Neurotoxins from Snake Venoms and α-Conotoxin ImI Inhibit Functionally Active Ionotropic γ-Aminobutyric Acid (GABA) Receptors*

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Background: Different snake venom three-finger toxins interact with various receptors, channels, and membranes.

Results: Here, we demonstrate that GABAα receptors are inhibited by α-cobratoxin, other long chain α-neurotoxins, nonconventional toxin from Naja kaouthia, and α-conotoxin ImI.

Conclusion: Some toxin blockers of nicotinic acetylcholine receptors also inhibit GABAα receptors.

Significance: Three-finger toxins offer new scaffolds for the design of GABAα receptor effectors.

Ionotropic receptors of γ-aminobutyric acid (GABAαR) regulate neuronal inhibition and are targeted by benzodiazepines and general anesthetics. We show that a fluorescent derivative of α-cobratoxin (α-Ctx), belonging to the family of three-finger toxins from snake venoms, specifically stained the α1β3γ2 receptor; and at 10 μM α-Ctx completely blocked GABA-induced currents in this receptor expressed in Xenopus oocytes (IC50 = 236 nM) and less potently inhibited α1β2γ2 = α2β2γ2 > α5β2γ2 > α2β3γ2 and α1β3δ GABAαRs. The α1β3δ2 receptor was also inhibited by some other three-finger toxins, long α-neurotoxin Ls III and nonconventional toxin WTX. α-Conotoxin ImI displayed inhibitory activity as well. Electrophysiology experiments showed mixed competitive and noncompetitive α-Ctx action. Fluorescent α-Ctx, however, could be displaced by muscimol indicating that most of the α-Ctx-binding sites overlap with the orthosteric sites at the β/α subunit interface. Modeling and molecular dynamic studies indicated that α-Ctx or α-bungarotoxin seem to interact with GABAαR in a way similar to their interaction with the acetylcholine-binding protein or the ligand-binding domain of nicotinic receptors. This was supported by mutagenesis studies and experiments with α-conotoxin ImI and a chimeric Naja oaxiana α-neurotoxin indicating that the major role in α-Ctx binding to GABAαR is played by the tip of its central loop II accommodating under loop C of the receptors.

Type A γ-aminobutyric acid receptor (GABAαR) is abundantly expressed across the nervous system. Most of the GABAαRs in the brain are constructed from α1–6 and β1–3 subunits co-assembled with γ1–3 or δ/ε/π/θ subunits. Interestingly, some β subunits, usually found in heteropentameric GABAαRs could also form constitutively active homopentameric ligand-gated ion channels. Channels with such properties were found in cultured neurons (1), and in fact the β3-homopentamer at the moment is the only GABAαR with known three-dimensional structure (2).

GABAαRs are targeted by diverse toxins of low molecular weight, such as the channel blocker picrotoxin (3), a polycysteinic compound oenanthotoxin (4), the GABA site antagonist bicuculline (5), and by GABA site agonists such as ibotenic acid and muscimol. Benzodiazepines are positive allosteric modulators of GABAαRs and were the world’s most prescribed drugs in the 1980s (6). It is almost impossible to introduce fluorescent labels into such molecules, and therefore, they cannot be used in live cell imaging studies. In contrast, peptide toxins can easily be tagged with fluorescent or radioactive labels. Many of them, for example α-conotoxins from Conus snails and three-finger toxins (TFTs) from snake venoms, target nicotinic acetylcholine receptors (nAChRs) and show high levels of selectivity between the different nAChR types (7). For example, α-bungarotoxin (α-Bgt) from multibanded krait venom binds with nanomolar affinity to the muscle-type and α7 receptors but not to the heteropentameric neuronal nAChRs (8, 9). Such peptide toxins also could be quite helpful in structural studies. The x-ray crystal structure of the extracellular domain of α1 (10)

*The abbreviations used are: GABAαR, type A γ-aminobutyric acid receptor; AChBP, acetylcholine-binding protein; α-Bgt, α-bungarotoxin; α-Ctx, α-co-bromatoxin; GlyR, glycine receptor; nAChR, nicotinic acetylcholine receptor; TFT, three-finger toxin; PDB, Protein Data Bank; NT II, neurotoxin II; Ls III, α-elapitoxin-Ls2a.
Polypeptide Neurotoxins Inhibit Functional GABA\(_A\) Receptors

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![Diagram of Structural diversity of three-finger neurotoxins. Top set represents sequence alignments. All TFTs have three loops stabilized by four disulfides, but some neurotoxins have one additional disulfide bond. Long (names highlighted green) and weak or nonconventional (names highlighted pink) neurotoxins have additional disulfide bonds in the second and the first loops, respectively, although short neurotoxins (name highlighted yellow) do not have additional disulfides. Bottom set represents typical spatial structures of three toxin groups as follow: long neurotoxin α-Ctx (PDB code 1CTX), short neurotoxin NT II (PDB code 1NOR), and nonconventional or weak toxin, here represented by the published structure of candoxin (PDB code 1JGK) that shares homology with WTX (N. kaouthia nonconventional toxin) and OWT (N. oxiana nonconventional toxin). Sequence alignments were produced via Clustal W algorithm; signal peptide sequences were removed manually, and top line shows numbering of α-Ctx residues. Backbones are shown in gray; β-strands are shown as arrows; cysteine residues, forming disulfide bonds are shown as yellow sticks on structures and highlighted orange in sequences, loop II arginine and valine residues in sequences are shown in blue and green, respectively.

and α9 (11) nAChR subunits were obtained in a complex with α-Bgt.

TFTs form an abundant group of proteins found in Colubridae, Elapidae, and Psammophiinae snake venoms. Members of this group possess no enzymatic activity and bind to receptors from different groups as follow: nAChRs (short and long chain α-neurotoxins), muscarinic acetylcholine receptors (African mamba-derived muscarinic toxins), L-type calcium channels (calciceptine from black mamba venom); and ASIC1, acid-sensitive ion channel (mambalgines). There are also three-finger cytotoxins (cardiotoxins) that interact with the cell plasma membrane (12).

Typical members of the long-chain group of TFTs, such as α-Bgt from Bungarus multicinctus as well as α-cobratoxin (α-Ctx) from Naja kaouthia cobra, neurotoxin NT I from Naja oxiana, and LsIII from marine krait Laticauda semifasciata, have an additional disulfide bond in loop II (Fig. 1, bottom left). This disulfide has been identified as a crucial feature for binding at the neuronal nAChR intersubunit sites (13, 14). Short neurotoxins, such as Naja oxiana neurotoxin II (NT II), acting exclusively on muscle nAChRs, do not have this additional disulfide (Fig. 1, bottom center). Another group, closely related to α-neurotoxins, is the group of so-called nonconventional or weak neurotoxins. In contrast to the previous two groups, weak toxins have an additional disulfide bond in the first loop (Fig. 1, bottom right), and show both weaker affinity toward nAChRs and some cross-reactions with other receptors (15). The toxins listed above block nAChRs with affinities that range from nanomoles to tens of micromoles/liter, and α-Bgt is the most active (Table 1). Interestingly, evidence for α-Ctx inhibition of T-type calcium channels through muscarinic receptors was recently provided (16), which may indicate that α-Ctx also has biological targets other than nAChRs.

Fluorescent α-Bgt is widely used in applications concerning nAChR location and expression (17–19). Moreover, the “bungarotoxin-binding site” comprising a fragment of the α1 nAChR could be incorporated into the extracellular loops of different receptors allowing imaging of the receptors on the surface of living cells with the fluorescently labeled α-Bgt, which in particular also has been done for the GABA\(_A\)R (20, 21). Using fluorophore-conjugated α-Bgt, about a decade ago the first evidence of α-Bgt binding to GABA\(_A\)R was obtained (22). However, it was suggested to be limited to the peculiar β3/β3-binding sites, whose existence in vivo in the wild-type organisms has not been unambiguously demonstrated. This work aroused our interest in searching for novel GABA\(_A\)R ligands among snake venom components structurally similar to α-Bgt.
namely among various TFTs, as well as among α-conotoxins, another class of nAChR inhibitors.

Here, we report specific labeling of the α1β3γ2 subtype of GABA<sub>R</sub> with a fluorescent derivative of α-Ctx, as well as inhibition of GABA-induced currents in a series of GABA<sub>R</sub>Ss by well known TFTs belonging to different structural groups and exhibiting anticholinergic properties; interaction of α-conotoxin Iml with α1β3γ2 GABA<sub>R</sub> was found as well.

**Experimental Procedures**

*Three-finger Toxin Preparations*—Ls III was from Latoxan (Valence, France). WTX, α-Ctx, and NT II were obtained as described previously (23–25) and OWT as described previously (26). NT I was obtained as described previously (27) with additional purification by reversed phase HPLC. α-Bgt was purified from *B. multicinctus* venom by combination of gel filtration, ion-exchange, and reverse-phase HPLC, and α-Bgt Val-31 analogue was used in this work. The structures of toxins obtained were confirmed by mass spectrometry. Chimeric NT II/I toxin with the tip of the second loop from NT I grafted to the NT II structure was obtained as described previously (14).

*Peptide Chemical Synthesis*—Peptide WCDACFSIRGKR from the α-Ctx loop II and α-conotoxin Iml was synthesized as described previously (28).

*Electrophysiology*—Oocytes were obtained from healthy mature (＞10 cm) *Xenopus* frogs. After manual separation, oocytes were injected with 2–3 ng of laboratory PCI vector containing DNA sequences encoding mouse GABA<sub>R</sub>Ss by well known TFTs belonging to different structural groups and exhibiting anticholinergic properties; interaction of α-conotoxin Iml with α1β3γ2 GABA<sub>R</sub> was found as well.

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obtained was incubated with DpnI enzyme to restrict the methylated DNA and then transformed into XL1-Blue supercompetent cells. The mutations were confirmed by DNA sequencing.

Results

Long-chain α-Ctx and Naja oxiana α-Neurotoxin NT I Compete with Fluorescent α-Bgt for GABA\(_{\alpha}\)-R-binding Site—First of all, we decided to check whether α-Ctx could displace a fluorescent α-Bgt derivative from α1β3 GABA\(_{\alpha}\)-R. We reproduced (Fig. 2A) fluorescent Alexa Fluor 555 α-Bgt binding to (presumably) the β3/β3-interface of α1β3 receptors first reported by McCann et al. (22). α-Ctx at 5 μM concentration competed with fluorescent α-Bgt and displaced it almost completely from the receptor. Interestingly, *Naja oxiana* NT I (see Fig. 1 for sequence alignments) was also an effective competitor; it diminished Alexa Fluor 555 α-Bgt binding by 91 ± 2% (experiments not shown).

Fluorescently Labeled α-Ctx Can Be Used for Imaging of Live Cells Expressing GABA\(_{\alpha}\)-R—To study α-Ctx competition with α-Bgt in detail, we tested the applicability of α-Ctx fluorescent derivative in cytochemical experiments for imaging of living cells heterologously expressing α1β3 and α1β3γ2 GABA\(_{\alpha}\)-R. α-Ctx bearing Alexa Fluor 546 fluorophore has been chosen due to similar spectral properties to commercially available Alexa Fluor 555 α-Bgt. It should be noted that we had no intention of registering simultaneously Alexa Fluor 555 α-Bgt and Alexa Fluor 546 α-Ctx binding. Both ligands (Alexa Fluor 555 α-Bgt and Alexa Fluor 546 α-Ctx) brightly stained cells expressing either α1β3 (Fig. 2, A and B) or α1β3γ2 subunit combinations (Fig. 2, C and D). The actual expression of the γ2-subunit in α1β3γ2 receptors was confirmed in patch clamp experiments with diazepam as discussed below (Fig. 4A). Binding of fluorescent toxins was then blocked by unlabeled toxins (α-Ctx or α-Bgt) to determine specificity of staining. Interestingly, only for α1β3-expressing cells stained by Alexa Fluor 555 α-Bgt (Fig. 2E), we detected no difference in efficiency of fluorescence block by 5 μM toxins (α-Bgt or α-Ctx). Fluorescence of α1β3-expressing cells stained by Alexa Fluor 546 α-Ctx, as well as α1β3γ2-expressing cells stained by either Alexa Fluor 555 α-Bgt or Alexa Fluor 546 α-Ctx, was more effectively blocked by 5 μM α-Ctx (Fig. 2, F–H).

Snake Venom Neurotoxins from Different Structural Groups Inhibit GABA\(_{\alpha}\)-R—To test whether α-Ctx and other toxins could inhibit α1β3γ2 GABA\(_{\alpha}\)-R, we performed two-electrode voltage clamp experiments. It was found that α-Ctx, as well as Ls III (long-chain α-neurotoxin from *Laticauda seminasciata*), and nonconventional toxin WTX from *Naja kaouthia* venom inhibit the GABA\(_{\alpha}\)-Rs in a dose-dependent manner (Fig. 3). Given at 10 μM Ls III and WTX blocked GABA-evoked response by 83 ± 12 and 31 ± 8%, respectively. Long-chain NT I and α-Bgt at 10 μM inhibited α1β3γ2 receptors only by 17 ± 5
and 19 ± 3%, respectively. No inhibition was observed for short-chain neurotoxin NT II and nonconventional toxin OWT, both from *N. oxiana* venom, at concentrations of 10 μM. Interestingly, α-Ctx (5 μM) also inhibited α1β3δ receptor (Fig. 3).

To characterize more broadly the α-Ctx potency and selectivity, we tested it on GABAAR with different subunit combinations. First of all, we investigated the potency of α-Ctx for inhibition of GABA-induced currents at α1β3 and α1β3γ2.

FIGURE 3. Effects of different three-finger toxins on GABA-evoked currents. Electrophysiological recordings demonstrate that three-finger toxins can inhibit GABAAR. GABA was applied alone or co-applied with the indicated toxin to oocytes injected with cDNA encoding α1, β3, and γ2 subunits. First peak of each set represents control (contr.) with a 1-s application of 10 μM GABA alone, corresponding to EC50. The concentration of co-applied toxin is indicated above the recording. The traces marked as wash represent the response to GABA after 5 min of oocyte washing with the buffer solution. The lower right inset shows inhibition of α1β3δ receptor by α-Ctx at 5 μM.

FIGURE 4. Inhibition of different GABAAR subtypes by α-Ctx and inhibition of Alexa Fluor 546 α-Ctx binding on α1β3γ2 by muscimol. A, inhibition of α1β3 and α1β3γ2 GABAARs by α-Ctx. The presence of γ subunit is confirmed by the effect of the benzodiazepine 1 μM diazepam (BZD) and differential sensitivity to 50 μM Zn2⁺ (see insets). Absence of a γ subunit influence on α-Ctx inhibition is consistent with the assumption that the α-Ctx-binding site is located at an interface between α and β subunits. B, this toxin manifests modest subtype selectivity; it inhibits α1β3γ2 receptor five times more potently than α2β2γ2. The potency of α-Ctx against diverse sets of GABAAR subunits was examined using the two-electrode voltage clamp method. To measure the effect of different α-Ctx concentration, α-Ctx was applied alone to the oocyte for 5 min before control GABA application and then was co-applied with GABA (10 μM, EC50). Current amplitudes obtained in the presence of α-Ctx were expressed as percentage of control amplitudes and plotted against logarithm of toxin molar concentration. C, GABA dose-response curve at α1β3γ2 receptors without α-Ctx (upper curve) and in the presence of 250 nM α-Ctx (lower curve). GABA EC50 is shifted to the right and the Hill slope is reduced, suggesting noncompetitive or some mixed competitive/noncompetitive type of inhibition. D, muscimol competes with Alexa Fluor 546 α-Ctx binding sites on orthosteric sites located at the β/α interface. Fluorescence data were normalized to the difference between mean stained cell fluorescence and mean cell fluorescence blocked by 10 μM α-Ctx (i.e. data represent specific α-Ctx binding). Data were fitted to a dose-response equation using the Origin 7.5 software.
receptors. As expected, GABA-induced currents at α1β3 receptors were completely inhibited by 50 μM Zn^{2+} in contrast to those at α1β3γ2 receptors (Fig. 4A, insets). The incorporation of a γ2 subunit in α1β3γ2 receptors was further demonstrated by their sensitivity to 1 μM diazepam. Interestingly, α-Ctx was able to completely inhibit diazepam-stimulated GABA currents, again demonstrating that α-Ctx is able to also block the actions of α1β3γ2 receptors (Fig. 4A, insets). As shown in Fig. 4A, α-Ctx exhibited a comparable potency for inhibition of these two receptor subtypes, indicating that the γ2 subunit does not influence the potency of α-Ctx.

Then we investigated the effects of α-Ctx at α1β2γ2, α1β3γ2, α1β2γ2, α2β3γ2, and α5β2γ2 receptors (Fig. 4B). α-Ctx exhibited a differential potency for inhibition of GABA-induced currents at the individual GABA_A receptors. IC50 values for each of them are shown in Table 2. The potency of α-Ctx depended on the types of α and β subunits present in the receptor, supporting the conclusion that the α-Ctx-binding site is located at an interface between α and β subunits. In contrast to the strong inhibition of α1β3γ2, long-chain Ls III (10 μM) inhibited α5β2γ2 and α2β2γ2 only by 13 ± 5 and 39 ± 9%, respectively (experiments not shown).

Electrophysiology Measurements Indicate That α-Ctx Inhibits GABA_R in a Mixed Competitive/Noncompetitive Manner—To shed light on the α-Ctx mode of inhibition, we compared the GABA dose-response dependence (for α1β3γ2 subtype) in the presence of 250 nM α-Ctx. As expected, GABA-induced currents at α1β3γ2 receptors were completely inhibited by 50 μM Zn^{2+} in contrast to those at α1β3γ2 receptors (Fig. 4A, insets). The incorporation of a γ2 subunit in α1β3γ2 receptors was further demonstrated by their sensitivity to 1 μM diazepam. Interestingly, α-Ctx was able to completely inhibit diazepam-stimulated GABA currents, again demonstrating that α-Ctx is able to also block the actions of α1β3γ2 receptors (Fig. 4A, insets). As shown in Fig. 4A, α-Ctx exhibited a comparable potency for inhibition of these two receptor subtypes, indicating that the γ2 subunit does not influence the potency of α-Ctx.

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TABLE 2

| Subunit combination | EC50 ± S.E. (95% confidence interval) | IC50 ± S.E. (95% confidence interval) |
|---------------------|-------------------------------------|-------------------------------------|
| αβ2γ2               | 35 ± 1 (33, 36)                     | 469 ± 23 (427, 515)                 |
| αβ3γ2               | 29 ± 7 (19, 44)                     | 236 ± 7 (223, 250)                  |
| αβ2γ2               | 45 ± 1 (43, 47)                     | 485 ± 39 (417, 564)                 |
| αβ3γ2               | 12 ± 1 (11, 13)                     | 1099 ± 57 (1016, 1088)              |
| α5β2γ2              | 20 ± 2 (17, 24)                     | 635 ± 91 (489, 825)                 |

FIGURE 5. A, structural similarity of α-conotoxin Iml, selective for α7 nAChR, to the tip of the α-Ctx loop II (magenta). Note that two positively charged arginines (highlighted red) are exposed in a very similar manner with respect to aromatic residues (tryptophan or phenylalanine, highlighted green) in both Iml (shown wheat) and loop II of α-Ctx (shown blue). Two different views (1st and 2nd rows) are provided for convenience. Amino acid sequences of α-conotoxins SIA and MII differ greatly from that of Iml (bottom). B, chemically synthesized WCDAFCSIRGKR peptide that mimics tip of α-Ctx loop II and α-conotoxin Iml at 100 μM inhibits 10 μM GABA-evoked current (upper left and right traces, respectively). SIA specific for muscle type nAChR and MII specific for heteromeric neuronal nAChRs have no effect on the currents at 100 μM.
nAChR. We decided to test two other conotoxins: SIA that binds to muscle nAChR and MII that binds to neuronal non α7 nAChRs. α-Conotoxin ImI (see Fig. 5A for structure) inhibited the α1β3γ2 GABA<sub>R</sub> at 100 μM by 45 ± 5% in terms of GABA-evoked peak current (Fig. 5B). α-Conotoxins SIA and MII failed to show inhibition of GABA<sub>R</sub> at 100 μM (Fig. 5B, bottom). This experiment also proves specificity of inhibition, and because 100 μM is a rather high concentration, we had to make some control experiments with peptides chemically similar to ImI at such a high concentration.

We also performed experiments with the synthetic peptide WCDAFCSIRGKR comprising the tip of loop I and loop II also formed contacts with that of the AChBP protomer (PDB code 1YI5). The structure obtained was subjected to 100 ns of molecular dynamics. The last 40-ns complex was stable, and its backbone root mean square deviation did not exceed 0.1 nm. By the end of simulation, the complex retained the overall “TFT to Cys-loop” binding mode known from the x-ray structures (Fig. 6A) with the toxin loop II buried under loop C of the receptor. Interestingly, α-Ctx residues Arg-33 and Arg-36 form a stable network of salt bridges with both Asp-27 and Glu-155 (Fig. 6B), which was present during all simulation times. The toxin C-terminal region and tips of loop I and loop II also formed contacts with both dimer subunits.

**Electrophysiological Analysis of Chimeric NT II/I**—To verify the model, we decided to test whether the loop II of the toxins plays a crucial role in functional inhibition of GABA<sub>R</sub>. In previous studies, short-chain toxin NT II with grafted loop II from long neurotoxin I was used to study the role of loop II in α-neurotoxin interaction with neuronal nAChRs (14). We used the same chimera in electrophysiological experiments on GABA<sub>R</sub>. Short-chain toxin NT II at 10 μM did not show any inhibition of the receptor but gained the inhibitory activity upon the grafting of loop II from long neurotoxin NT I (Fig. 7A). Chimeric NT II/I toxin and NT I generated a comparable inhibition of α1β3γ2 receptors (16 ± 3 and 19 ± 3%, respectively, Fig. 7B). Molecular dynamics simulations indicated that the model of NT II was unable to form a complex with the GABA<sub>R</sub> orthosteric intersubunit site in the manner similar to α-Ctx.
Polypeptide Neurotoxins Inhibit Functional GABA_A Receptors

FIGURE 7. Delineation of the active site in TFTs interacting with GABA_A Rs. A, loop II peptide fragment CDAWCGS of NT I was grafted to NT II structure, replacing the corresponding SDH sequence (see Fig. 1 for sequence alignment); B, normalized GABA-evoked currents (10 μM of GABA, α1β3γ2 GABA_A R) in the presence of wild-type NT I, NT II, and chimeric NT II/III; C, normalized GABA-evoked currents (10 μM of GABA, α1β3γ2 GABA_A R) in the presence of wild-type NT I and NT II/III chimeric toxin show current inhibition by 19 ± 3 and 16 ± 3%, respectively. Asterisks indicate significant inhibition (p < 0.01, n = 6, paired t-test). C, last frame of a 100-ns run of molecular dynamics simulation of NT II complex with β3/α1 extracellular domains. NT II failed to reproduce mode of binding shown by α1-Ctx model. D, molecular model of chimeric NT II/III toxin complex with β3/α1 extracellular domains. Note that the tip of the loop II (which was grafted to the short toxin NT II molecule from long NT I toxin) rests under the C loop of β3 subunit.

(Fig. 7C). In contrast, the model of NT II/III formed a stable complex with loop II resting under the C-loop of β3 extracellular domain model (Fig. 7D).

Single Point Mutations α1(S67K) and α2(K67S) Located at the Complementary Surface of the Respective α-Subunit Change Inhibition by α1-Ctx—Because we observed modest α1-Ctx selectivity toward α1β3γ2 (Table 2), we decided to test our model by introduction of α1→α2 and α2→α1 point mutants. Loop II of the toxin carries a significant positive charge, and we hypothesized that addition or deletion of a positive charge at position 67 of α1 and α2 subunits, located in close vicinity to the hypothetical toxin-binding site and occupied by a serine residue (Fig. 8A), might diminish affinity to α1-Ctx in the α1(S67K) mutant and increase affinity toward α2(K67S) mutant. Indeed, we found that the α1-Ctx IC_50 value for α1(S67K)β3γ2 GABA_A R (703 ± 39 nM) is closer to the α2-containing receptor (1099 ± 57 nM, Table 2) than the α1-containing receptor (236 ± 7 nM, Table 2), and vice versa the α1-Ctx IC_50 value for α2(K67S)β3γ2 GABA_A R (442 ± 54 nM) is closer to the α1-containing receptor than the α2-containing receptor (Fig. 8B). At the same time GABA EC_50 values were not affected by these mutations.

Discussion

There has been considerable progress in understanding the structure and function of GABA_A Rs (38). Notwithstanding, there are still significant gaps in our knowledge on peptide and proteins interacting with GABA_A Rs. In general, polypeptide ligands manifest higher selectivity to particular receptor subtypes as compared with low molecular weight organics. Several polypeptides interacting with GABA_A Rs have been reported earlier that include α1-Bgt (22), diazepam-binding inhibitor protein, and its processing fragments such as the octadecapeptide (39, 40). Given a plethora of different GABA_A R subunits, it seems surprising that there is such a paucity of known proteins interacting with the receptor. For example, a great deal of polypeptide toxins is known for nAChR, closely related to GABA_A R. Here, for a series of GABA_A R subtypes, we report specific fluorescent labeling and inhibition of GABA-induced ion currents by several protein toxins from snake venoms.

As a starting point, we reproduced α-Bgt binding to the α1β3 GABA_A R reported in Ref. 22 to obtain a verified test system of binding. As expected, long-chain α-neurotoxin α1-Ctx, closely related to α-Bgt, showed competition with α-Bgt binding. We then investigated whether a fluorescently labeled α1-Ctx, could stain cells expressing α1β3γ2 or α1β3 GABA_A R. We found that Alexa Fluor 546 α-Ctx, as well as Alexa Fluor 555 α-Bgt, were able to stain both types of cells (Fig. 2, A–D). Whereas α1-Ctx blocks Alexa Fluor 555 α-Bgt binding at α1β3-transfected cells (Fig. 2E) as effectively as α-Bgt, Alexa Fluor 555 α-Bgt or Alexa Fluor 546 α-Ctx staining of α1β3- or α1β3γ2-transfected cells was blocked more effectively by α1-Ctx (Fig. 2, F–H). This observation suggests that GABA_A Rs with different subunit combinations bear binding sites with slightly different properties.
To investigate how α-Ctx and other anticholinergic TFTs might influence GABAAR function, we performed electrophysiology studies of αβγδ, αβγτ, αβδτ, and αβδθ GABAAR subunit sets expressed in Xenopus oocytes. It was found that α-Ctx blocks αβγτ with a relatively high potency (IC50 = 236 nM, Fig. 4A and B) and other receptor subtypes with lower potencies, namely in submicromolar to low micromolar range (Fig. 4B). It is worth noting that α-Ctx manifests some selectivity toward α1 as compared with the α2 subunit only in the presence of the β3 subunit, whereas in the presence of the β2 subunit it does not show noticeable selectivity to any α subunit investigated. This suggests that the α-Ctx-binding site might be located at the interface between the β and α subunit. Other tested TFTs were less active, and their potencies were not measured. However, it is clear that Ls III and WTX are active in the micromolar range, whereas NT II and OWT do not show any inhibition of αβγτ receptor at 10 μM.

Electrophysiology measurements indicated that α-Ctx (for the αβγτ subtype) shifted GABA dose-response curve to the right, at the same time changes in the slope and maximally achieved current were observed (Fig. 4C). Such a picture is typical of noncompetitive antagonists. However, we found that muscimol, acting as an agonist of GABAAR, but not the allosteric modulator diazepam, displaced Alexa Fluor 546 (see Fig. 5A) of α-conotoxin ImI to the α-Ctx loop II (magenta, space-filling model). Inset shows that, according to our molecular modeling, by being mutated to lysine (as in α2 subunit) this residue comes close to the lysine of the α-Ctx loop II (dark blue). Inset shows that, according to our molecular modeling, by being mutated to lysine (as in α2 subunit) this residue comes close to the lysine of the α-Ctx loop II. Therefore, even molecules with structural similarity to the α-Ctx loop II could inhibit GABAAR. GABA was given at 10 μM, which is slightly less than EC50. Data points are expressed as percentage of control amplitudes (mean peak current ± S.E.).

The tip of loop II is the most important part of α-Ctx and other TFTs for binding to AChBP and nAChRs (41). To test whether this region is also involved in the interaction with GABAAR, we applied mutant NT II with loop II drafted from NT I. This chimeric toxin (14) seems to be a suitable candidate because we have not detected any inhibition of GABAAR by NT II at 10 μM, but we observed some inhibition by NT I at the same concentration. Upon grafting of the NT I loop II fragment bearing a disulfide loop to the short neurotoxin NT II, the latter became functionally active against the receptor, strongly supporting the key role of loop II in TFTs in the interaction with GABAAR. Thus, we conclude that the tip of the loop II of TFTs plays a crucial role in GABAAR binding and inhibition.

Previously, it was suggested that some structural similarity (see Fig. 5A) of α-conotoxin ImI to the α-Ctx loop II may contribute to its binding to the neuronal nAChRs (28). Electrophysiology revealed that ImI inhibited GABAAR at 100 μM (Fig. 5B), whereas muscle nAChR-specific SIA and heteromeric nAChR-specific MII, lacking α-Ctx-similar features, were ineffective (Fig. 5B, bottom). Therefore, even molecules with structural similarity to the α-Ctx loop II could inhibit GABAAR. These data indicated that α-conotoxin ImI represents another type of polypeptide compounds able to interact with GABAAR. Moreover, synthetic peptide representing the sequence WCDACFSIRGRK of the α-Ctx loop II tip also inhibited GABAAR (Fig. 5B). Despite the fact that we observe only weak inhibition at a relatively high concentration, short peptide GABAAR ligands on the base of loop II peptide or ImI could represent a good starting point for rational design of more specific ligands.

Our findings suggest that α-Ctx binds to the β/α interface and the loop II tip plays a crucial role in its binding. Given that, we constructed a molecular model of α-Ctx bound to the β3/α1 dimer of extracellular domains and performed 100-ns molecular dynamics studies in implicit solvent. Loop II residues 25–36 rested stably at the site under the loop C of the β3 subunit (Fig. 6A). With respect to β3 subunit backbone, root mean square deviation of these residues have not exceeded 0.15 nm, suggest-
Polypeptide Neurotoxins Inhibit Functional GABA<sub>A</sub> Receptors

ing that such a complex might exist in principle. Interestingly, we found that both Arg-33 and Arg-36 residues in α-Ctx form salt bridges with α-Ctx Asp-27 and β3 Glu-155 (Fig. 6B). Arg-36 residue is unique to α-Ctx among all tested toxins, and its substitution by hydrophobic residues in other toxins (Fig. 1) could explain their weaker GABA<sub>A</sub>R inhibiting activity. Molecular modeling of NT II interaction with the β3/α1 dimer revealed inability of this short toxin to form a complex with the orthosteric site of GABA<sub>A</sub>R (Fig. 7C). In contrast, a molecular model of the NT II/1 chimeric toxin showed properties very close to that of α-Ctx complex model with the β3/α1 dimer. In particular, loop II, grafted to the NT II scaffold from NT I, formed stable contacts with the C-loop of the β3 subunit extracellular domain (Fig. 7D).

It should be noted that previously we demonstrated that upon modification of α-Ctx with NHS ethers, the major product is the one with the label attached at Lys-23 (42). This lysine residue is in loop II, which according to our model takes part in receptor binding. However, it is not one of the deeply buried residues, and its modification does not necessarily disrupt α-Ctx binding, as indicated by the interaction of modified α-Ctx with the nAChRs (42).

In electrophysiological studies, we observed a somewhat more potent inhibition of α1β3γ2 over α2β3γ2 GABA<sub>A</sub>R. Because both α1 and α2 subunits share extremely high sequence homology, the respective receptors should differ only slightly in their extracellular parts. To test the above-described model, as a candidate for single-point mutagenesis we chose the Ser-67 residue (numbering of residues is given according to the homology model of extracellular domain) situated at the β/α1 interface. It is substituted by a lysine residue in the α2 subunit, and this positively charged residue could confer the difference in the α-Ctx affinity toward these two receptors, because α-Ctx loop II bears significant positive charge itself. This mutation was done, and the α-Ctx potency at α1(S67K)β3γ2 GABA<sub>A</sub>R was diminished, approaching that at α2β3γ2 (Fig. 8).

When our manuscript was ready for submission, two papers (43, 44) appeared, clearly demonstrating the perspective of TFTs for research on GABA<sub>A</sub>Rs. In Ref. 43, the authors analyzed in detail the interactions of α-Bgt and its fluorescent derivative with GABA<sub>A</sub>R in hippocampal neurons and with recombinant GABA<sub>A</sub>R subtypes, comparing their effects on ion currents and staining patterns in the presence of diverse ligands (agonists and antagonists) both with GABA<sub>A</sub>Rs and nAChRs. Their conclusion was that α-Bgt inhibits various functional GABA<sub>A</sub>R subtypes with different efficiency, with the α2β2γ2 receptor being inhibited most potently. Here, we show that the preferred target of α-Ctx is the α1β3γ2 receptor, indicating that diverse α-neurotoxins may have different affinity for distinct GABA<sub>A</sub>R subtypes and might be able to distinguish between various GABA<sub>A</sub>R subtypes. Concerning the mode of α-Bgt binding, Hannan et al. (43) described it as “a mixed inhibitory manner.” It is quite clear that further work is necessary to obtain a high resolution picture for recognition of GABA<sub>A</sub>R by both α-Bgt and α-Ctx.

Interestingly, in Ref. 43 it was also found that α-Bgt inhibited spontaneous channel openings in GABA<sub>A</sub>R composed of α4 and δ subunits, but in the presence of GABA the toxin behaved as a positive allosteric modulator. Such effects were not observed in this study, supporting our observation (Fig. 2) that different toxins, dependent on the receptor composition, might exhibit different effects. However, α-Bgt was found to bind at the orthosteric site (43). It is thus possible that binding of toxins to one of two orthosteric sites could lead to an allosteric change in the conformation of the second site and that the functional consequences of these allosteric interactions might be influenced by the type of additional (in this case δ and γ) subunits in the receptor (45).

The second above-mentioned paper (44) also deals with GABA<sub>A</sub>R potentiators by describing two TFTs from the coral snake venom that potentiate the GABA<sub>A</sub>R activity at low nanomolar concentrations. An undisputable advantage of these toxins is that they do not act on nAChRs and are the first, strictly speaking, GABA<sub>A</sub>R-specific TFTs. These toxins (MmTX1 and MmTX2) are not α-neurotoxins (like α-Ctx or α-Bgt) but belong to the nonconventional neurotoxins (additional 5th disulfide in loop I) like the GABA<sub>A</sub> receptor inhibiting toxin WTX identified in our study (Fig. 3). We and the authors of Ref. 44 hypothesized that TFTs should bind to GABA<sub>A</sub>R in a similar manner as α-neurotoxins bind to nAChRs. This is supported by their finding that the toxin interaction is decreased upon the H33S mutation in the central loop II of MmTX2 or upon double mutation G228E/Q231K in the receptor loop C. Concerning the binding interface of the toxin, this single mutation agrees with the conclusions we made based on the activity of the chimeric TFT. Our S67K and K67S mutations in the α1 subunit extra-domain and this positively charged residue could confer the difference in the α-Ctx affinity toward these two receptors, because α-Ctx loop II bears significant positive charge itself. This mutation was done, and the α-Ctx potency at α1(S67K)β3γ2 GABA<sub>A</sub>R was diminished, approaching that at α2β3γ2 (Fig. 8).

When our manuscript was ready for submission, two papers (43, 44) appeared, clearly demonstrating the perspective of TFTs for research on GABA<sub>A</sub>Rs. In Ref. 43, the authors analyzed in detail the interactions of α-Bgt and its fluorescent derivative with GABA<sub>A</sub>R in hippocampal neurons and with recombinant GABA<sub>A</sub>R subtypes, comparing their effects on ion currents and staining patterns in the presence of diverse ligands (agonists and antagonists) both with GABA<sub>A</sub>Rs and nAChRs. Their conclusion was that α-Bgt inhibits various functional GABA<sub>A</sub>R subtypes with different efficiency, with the α2β2γ2 receptor being inhibited most potently. Here, we show that the preferred target of α-Ctx is the α1β3γ2 receptor, indicating that diverse α-neurotoxins may have different affinity for distinct GABA<sub>A</sub>R subtypes and might be able to distinguish between various GABA<sub>A</sub>R subtypes. Concerning the mode of α-Bgt binding, Hannan et al. (43) described it as “a mixed inhibitory manner.” It is quite clear that further work is necessary to obtain a high resolution picture for recognition of GABA<sub>A</sub>R by both α-Bgt and α-Ctx.

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In conclusion, even such extensively studied objects as α-Ctx, which has been investigated since the 1970s, could hide some secrets, as demonstrated by its earlier overlooked inhibition of GABA<sub>A</sub>Rs. We showed that α-Ctx and NT I compete with α-Bgt for binding site(s) at the α1β3 receptor, and we found that α-Ctx, LsIII, and WTX inhibit the functional activity of α1β3γ2 GABA<sub>A</sub>R in the submicromolar to micromolar concentration ranges. In addition, the first peptide toxin α-conotoxin 1ml inhibiting GABA<sub>A</sub>R was identified. These facts together with previously published observations demonstrate that GABA<sub>A</sub>Rs are the target for α-conotoxin and diverse three-finger toxins.

Author Contributions—V. I. T., W. S., and Y. N. U. made study concepts and design. D. S. K., I. V. S., L. V. S., L. O. O., E. V. K., E. N. L., D. A. D., M. N. Z., I. A. I., I. E. K., V. G. S. and J. R. designed, performed, and analyzed the experiments and interpreted the data. D. S. K., I. V. S., E. N. L., L. O. O., and E. V. K. wrote the manuscript. M. N. Z., I. A. I., and V. G. S. provided technical assistance. W. S., V. I. T., and Y. N. U. revised the manuscript critically for important intellectual content and made a final approval of the version to be published. All authors reviewed the results and approved the final version of the manuscript.

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Polypeptide Neurotoxins Inhibit Functional GABA<sub>δ</sub> Receptors

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