**“Weak Toxin” from Naja kaouthia Is a Nontoxic Antagonist of α7 and Muscle-type Nicotinic Acetylcholine Receptors**

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A novel “weak toxin” (WTX) from Naja kaouthia snake venom competes with \(^{[125]}\)I-bungarotoxin for binding to the membrane-bound *Torpedo californica* acetylcholine receptor (AChR), with an IC\(_50\) of \(-2.2 \mu M\). In this respect, it is \(-300\) times less potent than neurotoxin II from *Naja oxicana* and α-cobratoxin from *N. kaouthia*, representing short-type and long-type α-neurotoxins, respectively. WTX and α-cobratoxin displaced \(^{[125]}\)I-bungarotoxin from the *Escherichia coli*-expressed fusion protein containing the rat α7 AChR N-terminal domain 1–208 preceded by glutathione S-transferase with IC\(_50\) values of 4.3 and 9.1 μM, respectively, whereas for neurotoxin II the IC\(_50\) value was >100 μM. Micromolar concentrations of WTX inhibited acetylcholine-activated currents in *Xenopus* oocyte-expressed rat muscle AChR and human and rat α7 AChRs, inhibiting the latter most efficiently (IC\(_50\) of \(-8.3 \mu M\)). Thus, a virtually nontoxic “three-fingered” protein WTX, although differing from α-neurotoxins by an additional disulfide in the N-terminal loop, can be classified as a weak α-neurotoxin. It differs from the short chain α-neurotoxins, which potently block the muscle-type but not the α7 AChRs, and is closer to the long α-neurotoxins, which have comparable potency against the above-mentioned AChR types.

The first representative of “weak toxins,” characterized by low toxicity, was isolated from *Naja melanoleuca* snake venom and sequenced in 1975 (1). Toxins of this type were later referred to as melanoleucin (2) or miscellaneous-type (3) toxins. In protein data banks (e.g. Swiss-Prot) they are classified together with some other toxins in the group of weak toxins. At present, around 15 amino acid sequences of such toxins are known. They consist of 62–68 amino acid residues, contain five disulfide bridges, and belong to the group of snake venom “three-fingered” toxins, whose characteristic feature is the presence of three disulfide-confined loops (“fingers”) (4, 5). However, most other three-fingered toxins, including cardiotoxins, muscarinic toxins, acetylcholinesterase inhibitors, and the so-called short chain α-neurotoxins, contain four disulfide bridges. A fifth disulfide bridge is present in the long chain α-neurotoxins, such as α-bungarotoxin (αBgt) from *Bungarus multicinctus* or α-cobratoxin from *Naja kaouthia* (CTX) and κ-bungarotoxin (κBgt), which together with short α-neurotoxins are potent antagonists of different classes of nicotinic acetylcholine receptors (AChRs) (for reviews see Refs. 6–8). In the long chain α- and κ-neurotoxins the fifth disulfide bridge is located in the central loop II, whereas in weak toxins the additional disulfide is in the N-terminal loop I (see Fig. 1B). The available data show that residues of loops I, II, and III of α-neurotoxins participate in binding to AChRs (for reviews see Refs. 4, 5, and 7). However, depending on the type of α-neurotoxin (short or long) and AChR (muscle-type or neuronal α7), the role of particular loops may vary. For example, there are numerous data demonstrating the involvement of identified residues in the central loop II of short chain and long chain neurotoxins in binding both to muscle-type (9–12) and α7 AChRs (13). Interestingly, a fifth disulfide in loop II of the long α-neurotoxins is essential for binding to α7 AChR but not to the *Torpedo* AChR (14). The residues of loop I in short α-neurotoxins were shown to be involved in binding to the *Torpedo* receptor (15), whereas loop I in long α-neurotoxins appears to be less important for the interaction with *Torpedo* and α7 AChRs (11, 13).

In the present work, we decided to check whether a weak toxin isolated from *N. kaouthia* venom (designated WTX) acts on AChRs. WTX was found to bind to the membrane-bound *Torpedo californica* AChR, although much less efficiently than a short chain neurotoxin, NT-II, or a long one, CTX. However, WTX and CTX were virtually equipotent in binding to the soluble N-terminal domain of the rat α7 AChR expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST). Subsequent electrophysiological experiments revealed that in the micromolar range WTX blocked human and rat α7 AChRs as well as rat muscle AChR (16).

**EXPERIMENTAL PROCEDURES**

**WTX and Other Toxins**

WTX was purified from *N. kaouthia* as described previously (17). The molecular mass of WTX was determined by matrix-assisted laser desorption ionization time of flight on a Vision 2000 (Thermo Bioanalysis Corp.) mass spectrometer. This preparation (see Fig. 1) was used for primary structure determination (17) and for preliminary testing of biological activity. Further experiments were done on the WTX preparations obtained after additional purification steps by ion exchange chromatography on a TSK SP-5PW column (see Fig. 4).

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§ The abbreviations used are: Bgt, bungarotoxin; CTX, α-cobratoxin; ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; WTX, weak toxin from *N. kaouthia*; NT-II, neurotoxin II from *Naja oxicana*; GST, glutathione S-transferase; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; HPLC, high performance liquid chromatography.
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CTX was obtained from N. kauithia venom as described previously (18). NT-II was purified from the N. oxtina venom by gel filtration (under the same conditions as those used for N. kauithia venom) followed by ion exchange chromatography on Bio Rex 70 in a gradient of ammonium acetate from 0.05 to 0.3 M (pH 7.5). αBgt was purchased from Sigma. Radioactive [125I]αBgt was prepared and purified as described previously (19).

ACR Preparations

Membrane-bound nicotinic acetylcholine receptor from T. californica electric organ was kindly provided by Prof. F. Hucho. A soluble fusion protein consisting of the N-terminal domain 1–208 of rat α7 AChR preceded by GST, whose denatured form was first mentioned in Ref. 20, was isolated after expression of the respective cDNA in E. coli (data to be published elsewhere). Briefly, a gene fragment corresponding to amino acids 1–208 was cloned into the pGEX-KG vector (21) at the BamHI/HindIII sites, and the recombinant plasmids were used to transform the E. coli strain JM107. The inclusion bodies containing the target protein were washed extensively with 1% Triton X-100 in the presence of 0.5 M NaCl, then dissolved in 8 M urea, 1 mM dithiothreitol, and used to prepare a keyhole limpet hemocyanin conjugate, which was isolated after expression of the respective cDNA in E. coli strain JM107. The inclusion bodies containing the target protein were washed extensively with 1% Triton X-100 in the presence of 0.5 M NaCl, then dissolved in 8 M urea, 1 mM dithiothreitol, and then dialyzed against 10 mM Tris-HCl, pH 8.0, 0.1% CHAPS, concentrated by ultrafiltration, and then dialyzed against 10 mM Tris-HCl, pH 8.0, 0.1% CHAPS.

Binding Experiments

Membrane-bound AChR from T. californica—Varying concentrations of competitors were added to 50 μl of membrane suspension (25 μg of protein/ml, 50 mM Tris-HCl buffer, pH 8.0), and the mixture was incubated for 1 h at room temperature in a total volume of 195 μl. Then 5 μl of 0.4 μM [125I]αBgt (specific activity 45 Ci/mmol) were added, and the samples were incubated for 1 h more. The mixture was quickly filtered through Whatman GF/F filters (preincubated in 0.25% polyethyleneglycol), washed four times with 1 ml of 50 mM Tris-HCl buffer, pH 8.0, and the radioactivity was determined in a liquid scintillation counter (Amersham Pharmacia Biotech).

Electrophysiological Experiments

Electrophysiological recordings were made using a dual electrode voltage clamp (TEC 00, NPI Electronic GmbH, Tamm, Germany) as described previously (25, 26). Cells were placed in a plexiglass chamber, voltage-clamped at −80 mV, and superfused with normal frog Ringer’s solution (in mM: NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10, pH 7.2, adjusted with NaOH). ACh stock solution (0.1 mM) was kept frozen and added to the test solutions on the day of the experiment. Cells were treated with WTX by incubating in the measuring chamber with a WTX-containing normal frog Ringer’s solution for 20 min with perfusion stopped.

RESULTS

The WTX isolated by sequential gel filtration, ion exchange, and reverse-phase chromatography is homogeneous by the criteria of the latter and has a molecular mass of 7613 Da (Fig. 1A). However, this mass is larger than that (7483 Da) calculated from the published sequence of the weak toxin CM-9a from N. kauithia venom (2). There are no tryptophan residues in CM-9a, whereas determination of the primary structure of WTX (17) detected a Trp residue in position 36 and also revealed two other differences from the CM-9a sequence: Lys-50 and Tyr-52 instead of Lys and Tyr-52 in the CM-9a sequence (17). The molecular mass calculated for this sequence practically coincides with that found experimentally. Therefore, WTX, whose sequence is depicted in Fig. 1B, can be considered as a new homologue of CM-9a. When tested for a capacity to compete with [125I]αBgt for binding to the membrane-bound T. californica AChR, WTX was found to be about 300 times less potent than CTX and NT-II, the long chain and short chain α-neurotoxins (Fig. 2A and Table I).
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In addition to the muscle-type AChRs, the long neurotoxins αBgt and CTX also bind to neuronal α7 AChR. To assess this possibility for WTX as well, we used a bacterially expressed protein containing the N-terminal domain (amino acid residues 1–208) of the rat brain α7 receptor preceded by GST. This chimera faithfully reproduces a number of α7 AChR features (Fig. 2B and Table I). It interacts with αBgt and CTX as well as with the α7-targeting α-conotoxin ImI but practically does not bind either NT-II, a short-chain α-neurotoxin, or α-conotoxin G1 acting on the Torpedo and other muscle-type AChRs. In competition with [125I]αBgt for binding to the chimerical protein, WTX was as active as CTX (Table I).

To test whether WTX binding can also lead to a functional block of the native α7 AChRs, electrophysiological investigations on the α7 AChR expressed in Xenopus oocytes were performed. Incubation for 20 min in the presence of 10 μM WTX caused an almost complete block of the subsequent ACh-evoked current (Fig. 3A). Such an inhibitory potency is much weaker than that of CTX or αBgt, which block α7 AChRs in the nanomolar range (13, 27, 28), but is of the same order of magnitude as the IC50 of α-conotoxin ImI in blocking the rat α7 AChR (26, 29). We decided to check whether WTX is closer to αBgt/CTX or to α-conotoxin ImI in the reversibility of its blocking effect on the rat α7 AChR.

Superfusion with control medium following WTX blockade showed very slow recovery, taking more than 1 h (Fig. 3A). It is similar to the slow dissociation of αBgt (29) or CTX (see Ref. 30 and Fig. 6), whereas α-conotoxin ImI could be washed out almost completely in 10 min.

In view of the facts that WTX is washed out from oocytes as slowly as CTX but in terms of acting concentrations is about 1000-fold less potent, it was necessary to address the possibility that the effects on α7 AChRs in oocytes ascribed to WTX in fact might have arisen if traces of CTX, even as low as 0.1%, were present in the WTX preparations. Therefore, additional experiments were done to check and, if necessary, to increase the degree of purity of WTX.

Fig. 1A shows only one peak in the mass spectrum of WTX purified by reverse-phase HPLC. No sequences other than that of WTX were detected on Edman degradation (17). However, matrix-assisted laser desorption ionization analysis of a model peptide [125I]αBgt showed only one peak in the mass spectrum of WTX (Fig. 1B). The peptide [125I]αBgt was not detected in oocytes by autoradiography (18). The possibility that α-conotoxin ImI, which has a high degree of homology to the N-terminal region of WTX, was present in the WTX preparations was also addressed. An attempt was made to partially digest WTX with trypsin. Trypsin digestion of the purified WTX resulted in a peptide of approximately 1000 Mr, which corresponded to the molecular weight of α-conotoxin ImI (Fig. 1C).

When the pooled fractions of the repurified WTX (eluting between 45 and 65 min) were lyophilized and applied again to the same column (Fig. 4, trace 2), no peak was detected at the position of CTX. Some other minor contaminating peaks were still present, which was not surprising in view of the relatively large amount of WTX applied onto the column and the extremely high sensitivity of UV detection.

We checked for the presence of CTX traces in the repurified WTX with the aid of polyclonal antibodies raised against reduced and carboxymethylated CTX. As seen from Fig. 5, these
antibodies allow the detection of very low concentrations of CTX. Although at high concentrations (≥0.01 mg/ml) WTX also reacts with these antibodies, the difference in the affinities makes possible the estimation of the upper limit of putative CTX admixture in the repurified WTX. A virtually complete inhibition of the \( \alpha_7 \) AChR expressed in oocytes was observed at 20 \( \mu \)M (0.15 mg/ml) repurified WTX (see below, Figs. 3B and 7). As seen from Fig. 5, to this concentration of WTX corresponds an OD of -0.16. If we assume that it is only the CTX admixture in WTX that interacts with antibodies, this amount should be as low as \(-8.8 \times 10^{-6}\) mg/ml (See Fig. 5), i.e. not more than 0.006% of the WTX. Moreover, if there is a slight cross-reactivity of antibodies with WTX, the admixture should be even lower, if present at all.

Thus, immunological data confirmed that after additional purification by ion exchange HPLC, there are no measurable traces of CTX in WTX, at least in the range that would force one to ascribe the biological activity of WTX to CTX admixtures. Some of the binding experiments (Fig. 2) and more detailed studies in oocytes (Figs. 3, 6, and 7) were done with the repurified samples of WTX.

Additional purification had no effect on the WTX binding either to the Torpedo membranes or to \( \alpha_7 \) AChR fusion protein. In Fig. 2 the experimental points for the repurified WTX (marked as diamonds and asterisks) fit the displacement curves obtained with the preparation that did not undergo such a treatment.

Electrophysiological experiments on the additionally purified WTX gave similar results as those described above; the WTX concentration required to achieve a virtually complete block was 20 \( \mu \)M (cf. Fig. 3, A and B). It is also seen (Fig. 3C) that at 20 \( \mu \)M WTX reduces by about 60% the ACh-induced current in the human \( \alpha_7 \) AChR. In the same concentration range it efficiently blocks the rat muscle AChR expressed in Xenopus oocytes (Fig. 3D). As mentioned above, long chain \( \alpha \)-neurotoxins like dBgt or CTX block the \( \alpha_7 \) and muscle AChR in the nanomolar range, as illustrated in Fig. 3E for CTX action on the human \( \alpha_7 \) AChR.

From the washout experiments shown in Fig. 3, A and B, and, in more detail, in Fig. 6, it is clear that additional purification did not affect the virtual irreversibility of the WTX action. In this respect, it is practically indistinguishable from
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αBgt or CTX and differs greatly from α-conotoxin ImI, which can be washed out in minutes (Fig. 6).

Fig. 7 shows the dose-response data illustrating the WTX action on the rat and human α7 AChRs. Accurate quantitative measurements of these blocking effects on oocytes are difficult, especially at low toxin concentrations, because of the long incubation times required for the WTX to manifest its activity. We were able to estimate the IC50 value for the WTX action on the rat α7 AChR as 8.3 μM, whereas for the qualitatively less potent action on human α7 AChR we can give a very rough estimation of the IC50 value as ~15 μM.

**DISCUSSION**

Our initial purpose was to isolate from the *N. kaouthia* snake venom a weak toxin, CM-9a, the only weak toxin previously found in this venom (2), and try to find its biological target. However, because the determined molecular mass of the corresponding fraction, designated by us as WTX, differed by 130 Da from that calculated from the published amino acid sequence of CM-9a (2), we had to determine the complete primary structure of WTX (17). Whereas CM-9a, like all previously described weak toxins from cobra venoms, contains no tryptophans, WTX has a Trp residue at position 36 and differs from CM-9a in two other positions: Lys-50/Tyr-50 and Tyr-52/Lys-52 (see Fig. 1). Therefore, WTX appears to be a new homologue of CM-9a. These minor alterations in the primary structure may simply be due to collecting the venoms from different snake populations. This structural difference can, however, influence the biological activity of the toxins. It is known that the toxicity (LD50) of weak toxins can vary from about 5 to 80 mg/kg (2, 31). We did not determine the LD50 for WTX, but in doses up to 2 mg/kg (intravenous injection) it was nontoxic to rats. Interestingly, a recently isolated γ-bungarotoxin, structurally related to weak toxins (32), has an LD50 of 0.15 mg/kg, which is comparable with those of α-neurotoxins, but its molecular target has not been yet characterized.

As an attempt to define the biological targets for weak toxins, we examined the activity of WTX against two types of nicotinic acetylcholine receptors. As seen from Fig. 2A and Table I, WTX binds specifically to the membrane-bound *Toxopsis* AChR, but about 300 times less effectively than CTX or NT-II. A comparison of WTX with these two α-neurotoxins was done also with an α7 AChR-related system using a fusion protein, GST-α7-(1–208). Although this protein binds CTX with only a moderate affinity (Fig. 2B and Table I), the following data allow us to consider it to be an appropriate model of the rat α7 AChR ligand-binding domain. It does discriminate, similarly to the intact α7 AChR, between the long and short α-neurotoxins (no inhibition of [125I]αBgt binding by short neurotoxin NT-II at 100 μM) as well as between the muscle-type and neuronal α-conotoxins (no inhibition by α-conotoxin G1 at 100 μM and an IC50 of 42.6 μM for neuronal α-conotoxin ImI). Under these circumstances, the comparable inhibitory potencies of WTX and CTX (IC50 of 4.3 and 9.1 μM, respectively) allowed us to anticipate that WTX would bind specifically to the native α7 AChRs.

Indeed, subsequent electrophysiological recordings demonstrated the capability of WTX to inhibit functional responses of α7 AChRs expressed in *Xenopus* oocytes (Figs. 3, 6, and 7). Although WTX and CTX are virtually indistinguishable in their binding to the fusion protein GST-α7-(1–208) (Fig. 2B), WTX is about 1000-fold less potent than CTX in blocking the ACh current on the native α7 receptors heterologously expressed in *Xenopus* oocytes. As described under “Results,” additional purification (Fig. 4) and immunological control experiments (Fig. 5) allowed us to rule out the possibility that the observed WTX effects on the ACh currents in oocytes might be caused by traces of CTX, if it were present even in as small amounts as 0.1%.

During the revision of this paper, a weak toxin from the *Naja naja atra* snake venom was shown to block cholinergic transmission in frog muscle preparations in the micromolar range (33), and a similar activity on a chick muscle was reported for a weak toxin from *Bungarus candidus* (34). However, these two weak toxins were not tested against α7 AChRs, and the authors did not set a task to rule out the presence of trace amounts of the respective α-neurotoxins.

WTX, despite acting on the α7 AChR at micromolar rather than nanomolar concentrations, as do αBgt or CTX, is very similar to these two long chain α-neurotoxins in the persistency of the produced block (Fig. 6). In this respect it differs from α-conotoxin ImI and its analogs, which are also active in the micromolar range but can be washed out within minutes (26, 29) (see Fig. 6).

A number of amino acid residues and certain conformational motifs essential for binding to respective AChRs have been identified both in α-neurotoxins and α-conotoxins (4, 5, 11, 13, 35). The additional disulfide bridge in the CTX and in α-bungarotoxin is a prerequisite for their binding to neuronal AChRs, that is to α7 and α3β2, respectively (14, 36). This disulfide fixes a helix-like scaffold in the α-neurotoxin central loop II (37). A similar scaffold could be distinguished in α-conotoxin ImI acting on the α7 AChR as well as in the other α-conotoxins targeting the neuronal AChRs, but not in α-conotoxin G1 blocking the *Torpedo* and muscle AChRs (38). Interestingly, there are no α-helices or helix-like segments in the N-terminal loop I of the three-fingered toxins. A helix was found in the CD59 protein (39), which, like other members of the Ly-6 family of immune system proteins (40, 41), has a fifth disulfide in the N-terminal loop I, rather than in the loop II as do the long-type α-neurotoxins. The “endogenous toxin” lynx-1 recently found in the nervous system (42) was supposed to have a helix turn in loop I, but the conclusion was based only on modeling studies. However, although confirming by x-ray analysis the β-structure-dominated three-fingered motif of buncladin, a presynaptic neurotoxin, no helical turns were found in its loop I, containing the additional disulfide (43). Only β-structure was detected by 1H-NMR studies of WTX (44). It seems that specificity and selectivity of a particular three-fingered toxin toward various AChRs or other targets depends not only on the chemical nature and distribution of functional groups (charged, hydrophobic, etc.) along the three-fingered scaffold, but also on the differences in the conformation and conformational mobility of those loops. In particular, long times required to restore the sensitivity of the α7 AChR to ACh after applying and washing out WTX might be associated with the conformational heterogeneity of WTX (44) and conformational changes that WTX might undergo itself on binding to the receptor or induce in the latter. Noteworthy allosteric effects of antagonists and agonists, some of them influencing the channel moiety, were described for the *Torpedo* AChR (45, 46).

In summary, the results obtained suggest that the biological target of WTX, a member of the family of weak toxins, may be nicotinic AChRs. Interacting with the *Torpedo*, muscle, and α7 AChRs at similar concentrations, WTX resembles the long chain α-neurotoxins more than the short ones in this respect. Although acting only at micromolar concentrations, WTX has an advantage of being practically nontoxic while producing a long lasting effect. In view of the pharmacological importance of neuronal AChRs (for review see Ref. 47), a search for selective and efficient markers and blockers of those receptors is still an important task. Our work shows that the three-fingered
proteins of the “weak-toxin structural subtype” are promising in this respect.

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