Minireview

Snake and snail toxins acting on nicotinic acetylcholine receptors: fundamental aspects and medical applications

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Received 17 October 2003; accepted 19 November 2003

First published online 15 December 2003

Edited by Maurice Montal

Abstract This review covers recent data on interactions of nicotinic acetylcholine receptors (AChR) with snake venom proteins (α- and κ-neurotoxins, 'weak' toxins recently shown to act on AChRs), as well as with peptide α-conotoxins from *Conus* snails. Mutations of AChRs and toxins, X-ray/nuclear magnetic resonance structures of α-neurotoxin bound to AChR fragments, and the X-ray structure of the acetylcholine-binding protein were used by several groups to build models for the α-neurotoxin–AChR complexes. Application of snake toxins and α-conotoxins for pharmacological distinction of muscle, neuronal and neuronal-like AChR subtypes and for other medical purposes is briefly discussed.

Key words: Nicotinic acetylcholine receptor; Snake toxin; α-Neurotoxin; α-Conotoxin

1. Introduction

Nicotinic acetylcholine receptors (AChR) are an abundant family of transmitter-gated ion channels classified into two major groups: muscle-type and neuronal (see reviews [1–3])[1]. The receptor from the *Torpedo* electric ray belongs to the first group and is composed of five subunits (two α, and one β, γ, and δ) arranged pseudo-symmetrically along a central axis. Mammalian muscle AChRs have a similar structure, with an ε subunit instead of γ in the mature form. Several versions of α (α2–α10) and non-α subunits (β2–β4) were found in neuronal AChRs which are heteromeric (combinations of α and β subunits) or homooligomeric (for example, built of five α7 subunits). Neuronal AChRs may be postsynaptic or presynaptic, the latter regulating the release of diverse neurotransmitters; neuronal-like AChR were also detected in non-neuronal tissues [2].

Current ideas on the structural organization of all AChRs [1] are largely based on experimental studies of the *Torpedo* AChR. It was the use of snake venom α-neurotoxins from cobras and kraits (see reviews [4–7]) that made possible isolation of preparative amounts of this receptor and subsequent comprehensive analysis. The aim of this review is to show how α-neurotoxins and other snake toxins are used today in AChR structural studies, for detection of pharmacologically distinct AChR subtypes and for other medical purposes. α-Conotoxins from poisonous marine snails *Conus*, another sophisticated tool in AChR research, will also be considered.

2. Sequences, three-dimensional structure and specificity of snake toxins and α-conotoxins acting on diverse AChRs

Short-chain α-neurotoxins from snake venoms (Table 1) contain 60–62 amino acid residues and four disulfide bridges, and the long-chain toxins 66–74 residues and five disulfides [4]. The spatial structure of both has a three-fingered fold: four disulfides make a hydrophobic core from which originate three loops forming a β-structure (Fig. 1). Some snake toxins not targeting AChRs have homologous cysteines and a similar spatial structure [6,7].

Short- and long-chain α-neurotoxins efficiently block both *Torpedo* and muscle AChRs, but only the long ones act on neuronal homooligomeric α7 AChRs. Heteromeric neuronal AChRs are not sensitive to α-neurotoxins, some of them are blocked by κ-bungarotoxins. The fifth disulfide in loop II of κ-bungarotoxins and of long α-neurotoxins is essential for their recognition of the respective neuronal AChRs [7].

Snake venoms contain proteins of low toxicity, named 'weak, miscellaneous, non-conventional toxins, or long neurotoxin homologs' [6–8]) having the fifth disulfide in the N-terminal loop I (Fig. 1). They have recently been shown to block muscle-type, α7 and α7-like AChRs [8–14]. Nuclear magnetic resonance (NMR) and X-ray analyses [12,15,16] revealed in weak toxins a three-finger fold. In spite of low toxicity, some weak toxins bind distinct AChRs irreversibly [11,12,14].

A loop I disulfide makes weak toxins structurally more similar than other three-finger toxins to the Ly6 proteins of the mammalian immune system and to a related protein lynx1 ('prototoxin') of the nervous system [17]. Membrane-anchored lynx1 is in proximity to neuronal α7 and α4β2 AChRs, modulating their properties. The disulfide in loop I is essential for lynx1 activity [17].

Waglerins (Table 1), neurotoxic peptides from the pit viper *T. wagleri*, block muscle AChRs. Whereas most α-neurotoxins bind with similar affinity to the two binding sites in the muscle-type AChRs (situated at the interfaces between α-sub-

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[1] Because of space limits, references are given mostly to recent original papers, earlier work being covered by reviews.

0014-5793/03/$30.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01454-6

Table 1
Representatives of snake and snail toxins acting on diverse AChRs

Toxin	Number of aa residues	S–S bonds	Target acetylcholine receptor
Snake venoms			
Short-chain α-neurotoxins (erabutoxins *L. fasciata*, α-toxins *N. nigricollis* and *N. mossambica*, neurotoxin II *N. oxiana*)	60–62	4	*Torpedo* ($\alpha_2\beta\gamma\delta$) and muscle ($\alpha_2\beta\gamma\delta$ or $\alpha_2\beta\varepsilon\delta$)
Long-chain α-neurotoxins (α-bungarotoxin *B. multicinctus*, α-cobratoxin *N. kaouthia*)	66–74	5	*Torpedo*, mammalian muscle; neuronal α7; α7-like Cl^--channels on *Aplysia* and *L. stagnalis* neurons
Long-chain κ-neurotoxins (κ-bungarotoxins *B. multicinctus*)	66	5	Neuronal α3β2 (α4β2)
'Weak' toxins (WTX *N. kaouthia*, candoxin *B. candidus*)	63–66	5	*Torpedo* and muscle, neuronal α7; α7-like Cl^--channels of *L. stagnalis*
Waglerins (from *T. wagleri*)	20–24	1	*Torpedo* and muscle
Marine snail *Conus* venoms			
α-Conotoxins G1, M1, SI, EI	13–18	2	*Torpedo* and muscle
α-Conotoxins (ImI and ImII *C. imperialis*, PnIB *C. pennaceus*, [A10L] PnIA)	12, 16	2	α7; α7-like Cl^- channels on *Aplysia* neurons
PnIA *C. pennaceus*, GIC *C. geographus*	16	2	α3β2
α-Conotoxin MII *C. magus*	16	2	α3β2, α6
α-Conotoxin AuIB *C. aulicus*	15	2	α3β4
αA-Conotoxins (PIVA *C. purpurascens*, EIVA *C. ermineus*)	25–30	3	*Torpedo* and muscle
ψ-Conotoxins (ψ-PIIIE and ψ-PIIIF *C. purpurascens*)	24	3	*Torpedo* (non-competitive block)

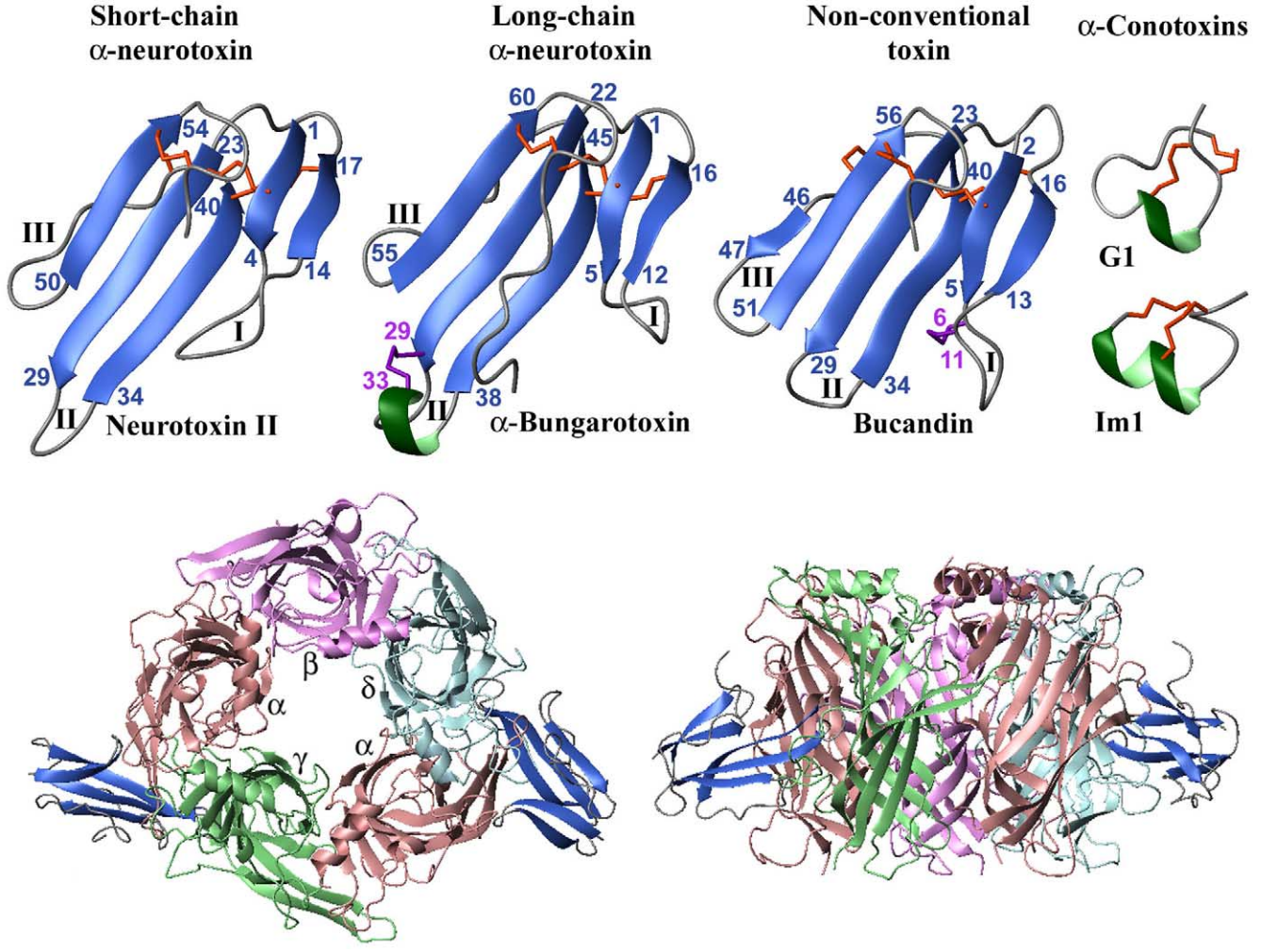

Fig. 1. Top: Ribbon presentation of the spatial structures of neurotoxin II *Naja oxiana* ([29], PDB code 1NOR), α-bungarotoxin *Bungarus multicinctus* ([30], PDB 1KFH); non-conventional toxin bucandin *Bungarus candidus* ([16], PDB 1IJC), α-conotoxin G1 *Conus geographus* ([31], PDB 1NOT), and α-conotoxin ImI *Conus imperialis* ([32], PDB 1IMI). β-Strands and helical regions are shown. The β-strand termini are marked by numbers. Additional disulfide bridges, characteristic for long-chain α-neurotoxins and non-conventional toxins, are colored in violet, and the corresponding cysteines are numbered. Roman numerals (I, II, and III) mark three loops of toxins. Bottom: Ribbon representation of the model of *Torpedo* AChR–α-bungarotoxin complex ([33], PDB 1LK1). Left to right: top and front views.

Table 2
Identified interactions between toxin and receptor residues

Toxin	Receptor or receptor fragment		
α-Bungarotoxin	*Torpedo* α1(182–202) [33]	α7 (178–196) [30]	HAP(187–199) [41]
T6	W187, Y189		HW187
A7		F186	
T8	W187, K185, L199		Y189
S9	W187, Y189	F186	
P10	W187		
I11	W187, Y189	F186	Y189
D30	Y190		R188, Y190
R36	W149, Y190, Y198	F186, Y194	S192
K38	T191, Y190	E188	E191
V39		Y187, Y194	
V40		Y187, E188	
H68	Y189, T191, C192		S193
Q71	T191, C192, P194		L194
α-Cobratoxin	*Torpedo* α1(181–198) [42]	α7–5HT3 [34][a]	
I9	Y189, T196		
T10	T191		
F29		W54	
I32	Y189		
R33	Y189	W148, Y187, P193, Y194	
K35	Y189	D163, F186	
R36	Y190	Y187, D196	
F65		F186, P193	
R68	Y190		
K69	Y190		
δ Toxin NmmI	Muscle AChR [37]		
K27	γE176, γW55, αV188		
R33	αL119, αY190, γW55		
R36	αV188, αY190		
Waglerin	Muscle AChR ($\alpha_2\beta\gamma\varepsilon$) [18] ε G57, D59, Y115, D173[b]		
α-Conotoxin M1	Muscle AChR($\alpha_2\beta\delta_2$) [35]		
A7, P6, Y12,	αY198		
Y12	δ S36, δ Y113, δ I178		
α-Conotoxin ImI	α7–5HT3 [27][a]		
R7	Y195		
α-Conotoxin PnIB	α7-5HT3 [28]c		
L10	W149		
α-Conotoxin MII	α3β2 AChR [36] α3 K185, α3 I188, β2 T59[b]		

[a]Chimera made of the α7 chick AChR (extracellular domain) and the rest of 5HT3 (serotonin) receptor.
[b]The receptor residues determining the high affinity are shown; their counterparts in the toxin have not been identified.

units and their 'non-α' counterparts), waglerins distinguish them, having much higher affinity for the α/ε site [18].

One group of α-conotoxins (Table 1) inhibit muscle-type AChRs, while others block distinct neuronal AChRs [19]. αA-conotoxins compete, like the respective α-conotoxins, with α-neurotoxins for binding to *Torpedo* receptor, while ψ-conotoxins are non-competitive blockers [19]. The spatial structures of α-conotoxins acting on various neuronal AChRs possess a similar fold differing somewhat from that of 'muscle' α-conotoxins (Fig. 1). ψ-Conotoxins have an extended conformation like μ-conotoxins blocking sodium channels [20].

α-Conotoxins allow researchers to distinguish diverse AChRs (Table 1) and even discriminate the two binding sites in the muscle-type AChRs, the difference in the affinities depending on a particular α-conotoxin and the AChRs species. α-Conotoxins differ in relative selectivity: α-conotoxin MII has been considered specific for α3β2 AChRs [19], but was recently shown to block also the α6-containing AChRs [21]. Similar to MII in affinity for α3β2 AChRs, α-conotoxin GIC is much more selective, its affinity for any other neuronal subtype being 100-fold lower, and for muscle AChR 10 000-fold lower [22]. α-Conotoxins may be isolated from venoms or first identified via analysis of cDNA and then obtained by peptide synthesis [22,23]. The latter provides unlimited possibilities for 'chemical mutagenesis', including insertion of non-natural amino acids [24]. The case of α-conotoxin PnIA/[A10L]PnIA (Table 1) illustrates a mutation-induced shift in selectivity [25,26].

Distinct α-conotoxins can act on the same AChR differently: PnIB, ImI and ImII all block the α7 AChR, the binding sites for the first two being non-identical but overlapping [27,28] (Table 2), while there is no overlap for ImII which, contrary to others, does not compete with α-bungarotoxin [23].

3. Interacting surfaces of toxins and receptors

There are no solved structures for AChR complexes with snake toxins or α-conotoxins. Below are discussed recent mutational analyses of pair-wise interactions of toxins with the whole-size receptors, as well as X-ray/NMR studies of α-neurotoxin complexes with the receptor fragments or with combinatorial peptides (Table 2).

The earlier work (reviewed in [5–7]) revealed that short-chain α-neurotoxins bind to muscle-type AChRs using loops I, II and III. For long α-neurotoxins loop III is less important, and additional contributions come from the toxin's C-terminus. Some residues in α-cobratoxin (W25, D27, R33,

F29, R36, F65) are essential for binding both to muscle and to neuronal α7 AChRs, whereas K23 and K49 are required only for the former, and C26–C30 and K35– for the latter [7]. Earlier results of affinity labeling [5,6] and recent mutational analyses [7] show that binding of different neurotoxins takes place at the interfaces of two α-subunits with δ and γ (or ε) in muscle-type receptors, and at the α/β and α/α interfaces in heteromeric and homooligomeric neuronal AChRs, respectively. Weak toxins apparently bind at subunit interfaces, one toxin (candoxin) distinguishing the two sites in muscle AChR [8]. The binding sites for α-neurotoxins, other antagonists and agonists are made by several fragments of α-subunits (A, B, C loops) and residues in the adjacent subunits (D, E and F loops) [3,34], loop C (fragment 180–200) being important for practically all kinds of ligands [18] (Table 2).

Synthetic α-conotoxin analogs made it possible to locate positions essential for binding, while AChR mutations identified the residues governing the affinity of the α/γ (α/ε) and α/δ sites [19]. Hydrophobic residues of α-conotoxin M1 are important for binding to muscle AChRs [35]. According to pairwise mutations, recognition of α7 AChR by PnIB is governed by hydrophobic interaction of toxin L10 with the receptor's W149 [28], whereas for ImI it is the R7–Y195 pair [27]. Among α-conotoxins acting on heteromeric neuronal AChRs, interactions of α-conotoxin MII with α3β2 AChRs were studied in most detail [36] (Table 2). Variability of functional groups grafted on the same scaffold (Fig. 1) allows diverse neuronal-type α-conotoxins to target distinct AChR subtypes. The same principle and a three-finger fold are employed by snake toxins against different AChRs or other targets (muscarinic receptors, acetylcholinesterase etc.) [6].

Table 2 shows that R33 from the central loop of α-cobratoxin and α-toxin NmmI interacts with several residues in the muscle and α7 AChRs. It was noted earlier that R33/F29 in α-cobratoxin have a topology similar to R7/W10 in α-conotoxin ImI which might be important for α7 AChR recognition [6]. Interestingly, the similarity in disposition of a pair of positively charged/aromatic residues in the scorpion and sea anemone toxins, differing in sequences and spatial structure, underlie their common mode of blocking the potassium channels [38].

The X-ray structure of the mollusk acetylcholine binding protein (AChBP) [39] provided the basis for visualizing the AChR–toxin interactions. Having at best 24% homology with extracellular N-terminal domains of AChR subunits, this protein contains virtually all residues essential for AChR binding of agonists and antagonists. Indeed, AChBP binds such compounds, including α-neurotoxins, and α-bungarotoxins resin was employed for isolating AChBP [40]. By molecular modeling the α7 AChR extracellular domain using the AChBP structure, a model of the α-cobratoxin complex with α7 AChR has been proposed that embraces abundant crosslinking and mutagenesis data [34].

The AChBP structure was also used for models of α-neurotoxin–AChR complexes based on the X-ray or NMR structures of α-neurotoxins with synthetic peptides [30,33,41,42]. Fragments from the region 180–200 of muscle-type α-subunits have long been known to bind these toxins, although much less efficiently than the complete receptors. Binding of α-bungarotoxin was also detected with the respective fragments of the α7 subunit [43]. Interestingly, an insertion of the α7 fragment into the α3 subunit made the α3β2 AChR sensitive to α-bungarotoxin [44]. Combinatorial peptide HAP (high affinity peptide showing some similarity to the region 187–199) bound α-bungarotoxin with nanomolar affinity, and the X-ray analysis of the complex revealed that the peptide conformation imitated the β-hairpin region 182–193 of the AChBP [41]. This finding as well as direct observations of intermolecular interactions (including H-bonds between the peptide and neurotoxin) (Table 2) were used for docking α-bungarotoxin to the AChBP [41]. A similar approach was employed for building the model of α-bungarotoxin complex with the *Torpedo* AChR starting from the H-NMR structure of the toxin complex with the α182–202 fragment [33]. In all analogous models [30,33,34,42], a portion of a toxin molecule penetrates between the subunits roughly at the half-height of the extracellular domain. (The idea of toxin penetration was earlier put forward based on photoaffinity labeling [5].)

Experiments stimulated by analyses of AChBP structure unraveled new toxin–AChR interactions: G152 and P193 form an H-bond in AChBP, and their simultaneous mutation in α7 AChR decreased the affinity for α-bungarotoxin 100-fold [45].

The current models of the toxin–AChR complexes will probably undergo some revision, since a recent publication by Unwin and co-authors [46] shows that the N-terminal extracellular domain of the *Torpedo* AChR includes a portion of the transmembrane fragment MI. It means that not only the distance of the bound toxin from the membrane surface may differ, but additional interactions with AChR appear possible which might affect the disposition of bound toxins.

4. Present and possible future applications of snake and snail toxins

Distinct AChRs are involved in many vitally important processes and their malfunctioning may be the cause or consequence of various diseases. Therefore, the determination of the density of AChR subtypes is of practical importance, and the appropriately labeled α-neurotoxins and α-conotoxins are used for this purpose, along with antibodies and other compounds. The cholinergic system is involved in cognitive functions, and the available data show a decrease in different neuronal AChRs as a result of Alzheimer's disease [2]. Recently, the involvement of α7 AChR in angiogenesis [47] and in the regulation of immune reactions via production of tumor necrosis factor [48] was demonstrated. In both cases either the respective knock-out mutants or blocking the receptors with α-bungarotoxin were used. The α7 AChRs are overexpressed in small cell lung carcinoma of smokers. In vitro experiments demonstrated that the malignant growth can be stopped by blocking these receptors with α-neurotoxins or α-conotoxins [49]. In this respect, weak toxins which have low toxicity but irreversibly block these receptors are of possible pharmacological use. There are already precedents when polypeptide or peptide toxins as such are used for medical purposes: for example, the Ca^{2+} channel blocker ω-conotoxin MVIIA (trade name Ziconotide) is on the market as treatment of chronic pain [50]. The literature data indicate both the involvement of distinct neuronal AChRs in analgesia [2] and analgesic activity of some α-conotoxins [51]. Therefore, there are reasons to expect drugs to be designed on the basis of naturally occurring or modified peptides or polypeptides targeting diverse AChRs.

Acknowledgements: The authors are grateful to Dr. I. Maslennikov for help in preparing Fig. 1.

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