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Computer modeling of binding of diverse weak toxins to nicotinic acetylcholine receptors

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Abstract

Weak toxins are the "three-fingered" snake venoms toxins grouped together by having an additional disulfide in the N-terminal loop I. In general, weak toxins have low toxicity, and biological targets have been identified for some of them only, recently by detecting the effects on the nicotinic acetylcholine receptors (nAChR). Here the methods of docking and molecular dynamics simulations are used for comparative modeling of the complexes between four weak toxins of known spatial structure (WTX, candoxin, bucandin, γ-bungarotoxin) and nAChRs. WTX and candoxin are those toxins whose blocking of the neuronal α7- and muscle-type nAChR has been earlier shown in binding assays and electrophysiological experiments, while for the other two toxins no such activity has been reported. Only candoxin and WTX are found here to give stable solutions for the toxin-nAChR complexes. These toxins appear to approach the binding site similarly to short α-neurotoxins, but their final position resembles that of α-cobratoxin, a long α-neurotoxin, in the complex with the acetylcholine-binding protein. The final spatial structures of candoxin and WTX complexes with the α7 neuronal or muscle-type nAChR are very similar and do not provide immediate answer why candoxin has a much higher affinity than WTX, but both of them share a virtually irreversible mode of binding to one or both these nAChR subtypes. Possible explanation comes from docking and MD simulations which predict fast kinetics of candoxin association with nAChR, no gross changes in the toxin conformation (with smaller toxin flexibility on α7 nAChR), while slow WTX binding to nAChR is associated with slow irreversible rearrangement both of the tip of the toxin loop II and of the binding pocket residues locking finally the toxin molecule. Computer modeling showed that the additional disulfide in the loop I is not directly involved in receptor binding of WTX and candoxin, but it stabilizes the structure of loop I which plays an important role in toxin delivery to the binding site. In summary, computer modeling visualized possible modes of binding for those weak toxins which interact with the nAChR, provided no solutions for those weak toxins whose targets are not the nAChRs, and demonstrated that the additional disulfide in loop I cannot be a sound criteria for joining all weak toxins into one group; the conclusion about the diversity of weak toxins made from computer modeling is in accord with the earlier phylogenetic analysis.

Keywords: Snake three-finger toxins; Weak toxins; Non-conventional toxins; Nicotinic acetylcholine receptor; Toxin-receptor complex

1. Introduction

The group of weak (or non-conventional) toxins (Nirthanan et al., 2003b) belongs to the superfamily of proteins called "three-finger toxins"—toxins from snake venoms adopting a flat, leaf-like shape formed by three adjacent loops that emerge from a globular core organized by the four conserved disulfide bridges (see for review Tsetlin and Hucho, 2004; Servent and Menez, 2001).

The name "weak toxins" was originally given to the group due to the low toxicity of its members. Weak toxins are characterized by a typically low toxicity (LD_{50} ~ 5–80 mg/kg as compared to LD_{50} ~ 0.04–0.3 mg/kg for α-neurotoxins). However, this group also includes γ-bungarotoxin (*Bungarus multicinctus*) with LD_{50} 0.15 mg/kg (Aird et al., 1999) and candoxin (*Bungarus candidus*) with LD_{50} 0.83 mg/kg (Nirthanan et al., 2002), whose toxicities are comparable to those of α-neurotoxins.

A large number of diverse three-finger toxins (short-chain α-neurotoxins, muscarinic toxins, cytotoxins and some other) contain four disulfide bridges (Fig. 1a and c). Weak toxins have five disulfides and in this respect they resemble the long-chain neurotoxins. However, the fifth disulfide in weak toxins

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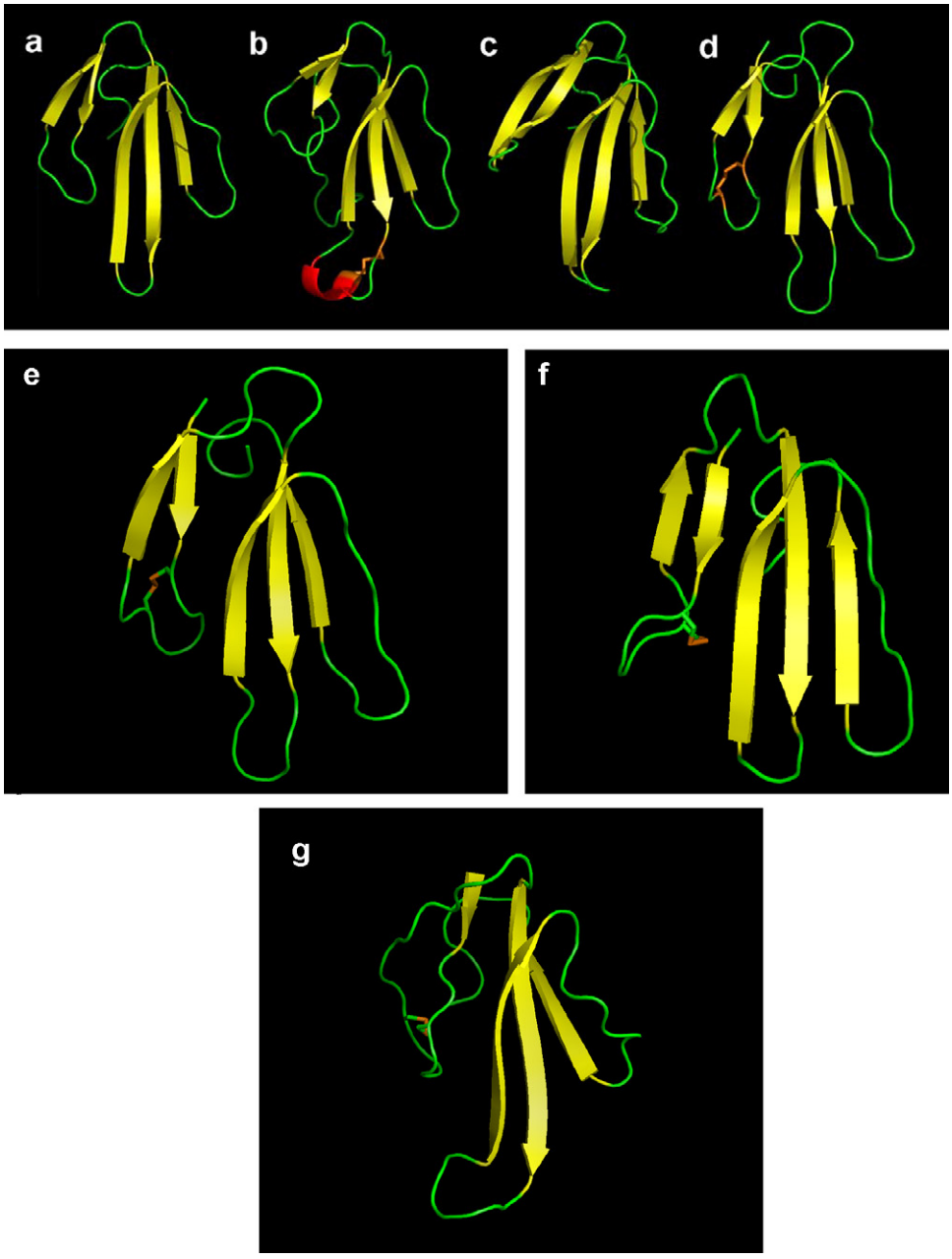

Fig. 1. Structures of three-finger toxins. (a) Short neurotoxin NTII from *Naja oxiana*, (b) long neurotoxin α-cobratoxin from *Naja kaouthia*, (c) muscarinic toxin 2 from *Dendroaspis angusticeps*, (d–g) weak toxins: (d) WTX, (e) candoxin, (f) bucandin, g–γ-bungarotoxin. The secondary structure is shown. Additional disulfides in loop II of the cobratoxin and loop I of weak toxin are demonstrated with orange sticks.

is located in the N-terminal loop I (Fig. 1d–g), while in long α- and κ-neurotoxins it is in the tip of the central loop II (Fig. 1b). To date about 30 weak toxins are known and still the new representatives of this group are appearing (Chang et al., 2003).

First data about biological activity were recently obtained for several weak toxins, namely WTX from *Naja kaouthia* (Utkin et al., 2001a,b; Ogay et al., 2005), NNA2 from *Naja atra* (Chang et al., 2000), synthetic toxin Wntx-5 from *Naja sputatrix* (Poh et al., 2002), γ-bungarotoxin from *B. multicinctus* (Aird et al., 1999; Chang et al., 2002) and candoxin from *B. candidus* (Nirthanan et al., 2003a). For all of them, with the exception of γ-bungarotoxin, antagonistic activity was found towards muscle-type and/or neuronal α7 nAChR. Similar activity towards nAChRs has been expected for all representatives of the group (Nirthanan et al., 2003a,b). However, some results indicating the phylogenetic heterogeneity of the group (Fry et al., 2003; Fry, 2005; Kryukova et al., 2005) and a variety of molecular targets (Chang et al., 2002; Ogay et al., 2005) of the weak toxins were obtained. In addition, much higher affinity of candoxin, as compared to WTX, for the nAChR of both types might suggest differences in binding mechanism. The available data indicate that interaction of weak toxins with their targets might have some distinctions from the interactions of α-neurotoxins.

One question concerns the role of the additional disulfide in the loop I: whether it influences the binding of weak toxins and hence might be a result of convergence of several evolutionary

distinctive groups of the three-finger toxins, or this feature was simply inherited from some ancestor and was not eliminated during the evolutional divergence of the proteins now unified in the artificial group of "weak toxins".

In this study we simulated the binding to the nAChRs of α7 and muscle types for four weak toxins, WTX, bucandin, candoxin and γ-bungarotoxin, which have at least partly established spacial structures and appear to differ considerably in biological activity. These simulations are also analyzed in light of earlier phylogenetic studies (Fry et al., 2003; Kryukova et al., 2005).

2. Experimental

2.1. Receptor models building

Models of the extracellular domains of the *Torpedo californica* nAChR, human and rat α7 nAChR subunits were constructed using the program MODELLER 8v0 (http://www.salilab.org/modeller) with the sequence alignment from LGIC database (http://www.ebi.ac.uk/compneur-srv/LGICdb/LGICdb.php), analogously to techniques described in Mordvintsev et al. (2005). The refined model described in Celie et al. (2005) was also used during α7 nAChR modeling. Each subunit was constructed separately and independently from others. The experimental structures were from Protein Data Bank (http://www.rcsb.org/pdb). Multiple requests to the Swiss-Model server (http://swissmodel.expasy.org) were used as control in several cases. The structure verification was carried out with WHAT_CHECK program (http://swift.cmbi.nl/gv/whatcheck/), then the structures were primarily relaxed (300 steps of steepest descent with cut-off 20 Å) with GROMOS'96 (Gunsteren et al., 1995) instruments included into the SPDBViewer 3.7 sp5 (http://swissmodel.expasy.org/spdbv/).

2.2. Toxin structures

The structures of candoxin, bucandin and γ-bungarotoxin (1JGK, 1IJC, 1MR6, respectively) were from PDB.

The refined model of WTX was constructed under MODELLER 7v7 on the basis of the known structures of α-neurotoxins, taking into account their phylogenetic relationship with WTX (Kryukova et al., 2005). The correctness of the model was checked using the NMR data for WTX (Eletsky et al., 2001). Flexibility of fragments and of the whole toxin molecule was determined in the TINKER program by the molecular dynamics methods in the force fields MM2 (Burkert and Allinger, 1982), AMBER'99 (Ponder and Case, 2003) and CHARMM'27 (original reference for CHARMM is Brooks et al., 1983) in the time interval from 2 ps to 10 ns. The system was filled up with up to 500 water molecules.

The "virtual" mutations in candoxin and WTX were introduced with SPDBViewer. The disulfide bridge was broken and, as a variation, cysteines were changed to serine residues. Thus, three toxin models were built: native toxin, toxin with free SH on cysteines 6 and 11, and toxin with cysteines 6 and 11 changed for serines. Further relaxation was done as described above for WTX molecule model.

2.3. Docking simulations

For protein–protein interactions simulation, the docking program HEX 4.5e (http://www.csd.abdn.ac.uk/hex/) was used. As during the docking process, multiple solutions of WTX structure and NMR structures of other toxins were used, the interactions between flexible ligand and rigid receptor being simulated. The structures obtained were visually analyzed using the SPDBViewer to reject obviously incorrect solutions. The solution was believed to be false if the toxin was bound to the channel region. The solution was also discarded if provided the position of the toxin in area where it could not be directly competitive with acetylcholine or its antagonists. This filtration was performed, since at least WTX and candoxin were shown to inhibit binding of long α-nerotoxins to the nAChRs. The selected docking solutions were submitted to molecular and stochastic dynamics routines.

The control docking was done in several cases with AUTODOCK 3.02 (http://www.scripps.edu/mb/olson/doc/autodock/) as it uses the algorithm different from that employed in HEX.

2.4. Molecular dynamics and counter-ion relaxation

Interactions of the toxins with the nAChRs were further studied in two molecular simulation packages under different conditions. The runs were for the systems comprising two subunits of the receptor and the toxin disposed according to the docking simulations. Stochastic dynamics (SD) in GROMACS 3.2.1 package (Lindahl et al., 2001) with OPLS–AA force field (Kaminski et al., 2001) and molecular dynamics (MD) in PUMA package (Lemak and Balabaev, 1995) with AMBER'99 force field were carried out with trajectories 1 ns long at a temperature of 300 K, dielectric permittivity $\varepsilon = 1$. Time step of integration procedures was taken as small as 1 fs. Radius of truncation for Coulomb interactions was 20 Å. In case of SD, periodic boundaries were applied to a system containing the subunits of the receptor, WTX and 14,778 molecules of TIP3P water with a Cl^- counter-ion. Coefficient of thermostating was 0.02 ps, Lennard–Jones interactions were cut-off at 20 Å without shift or switch functions. MD simulation in AMBER'99 was done only for a system without water and a counter-ion. Lennard–Jones interactions were calculated only up to 16 Å (at that, from 15 to 16 Å a polynomial switch function was applied). We applied collisional thermostat (Lemak and Balabaev, 1995) with the mean frequency of collisions 55 ps^{-1} and the mass of virtual particles 18 amu. Unlike Berendsen or Nosé–Hoover thermostats, it does not lead to physically incorrect dynamic behavior of the system (Golo and Shaitan, 2002; Golo et al., 2004).

3. Results and discussion

3.1. Structure of weak toxins

The chemical structure of weak toxins is characterized by the presence of the additional disulfide in loop I, and this very

feature is the essential criterion to single out them into a distinctive group of snake three-finger toxins. The tertiary structure of weak toxins (Eletsky et al., 2001; Parvathy et al., 2000; Torres et al., 2001; Kuhn et al., 2000—see Fig. 1d–g) is typical for representatives of the three-finger toxins superfamily. In the primary structures of weak toxins were found. All conservative amino acid residues responsible for chain folding of three-finger toxins (Karlsson, 1979), The exception is a substitution of usual for common neurotoxins Tyr25 (erabutoxin-b numbering) for Phe25, invariant among weak toxins (Endo and Tamiya, 1991). However, such a mutation in erabutoxin-b (Tyr25Phe) itself did not affect its folding and activity (Tremeaux et al., 1995).

Interestingly, not all amino acid residues conservative in α-neurotoxins and identified earlier as important for binding to different nAChRs subtypes (Antil et al., 1999; Antil-Delbeke et al., 2000; Fruchart-Gaillard et al., 2002; Hansen et al., 2005; Mordvintsev et al., 2005; Teixeira-Clerc et al., 2002), are present in the weak toxin sequences (Nirthanan et al., 2003b). Of these functionally important residues, candoxin has in homologous positions Trp29, Arg33, Arg36, Glu38 and Gly34 (these residues are essential for the α-neurotoxin binding to the muscle $\alpha_2\beta\gamma\delta$ receptors) and Trp25, Ala28, Arg33 and Arg36 (involved in binding of long-chain neurotoxins to the neuronal α7 nAChR). Bucandin, γ-bungarotoxin and weak toxins from *Naja* venoms have only two or three such residues functionally important for α-neurotoxins. It is worth mentioning that weak toxins lack the helix-like conformation cyclized by the fifth disulfide bridge present in the tip of the central loop of α/κ-neurotoxins and essential for binding to neuronal α7 or α3β2 receptors (see for review Nirthanan and Gwee, 2004; Tsetlin and Hucho, 2004; Tsetlin, 1999). Nevertheless, as mentioned above, candoxin and WTX do interact with α7 nAChR, and block the acetylcoline-induced currents.

In general the fold of weak toxins is the same as in other three-finger toxins. However, as the clade of weak toxins was shown to be phylogenetically heterogeneous (Fry et al., 2003; Fry, 2005; Kryukova et al., 2005), it is not surprising that the structures of different weak toxins were found to be obviously distinctive (compare candoxin, bucandin, WTX and γ-bungarotoxin on Fig. 1d–g). The structures of candoxin, bucandin and γ-bungarotoxin were described in detail in the corresponding papers (Parvathy et al., 2000; Torres et al., 2001; Kuhn et al., 2000) and are not discussed in detail here.

The correct model of WTX could be obtained only if the constraints were imposed according to the available NMR data (Eletsky et al., 2001). Information about the secondary structure of the molecule was essential for the model construction. Finally, the model correlating with these data was built and all the intramolecular contacts described previously were found to be present (Fig. 2). Moreover, a stabilization of the loop II with several additional predicted H-bonds was demonstrated (Tyr38 OH–Trp36 NE1, Ser35 OG–Arg32 NE2, His29 ND1–Arg32N and Arg31N). The position of the loop III relatively to the loop II was shown to be practically unchanged during molecular dynamics simulations. The loop I region confined by additional disulfide was found to be the most flexible (Fig. 2). The overall flexibility of the molecule did not exceed rmsd 1.38 Å.

When the additional disulfide in loop I was eliminated, the flexibility of the whole molecule increased markedly (rmsd 1.47 Å). The exchange of the cysteines 6 and 11 in WTX sequence to serine residues did not cause an additional increase. Similar, but smaller flexibility change was found for candoxin (rmsd 1.22 Å with the disulfide and rmsd 1.29 Å if disulfide was broken).

The WTX molecule was found to be more similar to candoxin (rmsd 1.04 Å) than to bucandin (1.15 Å) or γ-bungarotoxin (1.68 Å). When compared to other groups of snake three-finger toxins, WTX exhibited the highest structural similarity to the muscarinic toxins (1.38 Å for MT2–1FF4 by PDB see Segalas et al., 1995, for review see Jerusalinsky et al., 2000; Potter, 2001), but not to α-neurotoxins acting on nAChR (1.5–1.7 Å). This finding correlates with the phylogenetic analysis (Fry, 2005; Kryukova et al., 2005). The largest divergence in structures was found in the loop II tip, where the sequences of the mentioned toxins are the most different.

3.2. *Bucandin and γ-bungarotoxin do not form stable complexes with nAChR*

Bucandin is a non-toxic protein from *B. candidus* which does not produce any effect in dose up to 50 mg/kg i.v. or i.p. (Nirthanan et al., 2003a,b). Still by the structure, sequence homology and the cysteine scaffold it belongs to the group of weak toxins and was suggested to bind to nAChR as WTX or candoxin do (Nirthanan et al., 2003a,b). During docking simulations no solution which would be stable in molecular dynamics could be obtained. All the complexes produced by docking were considered as false and were discarded. Thus, bucandin should not bind to the nAChRs, at least to those of muscle or α7 types. The reason may be a sterical problem in reaching the binding site, because the loop I, containing the fifth disulfide (Fig. 1) is larger than the loop I in α-neurotoxins and does not permit the loop II to penetrate deeply into the pocket. Taking in account the absence of any action of the toxin injection in vivo (Torres et al., 2001), it is logical to suppose that bucandin is a "blind offshoot" of the evolution of the snake three-finger toxins, the molecule that has lost its function during the evolution but is still present in the "venome" (the term introduced in Fry, 2005). This explanation correlates with its detached location on the phylogenetic tree of the three-finger toxins (Fry et al., 2003; Fry, 2005; Kryukova et al., 2005).

γ-Bungarotoxin is also a three-finger protein from the *Bungarus* venom having the additional disulfide in loop I. Contrary to bucandin, it was shown to be lethal at a low dose (Aird et al., 1999). However, there is evidence that it binds to the muscarinic receptor (Chang et al., 2002), rather than to the nAChR. Indeed, during performed docking and molecular dynamics simulations no complex of γ-bungarotoxin and nAChR could be obtained. The phylogenetic position of γ-bungarotoxin is also very special and different from that of other weak toxins (Fry et al., 2003;

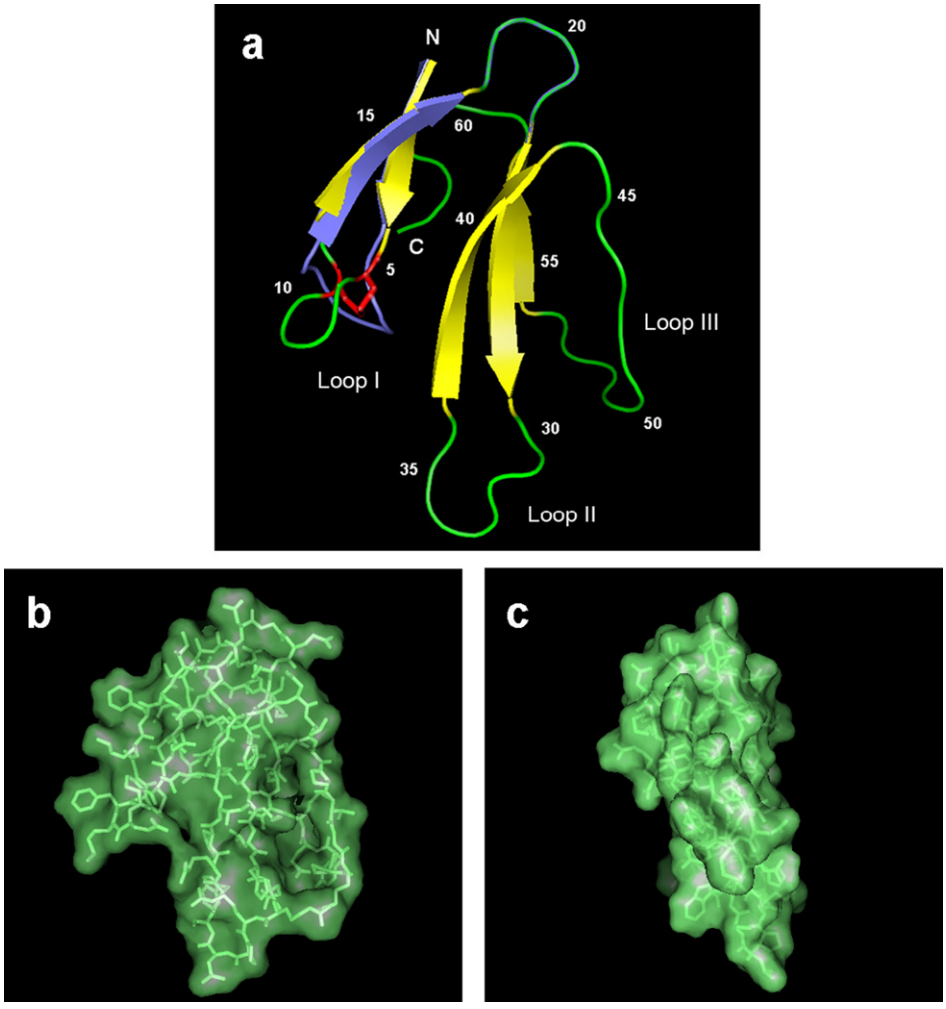

Fig. 2. Model of the WTX molecule. (a) Overall view of the molecule from the concave side. The secondary structure and additional disulfide in the loop I are shown. The N- and C-termini are marked. The two extreme positions of the flexible loop I are demonstrated. (b and c) molecular van der Waals surface of the WTX molecule from concave side and from the side of the loop I, respectively.

Kryukova et al., 2005). Thus, γ-bungarotoxin could be a unique and divergent case and it is not surprising that it appears not to interact with nAChR.

Thus, at least two known weak toxins were reported not to interact with α7 or muscle-type nAChR, and the lack of this activity is in accord with docking and molecular dynamics simulations. The fifth disulfide in the loop I does not facilitate binding of these toxins to nAChR, but might be important for the interaction with some other targets, if the latter exist.

3.3. WTX and candoxin binding to the nAChR

Candoxin, purified from the *B. multicinctus* venom, is the most homologous, from among all weak toxins, to the snake α-neurotoxins (see Fig. 3). As mentioned above, it has several amino acid residues shown to be critical for α-neurotoxin binding to the nAChR. Candoxin is a strong lethal toxin (LD_{50} 0.83 mg/kg), with clear physiological indication of nAChR-blocking function (Nirthanan et al., 2002, 2003a,b) and high affinity for the nAChR of α7 and muscle types.

```
WTX       LTCLNCPEMFCGKF--QICRNGEKICFKKLHQR---RPLSWRYIRGCADTCPVGKP-YEMIECCSTDKCNR-------
candoxin  MKCKICNFDTCRAGELKVCASGEKYCFKESWREARG----TRIERGCAATCPKGSVYGLYVLCCTTDDCN--------
bucandin  MECYRCGVSGCHLK--ITCSAEETFCYKWL------NKISNERWLGCAKTCTEIDTWNVYNKCCTTNLCNT-------
gbtx      MQCKTCSFYTCPNS--ETCPDGKNICVKRSWTAVRGDGPKREIRRECAATCPPSK-LGLTVFCCTTDNCNH-------
CTX       IRC---FITPDITS--KDC-PNGHVCYTKTWCDAFCSIRGKRVDLGCAATCPTVKT-GVDIQCCSTDNCNPFPTRKRP
NTII      LECHNQQSSQPPTT--KTC-SGETNCYKKWWSDH----RGTIIERGCG--CPKVKP-GVNLNCCRTDRCNN-------
MT7       LTCVKSNSIWFPTS--EDCPDGQNLCFKRWQYI---SPRMYDFTRGCAATCPKAEY-RDVINCCGTDKCNK-------
                  Loop I                Loop II              Loop III
```

Fig. 3. Sequence alignment of the weak toxins, short α-neurotoxin NTII from *N. oxiana*, long neurotoxin α-cobratoxin from *N. kaouthia* and muscarinic toxin MT7 from *D. angusticeps*. The cysteines common for all three-finger toxins are shown in green, the additional ones characteristic for weak and long-chain toxins—in rose. The loops I–III are marked with bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

WTX is a *N. kaouthia* toxin, one of the "proper" weak toxins, as follows from phylogenetic analysis (Fry et al., 2003; Kryukova et al., 2005). It is a non-toxic antagonist of α7 and muscle type nAChRs in vitro (Utkin et al., 2001a,b), with pronounced physiological effects observed in vivo on rats and mice (Ogay et al., 2005; Mordvintsev et al., unpublished). The effects are similar to those induced either by diverse nAChR-acting compounds, or by muscarinic toxins acting on G protein-copled muscarinic acetylcholine receptors. In addition, the sequence of WTX, like other "proper" weak toxins is much closer to three-finger muscarinic toxins than to α-neurotoxins. Thus, WTX, being structurally close to candoxin, might differ from it in potency and selectivity.

However, on modeling these two toxins form similar complexes with nAChR (Fig. 4). These two toxins are entering the binding pocket from the so-called "big cavity" (from the top-right of the C-loop, if looking to the external side of the receptor), like short α-neurotoxins (Mordvintsev et al., 2005) and α-conotoxins (Celie et al., 2005; Hansen et al., 2005; Ulens et al., 2006)—compare the complexes on Fig. 4. As in case of short α-neurotoxins, the convex side of both WTX and candoxin faces the complementary side of the interface and the loop III is the closest to the membrane (Figs. 4 and 5). The C-loop of the receptor principal side should be in an outward position before the toxin could enter the binding pocket. The C-loop is spontaneously fluctuating from and to the receptor body, as described earlier (Hansen et al., 2005; Gao et al., 2005; Unwin, 2005). After the C-loop is going out, the toxin molecule enters the pocket, due to the overall positive charge of the loop II, and provokes here a structural rearrangement. This conformational change could even lock the molecule into the binding site. This mechanism resembles the short neurotoxins binding to the muscle type nAChR (for details see Mordvintsev et al., 2005). However, the weak toxin molecule is finally placed 60–80° to the membrane plane and ~25° to the medial axis of the receptor (Figs. 4 and 5), this location being somewhat different from that of short α-neurotoxin.

In spite of general similarity of the WTX-nAChR and candoxin-nAChR complexes obtained by modeling, marked differences in details can be observed. This distinction is due to the difference in their sequences, especially in the loop II tip (Fig. 3). Candoxin emulates the positive charge of acetylcholine with its Arg35 on the tip of the loop II and fixes into the binding site of the nAChR (either α7 or muscle type) breaking the "aromatic cage" of the pocket, similarly to what *Naja oxiana* NTII does with its Arg31 and His32 (Mordvintsev et al., 2005) (see Table 1). This mechanism with primary role of π-cationic, strong ionic and H-bonds provides high energetic gain and leads to high affinity of the toxin and fast kinetics of the complex formation. The additional disulfide in loop I does not play a direct role in binding of candoxin, but the position of this loop and of the whole toxin molecule is different from that of α-neurotoxins. The loop I forms several strong contacts with the receptor residues and the disulfide could be crucial for correct orientation of these toxin residues during the binding. As the candoxin molecule is quite rigid, the orientation of the loop I should influence the position of the loop II and appropriate arrangement of the toxin residues

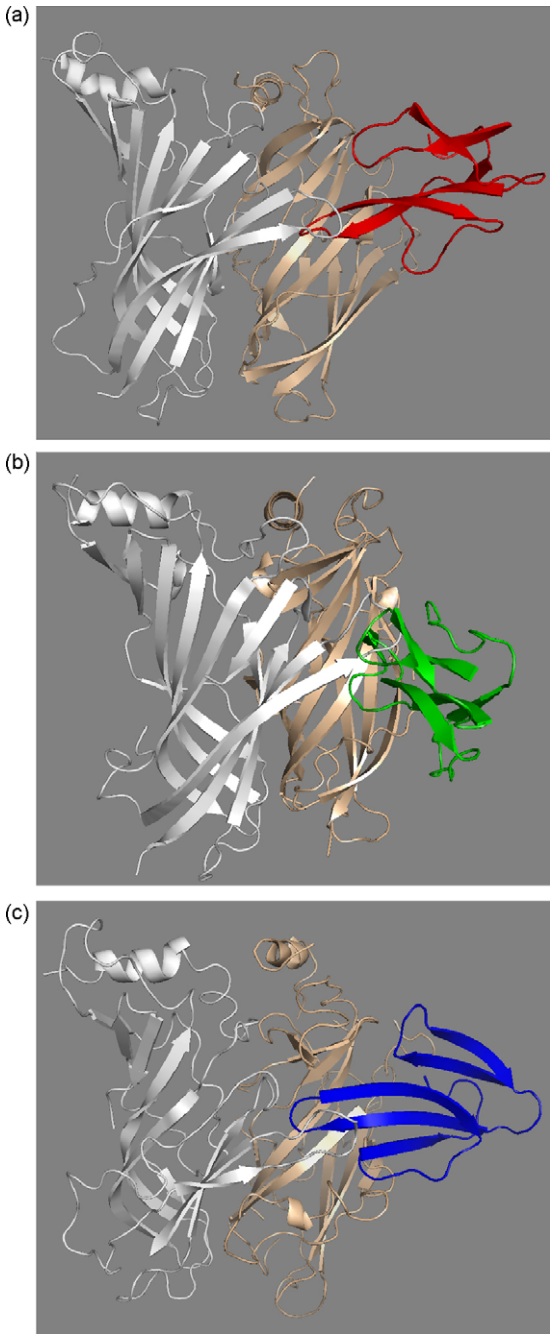

Fig. 4. Complexes of the snake toxins with receptors. (a) WTX with α7 nAChR, (b) α-cobratoxin with AChBP (Bourne et al., 2005), (c) α-neurotoxin NTII at the α–γ-interface of the *Torpedo californica* nAChR (Mordvintsev et al., 2005). Only extarcellular domains of two receptor subunits are shown (in white and wheat).

in the binding pocket. Thus, as follows from modeling, the loop I disulfide should play a pilot and corrector role during the binding. This conclusion is supported by the fact that the complex formed had a somewhat lower energy and slightly more solutions were produced if candoxin with removed disulfide 6–11 was used for docking, but there was no difference if the free cysteine or serine residues were kept in positions 6 and 11. During the MD simulations of the candoxin-nAChR complexes, no large differences were found in stability of the complexes with

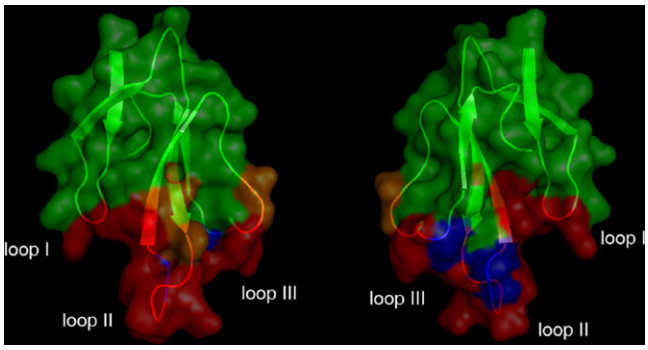

Fig. 5. Interacting surfaces of WTX. The molecular surface is shown. The residues interacting both with α7 and muscle-type nAChRs are shown in red. The residues interacting only with α7 are in blue, those interacting only with muscle-type AChR are in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

mutant or native candoxin. Thus, the fifth disulfide appears not to be essential for the complex stability.

As the disposition of candoxin in the binding site on α7–α7 and α–γ interfaces is very similar, the large difference in reversibility of the binding to α7 and muscle type nAChRs (Nirthanan et al., 2002) remains unclear. During the molecular dynamics simulations the position of candoxin in the binding site of the α7 nAChR was much less flexible and the energy gain (1550 kJ/mol × binding site) was a bit higher than that for α-γ interface (1350 kJ/mol × binding site). This finding may be one of the reasons for the experimentally found irreversibility of candoxin binding to the α7 nAChR (Nirthanan et al., 2003a,b).

Table 1
Amino acid residues participating in candoxin interaction with human α7 nAChR according to the modeling

Candoxin	AChR	
	+	−
Loop I		
Phe8		Val69, Gly74
Asp9	Asn25(OD1-ND2)	Gly74(N-O), Arg6(OD1-NZ)
Thr10	Asp26(OG-OD2)	
Arg12	Trp154(π-cation), Asp26(NH1/2-OD1)	
Loop II		
Glu29		Lys76(O-NZ)
Ser30		Lys76(OG-NZ)
Trp31	(Lys192)	
Arg32		His115, Gln117(NH-NE2), Val110(N-O), Asn111
Glu33	Tyr195	Gln117(O-NE2)
Ala34	Ser150	Gln117
Arg35	Trp149(π-cation), Ser150, Tyr151	Leu109
Gly36		Arg79
Thr37		Thr77, Asn111(OG-ND2)
lle39		Lys76
Loop III		
Ser51		(Tyr33)
Val52		Gln161(O-NE2)
Leu55		Asn111
Tyr56		Lys76(π-cation), Ser113(N-O)

+ and − designate the main and complementary binding surfaces formed by the adjacent subunits.

Modeling of WTX binding to nAChRs reveals some differences as compared to candoxin. The tip residues of the WTX loop II are quite distinctive from those of candoxin or short α-neurotoxins (see Fig. 3). The main difference is the lack of a single positive charge on the loop II tip of WTX which would have been analogous to the candoxin Arg35 or NTII Arg31. The tip of the loop II still has an impressive cumulative positive charge (see the sequence on Fig. 3—this area is rich in positively charged residues) and it is dragging WTX into the acetylcholine binding site composed of aromatic amino acid residues, in the same way as does candoxin Arg35, a driving force in its entering into the same pocket. The energy of such first stage WTX-nAChR interaction is not very high (about 500 kJ/mol × binding site). The main role at this stage should be ascribed to the van der Waals forces. They are not very intense, and could be easily broken, if a stronger contact would be formed as a result of reorganization. Thus, the cumulative positive charge of the tip of the loop II of WTX molecule is the driving force, slowly introducing the toxin into the pocket by the gradient of electrostatic interaction. The duration of such sliding should be considerable, and the initial complex has a high probability of being destroyed due to thermic fluctuations of the receptor and toxin molecules, or to a small difference in the energy gain via solvatation or exchange of the solvate microenvironment to the protein one. Thus, the affinity of WTX to the nAChR of α7 and muscle types should not be very high, as the possibility of the complex formation is relatively low. MD simulations show that once entered deeply into the pocket, the toxin undergoes some conformational reorganization of sufficiently long duration (∼650 ps—Fig. 6) affecting the tip of the loop II. As the result of the rearrangement the energy gain increases markedly, strong ionic and H-bonds are formed, in part replacing initial weak van der Waals bonds, expanding the binding platform of the toxin and locking it into the pocket. A very stable complex is built up (Figs. 5 and 6, Tables 2 and 3). During the SD and MD simulations over the docking solution, best approximating the real position of the WTX molecule in the pocket, all elements of the secondary structure were well preserved and most of the contacts between the WTX and nAChR residues found by docking simulation were retained. The kinetics of changing of distances characterizing these contacts is presented in Fig. 6. Since OPLS-AA simulation implied aqueous medium, the structure of the toxin and receptor subunits virtually did not change. In AMBER'99, the shape of the macromolecules did not change either, but their sizes reduced,a little, so the overall structure became more compact. At the same time, loop II protruded into the pocket between the receptor subunits. Thus, WTX is entering the nAChR pocket as a rigid-body, and only the relative position of the loop II is slightly changed during MD simulations (the illustrating media file may be obtained from the corresponding author).

Disulfide bridges of the weak toxin, including the "additional" one of the loop I, maintain the shape of the molecule but appear not to be involved directly in the process of binding. The molecule with removed disulfide in loop I showed practically the same range of energies of the complex forma-

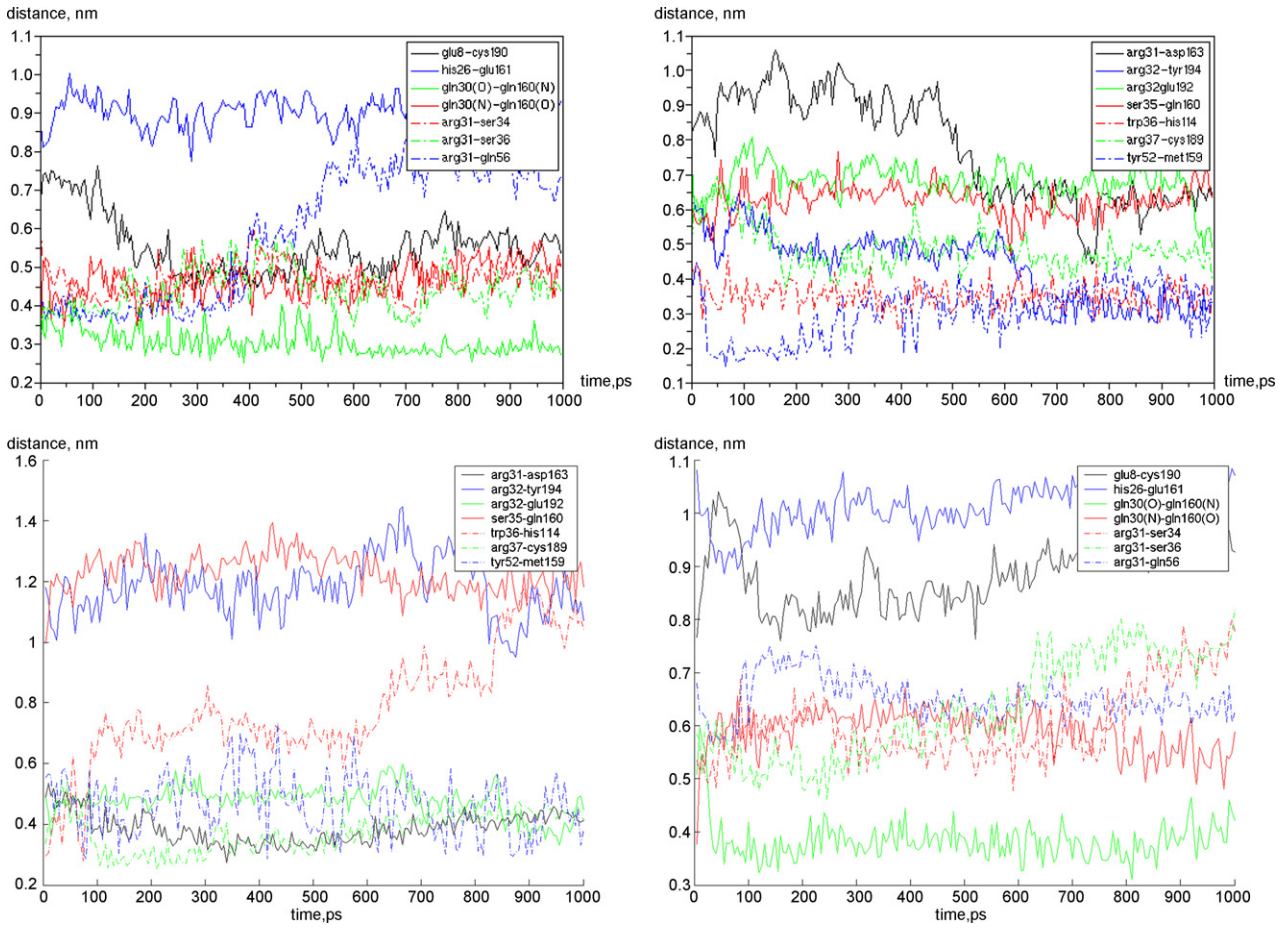

Fig. 6. Distances between some group of atoms in receptor subunits and WTX. The contacts are taken according to Table 2. (a and b) are by OPLS-AA force field, (c and d) are by AMBER'99.

Table 2
Amino acid residues, participating in WTX interaction with human α7 nAChR according to the modeling

WTX	AChR	
	+	−
Loop I		
Glu8	Cys190(OE2-SG1)	
Met9		Lys75
Loop II		
His26	Cys189(7A)	Glu161, Asp163 HOH
Gln30		Gln160
Arg31		Ser34, Ser36(OG), Trp170(pi), Gln56?, Asp163(O)
Arg32	Tyr194, Glu192 (via HOH)	
Pro33	Tyr187	Trp54VdW
Leu34	Ser149	VdWGln116, Leu118, Gln56
Ser35		Gln160
Trp36		His114
Arg37	Cys–Cys, Cys189(NZ-O)	
Loop III		
Lys50		Gln160(NZ-O)
Tyr52		Met159(O), Gln160(O)

Table 3
Amino acid residues, participating in WTX interaction with *Torpedo californica* nAChR (α-gamma interface) according to the modeling

WTX	AChR	
	α	γ
Loop I		
Glu8	Cys191	
Met9	Thr190(SD-O)	
Loop II		
His26		Gln57(OE2), Ser161(OG), Ala162(O)
Gln30		Tyr117, Gln59(O-NE2)
Arg31	Trp148 (not ionic)	Asn107(O), Tyr117(O)
Arg32	Tyr197(OH), Asp194(H-bond OD2), Cys191(SG)	
Pro33	Trp148? Thr149	
Leu34	Asp151?	
Arg37		Glu164, 166, 169
lle39		Ala160, Glu164
Loop III		
Val48		Gln159
Gly49		Gln159
Lys50		Asp30, Thr32, Tyr117?
Tyr52		Asn61

tion. However, upon this modification the flexibility of the WTX molecule increases, which prolongs the complex formation and, consequently, diminishes possibility of its formation. The additional disulfide in the loop I, as the flexible loop I itself, generates some sterical problems while the toxin is entering the pocket. As in the case of candoxin, this disulfide does not directly interact with the receptor residues, but its presence should lead to specific orientation of the toxin molecule during the penetration to the binding site. One of the prima facie for this is the general similarity of the candoxin and WTX complexes with nAChRs. Thus, the additional disulfide of these two toxins exerts a real (but not strictly essential) impact on binding, influencing correct position of the toxin in the binding site of the receptor. Intriguingly, these toxins were shown to be phylogenetically divergent (Fry et al., 2003; Kryukova et al., 2005).

Modeling provides explanations to some features of the WTX binding to the nAChR found experimentally (Utkin et al., 2001a). The energetically poor and kinetically slow first stage of the binding, deduced from docking and MD simulations, may be the reason of the low affinity of the toxin. However, the predicted subsequent conformational changes (not observed for candoxin biding) led to the extremely rigid and stable complex. By the MD, the complex stays practically unchanged and shows no tendency for degradation, at least in nanosecond range (Fig. 6). This may underlie practically irreversible binding seen in the electrophysiology experiments on the nAChR in oocyte system (Utkin et al., 2001b).

Tables 2 and 3 show the intermolecular contacts in the final complexes of WTX with nAChR of α7 and muscle types. The essential are the ionic interactions between positively charged residues of the toxin and the receptor surface. It is of particular interest that the WTX complexes with nAChR of α7 and muscle types are very similar and the order of events in binding to both these receptors is analogous (Fig. 5). Still some differences could be observed which might result in pharmacological distinctions for the WTX interaction with nAChRs of these two subtypes.

3.4. Additional disulfide—trace of evolution or a functional element ?

Our modeling confirms that the group of weak toxins is heterogenic and artificial, as earlier suggested from the phylogenetic analysis (Fry et al., 2003; Kryukova et al., 2005). The modeling provided the additional evidence for this heterogeneity, since different weak toxins were found to interact distinctively with their molecular targets. Two of the four weak toxins studied by us could not adopt any adequate position on the nAChR. One of them, γ-bungarotoxin, is known to be competitive to muscarinic agents on muscarinic receptors (Aird et al., 1999). The second one, bucandin, is biologically inactive and seems to be a flotsam of the evolution. Other two evolutionary distinct weak toxins, candoxin and WTX, are predicted to form very similar complexes with muscle and α7 nAChRs. The additional disulfide in the loop I is playing a role of a pilot in correct orientation of these two toxins in the binding site, but does not participate in binding directly. Thus, four weak toxins bearing this disulfide in the loop I have different activity and even differ in details of structural organization of the molecule (see Fig. 1). In addition, they all have been earlier shown to be phylogenetically divergent. As known from theory of evolution, such a conservation of any characteristic in a number of different proteins (or species) is proper to the element inherited from some common ancestor, but is not a convergence feature.

4. Conclusions

The computer modeling of the binding of several weak toxins to nAChRs showed functional heterogeneity of the snake three-finger toxins having the additional fifth disulphide in the loop I. Only two of four tested toxins (WTX and candoxin) could produce stable complexes with nAChR of α7 and muscle types, while two other (bucandin and γ-bungarotoxin) failed. This result is in accord with the available biochemical and phylogenic data. WTX and candoxin bind nAChRs via the mechanism generally resembling the short α-neurotoxins binding, but the final position of the toxin is more alike this characteristic for long α-neurotoxins. The additional disulfide of weak toxins does not play a crucial role in binding, but influences the orientation of the toxin while it enters the binding pocket. The modeling provided a possible explanation for a large difference in the affinity of WTX and candoxin for AChRs.

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References

Aird, S.D., Womble, G.C., Yates III, J.R., Griffin, P.R., 1999. Primary structure of gamma-bungarotoxin, a new postsynaptic neurotoxin from venom of *Bungarus multicinctus*. Toxicon 37 (4), 609–625.

Antil, S., Servent, D., Menez, A., 1999. Variability among the sites by which curaremimetic toxins bind to Torpedo acetylcholine receptor, as revealed by identification of the functional residues of a-cobratoxin. J. Biol. Chem. 274, 34851–34858.

Antil-Delbeke, S., Gaillard, C., Tamiya, T., Corringer, P.J., Changeux, J.P., Servent, D., Menez, A., 2000. Molecular determinants by which a long chain toxin from snake venom interacts with the neuronal a7-nicotinic acetylcholine receptor. J. Biol. Chem. 275, 29594–29601.

Bourne, Y., Talley, T.T., Hansen, S.B., Taylor, P., Marchot, P., 2005. Crystal structure of a Cbtx-AChBP complex reveals essential interactions between snake alpha-neurotoxins and nicotinic receptors. EMBO J. 24 (8), 1512–1522.

Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S., Karplus, M., 1983. CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. J. Comp. Chem. 4, 187–217.

Burkert, U., Allinger, N.L., 1982. Molecular Mechanics. ACS Monograph 177. American Chemical Society, Washington, DC.

Celie, P.H., Kasheverov, I.E., Mordvintsev, D.Y., Hogg, R.C., van Nierop, P., van Elk, R., van Rossum-Fikkert, S.E., Zhmak, M.N., Bertrand, D., Tsetlin, V.I., Sixma, T.K., Smit, A.B., 2005. The AChBP—α-conotoxin structure: toward receptor-selective ligands for nicotinic receptors. Nat. Struct. Biol. 7, 582–588.

Chang, L.-S., Chung, C., Wu, B., Yang, C., 2002. Characterization and gene organization of Taiwan banded krait (*Bungarus multicinctus*) γ-bungarotoxin. J. Prot. Chem. 21 (4), 223–229.

Chang, L.-S., Chung, C., Liou, J.-C., Chang, C.-W., Yang, C.-C., 2003. Novel neurotoxins from Taiwan banded krait (*Bungarus multicinctus*) venom: purification, characterization and gene organization. Toxicon 42, 323–330.

Chang, L.-S., Lin, S.-R., Wang, J.-J., Hu, W.-P., Wu, B.-N., Huang, H.-B., 2000. Structure–function studies on Taiwan cobra long neurotoxin homologue. Biochim. Biophys. Acta 1480, 293–301.

Eletsky, A.V., Maslennikov, I.V., Kukhtina, V.V., Utkin, Y.N., Tsetlin, V.I., Arseniev, A.S., 2001. Structure and conformational heterogeneity of the weak toxin from the cobra *Naja kaouthia* venom. Bioorg. Khim. 27, 89–101.

Endo, T., Tamiya, N., 1991. Structure–function relationships of postsynaptic neurotoxins from snake venoms. In: Harvey, A.L. (Ed.), Snake Toxins. Pergamon Press, New York, pp. 165–222.

Fruchart-Gaillard, C., Gilquin, B., Antil-Delbeke, S., Le Noveve, N., Tamiya, T., Corringer, P.-J., Changeux, J.-P., Menez, A., Servent, D., 2002. Experimentally based model of a complex between a snake toxin and the α7 nicotinic receptor. Proc. Natl. Acad. Sci. U.S.A. 99, 3216–3221.

Fry, B.G., 2005. From genome to "venome": molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Res. 15 (3), 403–420.

Fry, B.G., Wuster, W., Kini, R.M., Brusic, V., Khan, A., Venkataraman, D., Rooney, A.P., 2003. Molecular evolution and phylogeny of elapid snake venom three-finger toxins. J. Mol. Evol. 57 (1), 110–129.

Gao, F., Bren, N., Burghardt, T.P., Hansen, S., Henchman, R.H., Taylor, P., McCammon, J.A., Sine, S.M., 2005. Agonist-mediated conformational changes in acetylcholine-binding protein revealed by simulation and intrinsic tryptophan fluorescence. J. Biol. Chem. 280 (9), 8443–8451.

Golo, V.L., Salnikov, V.N., Shaitan, K.V., 2004. Harmonic oscillators in the Nose-Hoover environment. Phys. Rev. E 70, 046130.

Golo, V.L., Shaitan, K.V., 2002. Dynamic attractor for the Berendsen thermostat and the slow dynamics of biomacromolecules. Biofizika 47, 611–617.

Gunsteren, W.F., van Hünenberger, P.H., Mark, A.E., Smith, P.E., Tironi, I.G., 1995. Computer simulation of protein motion. Comput. Phys. Commun. 91, 305–319.

Hansen, S.B., Sulzenbacher, G., Huxford, T., Marchot, P., Taylor, P., Bourne, Y., 2005. Structures of Aplysia AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. EMBO J. 24 (20), 3635–3646.

Jerusalinsky, D., Kornisiuk, E., Alfaro, P., Quillfeldt, J., Ferreira, A., Rial, V.E., Duran, R., Cervenansky, C., 2000. Muscarinic toxins: novel pharmacological tools for the muscarinic cholinergic system. Toxicon 38 (6), 747–761.

Kaminski, G.A., Friesner, R.A., Tirado-Rives, J., Jorgensen, W.L., 2001. J. Phys. Chem. B 105, 6474–6487.

Karlsson, E., 1979. Chemistry of protein toxins in snake venoms. In: Lee, C.Y. (Ed.), Snake Venoms, Handbook of Experimental Pharmacology, vol. 52. Springer, Berlin, pp. 159–212.

Kryukova, E.V., Mordvintsev, D.Yu., Utkin, Yu.N., Tsetlin, V.I., 2005. Antibodies against native *Naja kaouthia* weak toxin WTX interacts with weak toxins and several other 3-finger toxins, but not with their denatured forms. Toxicon 46 (1), 24–30.

Kuhn, P., Deakon, A.M., Comoso, S., Rajaseger, G., Kini, R.M., Uson I., Kolatkar, P.R., 2000. The atomic resolution structure of bucandin, a novel toxin isolated from the Malayan krait, determined by direct methods. Acta Crystallogr. Sect. D 56, 1401–1407.

Lemak, A.S., Balabaev, N.K., 1995. A comparison between collisional dynamics and Brownian dynamics. Mol. Simul. 15, 223–231.

Lindahl, E., Hess, B., van der Spoel, D., 2001. GROMACS 3.0: a package for molecular simulation and trajectory analysis. J. Mol. Mod. 7, 306–317.

Mordvintsev, D.Y., Polyak, Y.L., Levtsova, O.V., Tourleigh, Y.V., Kasheverov, I.E., Shaitan, K.V., Utkin, Y.N., Tsetlin, V.I., 2005. A model for short α-neurotoxin bound to nicotinic acetylcholine receptor from Torpedo californica: comparison with long-chain α-neurotoxins and α-conotoxins. Comput. Biol. Chem. 29 (6), 398–411.

Nirthanan, S., Charpantier, E., Gopalokrishnakone, P., Gwee, M.C.E., Khoo, H.E., Cheah, L.S., Bertrand, D., Kini, R.M., 2002. Candoxin, a novel toxin from Bungarus candidus, is a reversible antagonist of muscle ($\alpha_2\beta\gamma\delta$) but a poorly reversible antagonist of neuronal α7 nicotinic acetylcholine receptors. J. Biol. Chem. 277, 17811–17820.

Nirthanan, S., Charpantier, E., Gopalokrishnakone, P., Gwee, M.C.E., Khoo, H.E., Ceah, L.S., Kini, R.M., Bertrand, D., 2003a. Neuromuscular effects of candoxin, a novel toxin from the venom of the Malayan krait (*Bungarus candidus*). Br. J. Pharmacol. 139, 832–844.

Nirthanan, S., Gopalokrishnakope, P., Gwee, M.C., Khoo, H.E., Kini, R.M., 2003b. Non-conventional toxins from Elapid venoms. Toxicon 41 (4), 397–407.

Nirthanan, S., Gwee, M.C., 2004. Three-finger alpha-neurotoxins and the nicotinic acetylcholine receptor, forty years on. J. Pharmacol. Sci. 94 (1), 1–17.

Ogay, A.Y., Rzhevsky, D.I., Murashev, A.N., Tsetlin, V.I., Utkin, Y.N., 2005. Weak neurotoxin from *Naja kaouthia* cobra venom affects haemodynamic regulation by acting on acetylcholine receptors. Toxicon 45 (1), 93–99.

Parvathy, V.R., Chary, K.V.R., Kini, R.M., Govil, G., 2000. NMR studies of a neurotoxin (candoxin) from *Bungarus candidus*—presence of a predominantly beta-sheeted structure. Curr. Sci. 79, 219–221.

Poh, S.L., Mourier, Gr., Thai, R., Armugam, A., Molgo, J., Servent, D., Jeyaseelan, K., Menez, A., 2002. A synthetic weak neurotoxin binds with low affinity to Torpedo and chicken α7 nicotinic acetylcholine receptor. Eur. J. Biochem. 269, 4247–4256.

Ponder, J.W., Case, D.A., 2003. Force fields for protein simulations. Adv. Prot. Chem. 66, 27–85.

Potter, L.T., 2001. Snake toxins that bind specifically to individual subtypes of muscarinic receptors. Life Sci. 68 (22/23), 2541–2547.

Segalas, I., Roumestand, C., Zinn-Justin, S., Gilquin, B., Menez, R., Menez, A., Toma, F., 1995. Solution structure of a green mamba toxin that activates muscarinic acetylcholine receptors, as studied by nuclear magnetic resonance and molecular modeling. Biochemistry 34, 1248–1260.

Servent, D., Menez, A., 2001. Snake neurotoxins that interact with nicotinic acetylcholine receptors. In: Massaro, E.J. (Ed.), Handbook of Neurotoxicology, vol. 1. Humana, Totowa, NJ, pp. 385–425.

Teixeira-Clerc, F., Menez, A., Kessler, P., 2002. How do short neurotoxins bind to a muscular-type nicotinic acetylcholine receptor? J. Biol. Chem. 277, 25741–25747.

Torres, A.M., Kini, R.M., Nirthanan, S., Kuchel, P.W., 2001. NMR structure of bucandin, a neurotoxin from the venom of the Malayan krait (*Bungarus candidus*). Biochem. J. 360, 539–548.

Tremeaux, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.C., Menez, A., 1995. Genetic engineering of snake toxins. The functional site of erabutoxin a, as delineated by site-directed mutagenesis, includes variant residues. J. Biol. Chem. 270, 9362–9369.

Tsetlin, V., 1999. Snake venom alpha-neurotoxins and other 'three-finger' proteins. Eur. J. Biochem. 264 (2), 281–286.

Tsetlin, V.I., Hucho, F., 2004. Snake and snail toxins acting on nicotinic acetylcholine receptors: fundamental aspects and medical applications. FEBS Lett. 557 (1–3), 9–13.

Ulens, C., Hogg, R.C., Celie, P.H., Bertrand, D., Tsetlin, V., Smit, A.B., Sixma, T.K., 2006. Structural determinants of selective {α}-conotoxin binding to a nicotinic acetylcholine receptor homolog AChBP. Proc. Natl. Acad. Sci. U.S.A. 103 (10), 3615–3620.

Unwin, N., 2005. Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J. Mol. Biol. 346 (4), 967–989.

Utkin, Yu.N., Kukhtina, V.V., Maslennikov, I.V., Eletsky, A.V., Starkov, V.G., Weise, C., Franke, P., Hucho, F., Tsetlin, V.I., 2001a. First tryptophan-containing weak toxin from cobra venom. Toxicon 39, 921–927.

Utkin, Yu.N., Kukhtina, V.V., Kryukova, E.V., Chiodini, F., Bertrand, D., Methfessel, C., Tsetlin, V.I., 2001b. Weak toxin from *Naja kaouthia* is a nontoxic antagonist of α7 and muscle-type nicotinic acetylcholine receptors. J. Biol. Chem. 276, 15810–15815.