (7), Ala 1.0 (1), Val 1.6 (2), Met 0.2 (1), Ile 1.4 (4), Leu 0.0 (0), Phe 0.0 (0), His 0.0 (0), Lys 4.8 (6), and Arg 3.7 (4); those not determined included 1/2-Cys (8), Tyr (2), and Trp (1). The amino acid composition of toxin 2 was as follows: Asp 4.9 (5), Thr 6.4 (8), Ser 5.5 (7), Glu 5.1 (5), Pro 0.8 (1), Gly 5.9 (6), Ala 1.0 (1), Val 1.9 (2), Met 0.6 (1), Ile 3.9 (5), Leu 0.0 (0), Tyr 1.7 (2), Phe 0.0 (0), His 0.0 (0), Lys 5.9 (6), and Arg 3.8 (4); those not determined included 1/2-Cys (8) and Trp (1).

The N-terminal sequence of toxin 3 was determined directly to be LTCYMNPSGTMVXKEXETMXYQL, with three additional segments also determined (SSGTSYPDVLK, LGEDNXNVVEGNEXF, and ICTS), but the full overlapping sequence was not obtained.

Inhibition of [^{125}I]-α-Bungarotoxin Binding to Skeletal Muscle. The α-neurotoxins are known to bind with high affinity to AChRs located in skeletal muscle. We therefore determined whether taipan toxins 1 and 2 inhibited the binding of [^{125}I]-α-bungarotoxin to AChRs in homogenates of chick skeletal muscle. The results demonstrated that both taipan toxins were potent antagonists in this assay, as indicated by IC_{50} values of 2.4 ± 0.4 nM for taipan toxin 1 and 2.5 ± 0.5 nM for taipan toxin 2 (all values are mean ± SEM; Figure 3). However, the taipan toxins were not as potent in this muscle receptor assay as nonradiolabeled α-bungarotoxin ($IC_{50} = 0.52 \pm 0.22$ nM) or the short-chain α-neurotoxins erabutoxin a and erabutoxin b ($IC_{50} = 0.52 \pm 0.07$ nM for erabutoxin a and 0.65 ± 0.16 nM for erabutoxin b). Taipan toxin 3 exhibited no potency in the muscle receptor assay at concentrations up to 5 μM (Figure 3).

Inhibition of [^{125}I]-α-Bungarotoxin Binding to Brain AChRs. One subclass of neuronal AChRs is very sensitive to blockade by α-bungarotoxin, and [^{125}I]-α-bungarotoxin binds with high affinity to these receptors in chick optic lobe

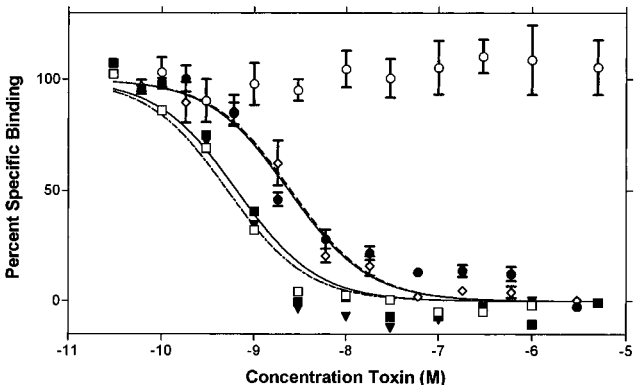

FIGURE 3: Inhibition of [^{125}I]-α-bungarotoxin binding to chick skeletal muscle AChRs. Incubation with 10 nM radiolabel for 7 min followed a 2 h preincubation with the indicated concentrations of neurotoxins. The data are presented as percentage of control specific binding and represent mean values obtained from either two (no error bars) or three (error bars are ± SEM) separate experiments for each toxin. Lines are nonlinear regression computer fits used to determine IC_{50} values, and assumed a one-site competition model: (▼) α-bungarotoxin, (□) erabutoxin a, (■) erabutoxin b, (●) taipan toxin 1, (◇) taipan toxin 2, and (○) taipan toxin 3.

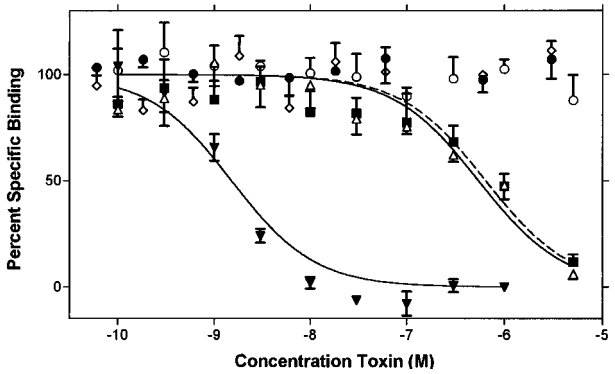

FIGURE 4: Inhibition of [^{125}I]-α-bungarotoxin binding to chick optic lobe neuronal AChRs. Incubation with 10 nM radiolabel for 5 min followed a 2 h preincubation with the indicated concentrations of neurotoxins. The data are presented as percentage of control specific binding and represent mean values obtained from three or four (error bars are ± SEM) separate experiments for each toxin. Lines are nonlinear regression computer fits used to determine IC_{50} values and assumed a one-site competition model: (▼) α-bungarotoxin, (△) erabutoxin a, (■) erabutoxin b, (●) taipan toxin 1, (◇) taipan toxin 2, and (○) taipan toxin 3.

homogenates (K_d = 1.7 nM; Wolf et al., 1988), providing a convenient assay for determining whether taipan toxins recognize these brain receptors. In this assay, nonradiolabeled α-bungarotoxin had an IC_{50} of 1.5 ± 0.5 nM (Figure 4). The erabutoxins also inhibited [^{125}I]-α-bungarotoxin binding to brain homogenates but were over 350-fold less potent, with IC_{50} values of 550 ± 150 nM for erabutoxin a and 650 ± 170 nM for erabutoxin b. In contrast, taipan toxins 1 and 2 exhibited no detectable ability to block [^{125}I]-α-bungarotoxin binding to brain receptors. At the highest concentration of the taipan toxins tested (3 μM), no reduction was observed in [^{125}I]-α-bungarotoxin specific binding (Figure 4). Taipan toxin 3 was also ineffective in this assay at 5 μM.

Inhibition of [^{3}H]Nicotine Binding to High-Affinity Nicotine Receptors in Brain. There is a major subclass of AChRs in brain that have a high affinity for nicotine and other nicotinic agonists but exhibit little or no affinity for α-bungarotoxin or κ-bungarotoxin. These receptors can be identified by the high-affinity binding of [^{3}H]nicotine (K_d = 3.1 nM; Wolf et al., 1988) to chick optic lobe homogenates. We tested the ability of the taipan toxins and the erabutoxins to inhibit [^{3}H]nicotine binding to brain and found no evidence of inhibition at the following concentrations (values in parentheses are percent of control specific binding and are means of duplicate determinations): 3 μM taipan toxin 1 (101%), 3 μM taipan toxin 2 (103%), 5 μM erabutoxin a (105%), and 5 μM erabutoxin b (96%). Likewise, α-bungarotoxin exhibited little or no affinity for the [^{3}H]nicotine site at a concentration of 10 μM (90%).

DISCUSSION

The present study demonstrates for the first time that the venom of the taipan, *O. scutellatus*, contains α-neurotoxins in addition to previously characterized toxins that have quite different mechanisms of action (Possani et al., 1992a,b). Taipan toxins 1 and 2 are toxic to rats, having LD_{50} values within the range previously reported for other α-neurotoxins (Endo & Tamiya, 1991). The fundamental pharmacological property of all α-neurotoxins is high-affinity binding to AChRs located in skeletal muscle and electric organ. We find that taipan toxins 1 and 2 inhibit the binding of [^{125}I]-α-bungarotoxin to muscle AChR, but with a potency that is 5-fold lower than that of α-bungarotoxin or erabutoxin a. A third novel toxic peptide identified in this venom, toxin 3, had only limited amino acid sequence homology with the α-neurotoxins and was completely ineffective in all of the nicotinic receptor assays performed.

Taipan toxins 1 and 2 did not inhibit [^{125}I]-α-bungarotoxin binding to AChRs in brain, even at micromolar concentrations. This was initially quite surprising, since α-bungarotoxin displays nanomolar affinity for these neuronal AChRs. However, when erabutoxin a and erabutoxin b, both well-characterized short-chain α-neurotoxins, were tested in this assay, they were found to be over 350-fold less potent than α-bungarotoxin. This is strikingly different than the results in the skeletal muscle assay, where the erabutoxins had affinities for the muscle AChR that were comparable to that of α-bungarotoxin. We therefore find that a distinctive feature of neuronal α-bungarotoxin-sensitive AChRs is a high affinity for the long-chain α-neurotoxins α-bungarotoxin (K_d = 1.7 nM; Wolf et al., 1988) and α-cobratoxin (K_d = 0.7 nM; Tindall et al., 1978) but a much lower affinity for short-chain α-neurotoxins. Taipan toxins 1 and 2 are at least 10-fold less potent at these brain AChRs than the structurally very similar erabutoxins.

Previous studies using assays similar to those of the present study have shown that some nicotinic antagonists, including d-tubocurarine and dihydro-β-erythroidine, have nearly identical affinities for α-bungarotoxin-sensitive AChRs in chick optic lobe and chick muscle receptors, while other antagonists, including decamethonium and gallamine, are over 100-fold more potent at binding to muscle receptors (Wang et al., 1978). In contrast, methyllycaconitine, a diterpenoid purified from *Delphinium*, is an antagonist that is 200-fold more potent at binding to α-bungarotoxin-sensitive AChRs in optic lobe than to muscle receptors (Yum et al., 1996). Among agonists, acetylcholine and carbachol are more potent at muscle receptors, while nicotine is 12-fold more potent at optic lobe receptors (Wang et al., 1978).

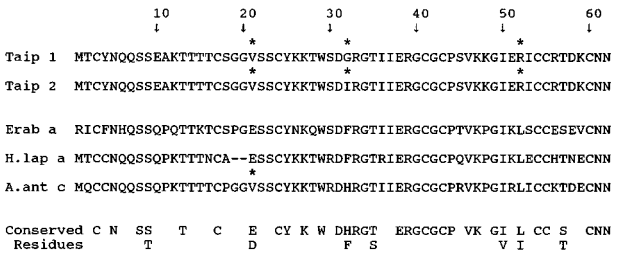

FIGURE 5: Comparison of amino acid sequences of new taipan α-neurotoxins with those of other short α-neurotoxins. Conserved residues indicated at the bottom of the figure are based on the complete amino acid sequences of 46 known short α-neurotoxins, including three toxins shown in the figure (Endo & Tamiya, 1991). Asterisks indicate sequence positions where toxins exhibit nonconserved amino acids. Abbreviations: Taip 1, taipan toxin 1; Taip 2, taipan toxin 2; Erab a, erabutoxin a; H.lap a, *Hydrophis lapemoides* a; A.ant c, *Acanthophis antarcticus* c.

Thus, α-bungarotoxin-sensitive receptors in both tissues exhibit a nicotinic pharmacology, but there are certain ligands that readily distinguish these receptor subtypes. The taipan toxins and the erabutoxins, with over a 350-fold preference for the muscle receptors, are most similar to the antagonists decamethonium and gallamine in their strong preference for AChRs in skeletal muscle. The different subunit compositions of brain and muscle AChRs are responsible for these pharmacological distinctions, and thus, drugs that discriminate between these receptor subunits may provide valuable insight into the structural differences between AChRs.

The binding of taipan toxins to a second subtype of neuronal AChRs was examined through inhibition assays with [^{3}H]nicotine. None of the α-neurotoxins tested, including the taipan toxins, had any effect on [^{3}H]nicotine binding to brain homogenates at micromolar concentrations, a finding consistent with previous studies examining the actions of α-neurotoxins on these AChRs (Schneider et al., 1985; Whiting & Lindstrom, 1986; Wolf et al., 1988).

The complete amino acid sequences of taipan toxins 1 and 2 are compared with those of selected short-chain α-neurotoxins in Figure 5. This family of toxic proteins exhibits considerable sequence homology. Compared with all known short-chain α-neurotoxins, both taipan toxins display 31 out of 34 highly conserved amino acids at the appropriate sequence positions, including all eight cysteines forming intrachain disulfide bonds (Endo & Tamiya, 1991). Thus, there are only three nonconservative substitutions which might account for the reduced affinities of these toxins at α-bungarotoxin-sensitive muscle and brain AChRs.

The first of these substitutions, Val-21 in place of a glutamate or aspartate, has previously been reported in a single short-chain toxin, *Acanthophis antarcticus* c, also from an Australian snake. Since this toxin does not appear to have reduced potency at muscle AChRs (Kim & Tamiya, 1981), this substitution is unlikely to be responsible for the reduced potency of the taipan toxins. Furthermore, it has been reported that the sequence region 17−24 in erabutoxin a is not critical for binding to the muscle receptor (Trémeau et al., 1995).

A second nonconservative substitution, Arg-52 in place of a leucine or isoleucine residue, is also not likely to significantly alter toxin potency. While a positively charged residue has not previously been reported at this sequence position, arginine and lysine residues are frequently found at the adjacent, nonconserved position 51. Furthermore, a recent recombinant study of erabutoxin a found that mutating Leu-52 to an alanine did not alter the affinity of erabutoxin a for the muscle AChR (Pillet et al., 1993).

The third nonconservative substitution is at position 32, where Taipan toxin 1 has a glycine and taipan toxin 2 has an isoleucine, in place of either a phenylalanine or histidine found at this position in all 46 previously reported short-chain α-neurotoxins. This is a critical position in the sequences of α-neurotoxins, since it is adjacent to the invariant Arg-33 that has been shown to be crucial for optimum functional activity in numerous studies (Endo & Tamiya, 1991; Pillet et al., 1993). The generally conserved histidine and phenylalanine residues bear some structural similarities, including heterocyclic ring structures, that are not shared by the residues present at this position in the taipan toxins. These nonconservative substitutions may thus result in reduced affinities of the taipan toxins for muscle and brain AChRs. Support for this hypothesis comes from the recombinant study of erabutoxin a. When Phe-32 in erabutoxin a was replaced with a leucine, binding affinity to the muscle receptor declined 7-fold compared with that of native toxin (Pillet et al., 1993). This loss of activity can be compared to the 5-fold difference in potencies for the muscle AChR measured in the present study between erabutoxin a and the two taipan toxins.

Taipan toxins 1 and 2 can be considered naturally occurring α-neurotoxin point mutants that provide a correlation of a discrete structural change with altered functional properties. Since these are the only α-neurotoxins identified in taipan venom, and since the toxins have different nonconserved substitutions at position 32, the possibility should be considered that these mutations represent some unknown advantage for the taipan in the capture of prey. It is known that some animals, including certain lizards, snakes, the mongoose, and the hedgehog, are very resistant to the effects of α-neurotoxins due to mutations in their muscle nicotinic receptors (Barchan et al., 1995). While the mutations in taipan toxins 1 and 2 reduce potency at chick muscle and brain receptors, they might have the opposite effect on nicotinic receptors in a particular α-neurotoxin-resistant prey species. Selective evolutionary mutations in some α-neurotoxins have led to a subfamily of postsynaptic neurotoxins termed the κ-neurotoxins that have a unique pharmacological specificity (Grant & Chiappinelli, 1985; Grant et al., 1988). The κ-neurotoxins are potent antagonists at several α-neurotoxin-resistant neuronal nicotinic receptors containing α3 or α4 subunits but have markedly reduced affinity for many muscle nicotinic receptors (Chiappinelli, 1993). Thus, the heterogeneity of nicotinic receptors can influence the evolution of snake toxins directed against them.

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