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Solution conformation of a neuronal nicotinic acetylcholine receptor antagonist α-conotoxin OmIA that discriminates α3 vs. α6 nAChR subtypes

Seung-Wook Chi [a], Do-Hyoung Kim [a], Baldomero M. Olivera [b], J. Michael McIntosh [b,c], Kyou-Hoon Han [a,*]

[a] *Molecular Anti-Cancer Research Center, Division of Molecular Therapeutics, Korea Research Institute of Bioscience and Biotechnology, Yusong P.O. Box 115, Daejon, Republic of Korea*
[b] *Department of Biology, University of Utah, Salt Lake City, UT 84112, USA*
[c] *Department of Psychiatry, University of Utah, Salt Lake City, UT 84112, USA*

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Abstract

α-Conotoxin OmIA from *Conus omaria* is the only α-conotoxin that shows a ~20-fold higher affinity to the α3β2 over the α6β2 subtype of nicotinic acetylcholine receptor. We have determined a three-dimensional structure of α-conotoxin OmIA by nuclear magnetic resonance spectroscopy. α-Conotoxin OmIA has an "ω-shaped" overall topology with His^5-Asn^{12} forming an α-helix. Structural features of α-conotoxin OmIA responsible for its selectivity are suggested by comparing its surface characteristics with other functionally related α4/7 subfamily conotoxins. Reduced size of the hydrophilic area in α-conotoxin OmIA seems to be associated with the reduced affinity towards the α6β2 nAChR subtype.

Keywords: α-Conotoxin; Solution structure; Peptide; Nicotinic acetylcholine receptor; NMR

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels activated by the endogenous neurotransmitter acetylcholine and the tobacco plant toxin nicotine [1–3]. In mammals, eleven different neuronal nAChR subunits have been cloned. These homologous subunits combine in a pentameric complex to form an ion channel. The receptor subunits are widely expressed throughout the nervous system; nevertheless, there is distinct regional expression of various subunits. Different subunits may associate to form a variety of possible subunit combinations. The subunit compositions of nAChRs determine a number of pharmacological and functional properties, including affinity for agonist and antagonist, agonist efficacy, and desensitization rates [1]. Understanding the detailed ligand-receptor interaction mode for nAChRs has been greatly hampered due to the absence of a high-resolution structure of nAChRs. Two strategies have been used to study the mechanism of ligand-nAChR interaction under such circumstances. The first is to delineate receptor residues that interact with ligands through receptor modification. Several potential ligand-binding residues have been suggested from studies using mutant receptors and affinity labeled receptors [4–7]. Ligand binding modes of these residues have been confirmed in recent homology modeled structures of nAChRs [8] and the X-ray structures of acetylcholine binding protein (AChBP) bound to α-conotoxins [9–11] or α-cobratoxin [12]. Another strategy for studying nAChR-ligand interactions in the absence of nAChR structures has been to identify potential receptor-binding pharmacophores within various ligands including nAChR-targeting conotoxins [13–20].

* Corresponding author. Fax: +82 42 860 4259.
E-mail address: khhan600@kribb.re.kr (K.-H. Han).

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Table 1
Specificity of α4/7 subfamily α-conotoxins and their activity for α3β2 and α6β2 nAChRs

Name[a]	Sequence[b]	IC_{50} (nM)		Specificity[c]
		α3/β2	α6/β2	
OmIA	GCCSHPACNVNNPHICG*	11	201	α3/β2 > α7 > α6/β2
GIC	GCCSHPACAGNNQHIC*	1.1	~3	α3/β2 ≅ α6/β2 > α4/β2 ≅ α3/β4
MII	GCCSNPVCHLEHSNLC*	2.2	0.39	α6/β2 ≅ α3/β2 > α4/β2 ≅ α3/β4
PnIA	GCCSLPPCAANNPDYC*	9.56	ND	α3/β2 > α7 > α3/β4
MII[E11A]	GCCSNPVCHLAHSNLC*	8.72	0.16	α6/β2 > α3/β2
PIA	RDPCCSNPVCTVHNPQIC*	74.2	0.95	α6/β2 > α3/β2 > α3/β4
PnIB	GCCSLPPCALSNPDYC*	1970	ND	α7 > α3/β2
PnIA[N11S]	GCCSLPPCAASNPDYC*	241	ND	

ND, not determined.
[a] The first capital letter indicates the species origin.
[b] The asterisk indicates an amidated C-terminus.
[c] The references are as follows: OmIA [29], GIC [44], MII, MII[E11A] [16], PIA [31], PnIA, PnIB, PnIA[N11S] [17].

Conus is a large genesis of predatory snails that feed on fish, snails, and marine worms [21]. Their venoms contain hundreds of different peptides that are notable for their small size, potency, and subtype selectivity. One of the most common types of *Conus* peptides is the α-conotoxin that acts at the muscle nAChR subtype, α7-homomers, α3β2, α6β2β3, α6β4, α3β4, and α9α1 subunit combinations [17,22–24]. Extensive structural characterization and comparison of the nAChR targeting conotoxins have suggested residues that might be important for their subtype recognition specificities [18–20,25–28]. In particular, the structural studies on the α4/7-conotoxins have shown that these toxins with a same backbone topology can display a widely different receptor recognition profiles by relying on subtle differences in their surface properties [15,18,27,28] (Table 1), suggesting that precise delineation of structural factors that are responsible for specific recognition of a particular receptor subtype calls for a large and reliable structural database on α-conotoxins.

α-Conotoxin OmIA is a newly discovered peptide from *Conus omaria*, showing nanomolar affinity for *Lymnaea* and *Aplysia* AChBPs in addition to α3β2, α7, and α6β2 nAChR subtypes [29]. In particular, it is the only α-conotoxin displaying a ~20-fold higher affinity to the α3β2 nAChR subtype over the closely related α6β2 subtype. Out of more than 20 nAChR-targeting conotoxins identified to date, only two native α-conotoxins MII and PIA can distinguish the two nAChR subtypes, but unlike α-conotoxin OmIA, MII, and PIA favor the α6β2 over the α3β2 subtype by ~5- and ~80-fold, respectively. Thus, α-conotoxin OmIA represents a promising candidate for designing α3β2 selective ligands. As the α3β2 nAChR subtype is implicated in human dopaminergic modulation, the ability to manipulate this nAChR subtype may be of therapeutic benefit with respect to Parkinson's disease [30]. Here, we describe the three-dimensional solution structure of α-conotoxin OmIA.

Materials and methods

Peptide synthesis and purification. α-Conotoxin OmIA, originally purified from the venom of *Conus Omaria*, was synthesized and purified according to the previously described protocol [31].

NMR experiments. Samples for the NMR studies were prepared in 90% H_2O/10% ^2H_2O or in 100% ^2H_2O with a final concentration of 4.8 mM at pH 4.1. The pH was measured as a direct reading from a combination microelectrode calibrated at two reference pHs. All NMR experiments were performed using a Varian UNITY INOVA 600 spectrometer at 25 and 15 °C in order to obtain unambiguous resonance assignment. For TOCSY experiments [32], mixing times of 65–87 ms were applied. Spectral widths were 6 kHz in both dimensions. Typical 2D data consist of 2048 complex points in the t_2 dimension with 256 complex t_1 increments.

Structure calculations. Inter-proton distance restraints used for structure calculation were derived primarily from the NOESY spectrum recorded with a mixing time of 200 ms obtained at 15 °C. The NMRView 5.0.4 program (Merck & Co Inc., NJ) was used for peak picking and quantification of

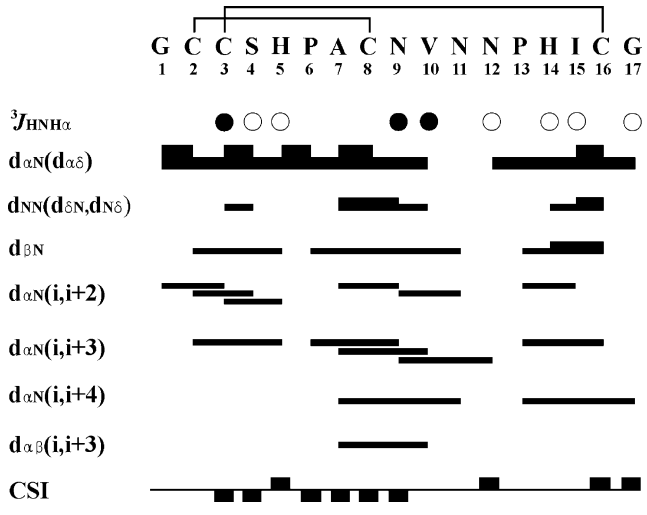

Fig. 1. Amino acid sequence of α-conotoxin OmIA and a summary of short- and medium-range NOEs, $^3J_{HN\alpha}$, and chemical shift index (CSI) for H_α protons. Thickness of bar represents relative strength (strong, medium, and weak) of NOEs. Filled circles are drawn when $^3J_{HN\alpha} < 6$ Hz and open circles when $^3J_{HN\alpha} > 8$ Hz. The filled squares above and below the horizontal line represent CSI values of +1 and −1, respectively. Note the presence of α-helix from His^5-Asn^{12}, a common feature in the α4/7-conotoxin subfamily.

NOE volumes. A total of 152 NOE crosspeaks were manually assigned and 9 dihedral angle restraints were derived from coupling constants. Backbone torsion angle ϕ was set to $-120°$ ($\pm 20°$) when ${}^{3}J_{HNH\alpha} > 8$ Hz and to $-65°$ ($\pm 20°$) if ${}^{3}J_{HNH\alpha} < 6$ Hz. Using these experimental datasets, structure calculation was performed using ARIA (version 1.1) as described [33]. Floating chirality assignment [34,35] was employed for all methylene and isopropyl groups. The final iteration was done using a total of 290 unambiguous NOE-derived inter-proton distance restraints [121 intraresidue, 106 sequential, 45 medium-range ($|i-j| < 4$), and 18 long-range ($|i-j| \geqslant 4$) inter-proton restraints] as well as 9 dihedral angle restraints. Out of 50 structures calculated, 20 low energy structures of α-conotoxin OmIA were selected and a quality analysis of them was performed using the program PROCHECK [36]. The atomic coordinates of α-conotoxin OmIA have been deposited in the Protein DataBank under Accession No. 2GCZ.

Results

NMR spectroscopy

NMR resonance assignment for α-conotoxin OmIA was achieved using homonuclear two-dimensional NMR methods according to the standard sequential resonance assignment strategy [37]. Due to their unique "back-transfer" crosspeaks, unambiguous assignment of His^{5} and His^{14} was possible in TOCSY spectra without relying on the NOE connectivity information. Also, Ser^{4}, Pro^{6}, Val^{10}, Pro^{13}, and Ile^{15} were assigned based only on the COSY and TOCSY spectra following their characteristic coherence transfer patterns. For the other residues, classification of spin systems in a TOCSY spectrum along amide NH resonances preceded the sequential resonance assignment procedure. Strong sequential $d_{\alpha\delta}$ type NOE crosspeaks representing inter-proton distances of less than 2.5 Å were clearly observed for Pro^{6} and Pro^{13}, indicating that they are in trans configuration [38]. Shown in Fig. 1 are short- and medium-range NOEs used for resonance assignment, ${}^{3}J_{HNH\alpha}$, and chemical shift index (CSI) [39] along the amino acid sequence of α-conotoxin OmIA.

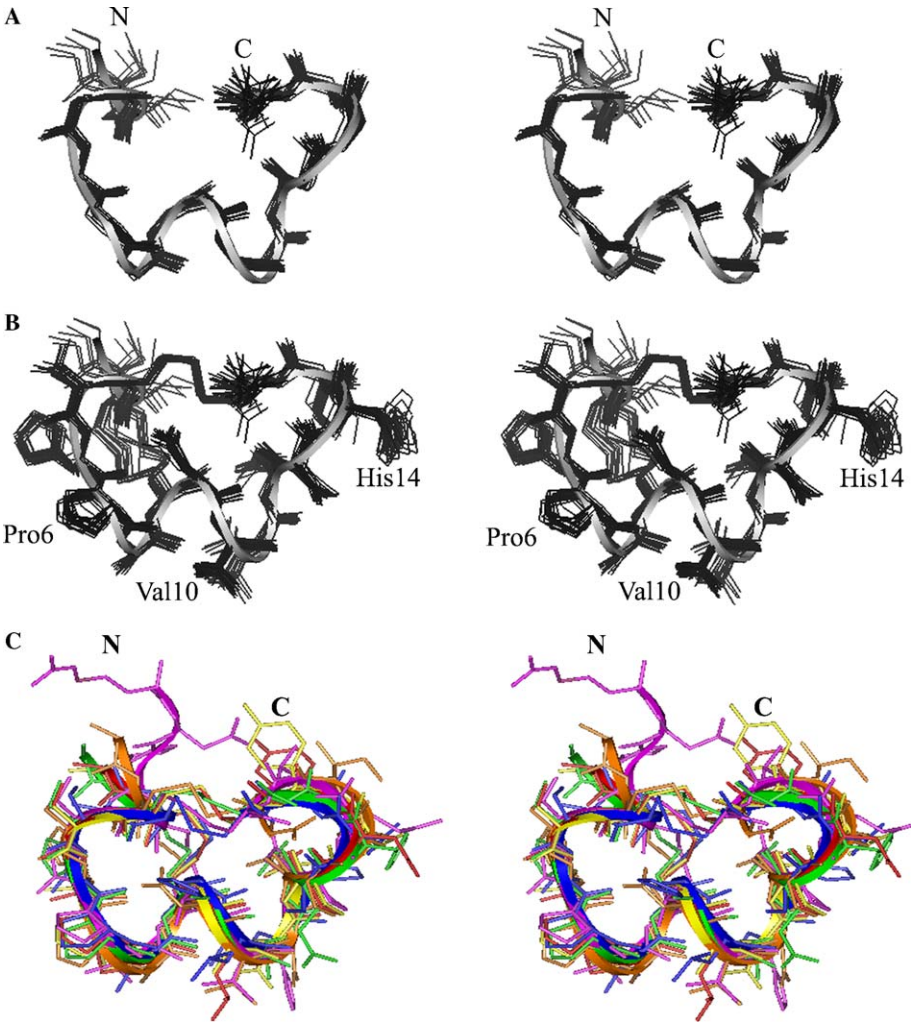

Fig. 2. A stereo view of the final 20 structures for α-conotoxin OmIA. (A) Backbone only. (B) Backbone and heavy chains. A ribbon is drawn to trace the backbone. The N- and C-terminus are labeled with capital letters N and C, respectively. For visual clarity only selected residues are labeled. (C) Backbone superposition of six different α3β2 nAChR targeting α-conotoxins; OmIA (orange), PIA (magenta), MII (blue), GIC (green), PnIA (yellow), and PnIB (red). Superposition is made over residues 2–16 of α-conotoxin OmIA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Structural description of α-conotoxin OmIA

Fig. 2 shows the final ensemble of 20 superimposed structures of α-conotoxin OmIA. The structure is well determined with backbone and heavy atom RMSDs over residues 2–16 of 0.36 and 0.75 Å, respectively. The overall statistics for structure calculation is summarized in Table 2. The quality evaluation by PROCHECK [36] indicates that 76.2% and 23.8% of backbone dihedral angles of all non-glycine and non-proline residues reside within the most favored region and additional allowed region of the Ramachandran plot, respectively. The backbone fold of α-conotoxin OmIA is ω-shaped, similar to that found in other α4/7-conotoxins (Fig. 2C). Secondary structure analysis by PROMOTIF [40] shows that the residues Cys^2-His^5 and Asn^{12}-Ile^{15} constitute type I β-turns and the residues Pro^{13}-Cys^{16} and His^{14}-Gly^{17} form consecutive type IV β-turns. At the bottom of the ω-shaped fold, a two-turn α-helix is composed of residues from His^5 to Asn^{12}. A hydrophobic face on the α-helix consists of Pro^6, Ala^7, and Val^{10} and a hydrophilic face at the other side comprises His^5, Cys^8, Asn^9, Asn^{11}, and Asn^{12}. The amphipathic nature of this α-helix is a common feature among the α4/7-conotoxin members. The hydrophobic face, details of which differ among different toxins, is believed to interact with the hydrophobic ligand-binding pocket of the nAChR. Shown in Fig. 2C is the superposition of the backbone of α-conotoxin OmIA with those of five other α3β2 nAChR targeting α-conotoxins. The backbone RMSDs over residues 2–16 are 0.74 Å with PnIB (PDB Accession No.: 1AKG), 0.76 Å with PnIA (1PEN), 0.79 Å with GIC (1UL2), 1.12 Å with PIA (1ZLC), and 1.20 Å with MII (1M2C), respectively.

Table 2
NMR structural determination statistics of α-conotoxin OmIA for an ensemble of 20 structures

RMS deviations from experimental restraints[a]	
NOE distance restraints (Å)	0.374 ± 0.006
Dihedral angle restraints (deg)	0.753 ± 0.210
Number of NOE distance restraints	290
Intraresidue	121
Sequential	106
Medium range (<4)	45
Long range (⩾4)	18
Ramachandran plot regions (%)	
Residues in most favored region	76.2
Residues in additional allowed region	23.8
Residues in generously allowed region	0.0
Residues in disallowed region	0.0
Angular order parameters	
Phi	0.955 ± 0.149
Psi	0.977 ± 0.047
Chi 1	0.739 ± 0.315
RMS deviations from the average structure (residues 2–16) (Å)	
Backbone atoms[b]	0.36 ± 0.10
Heavy atoms	0.75 ± 0.15

[a] Values where applicable are means ± standard deviations.
[b] Backbone atoms are N, C^{α}, C′, and O.

Discussion

Generation of selective ligands that can effectively discriminate different nAChR subtypes should provide potentially novel avenues for treating a variety of diseases including memory disorders, impaired cognition, depression, schizophrenia, and nicotine addiction. The ability of a ligand to discriminate among nAChRs containing α6 and α3 subunits that share a sequence identity of ~80% in the ligand-binding extracellular domain [41] would be particularly valuable for assessing the potential roles of these receptor subtypes in Parkinson's disease. Some α4/7 subfamily conotoxins have features that can be potentially utilized to produce such ligands as they show a broad spectrum of affinity to nAChRs composed of α3 and α6 subunits (Table 1). Three native members of α4/7-conotoxins, MII, GIC, and PIA target nAChRs containing both α6 and α3 subunits. Among the three, α-conotoxins MII and GIC do not distinguish between the two subtypes whereas α-conotoxin PIA not only can but binds preferentially to the α6β2 over the α3β2 subtype of nAChR by nearly two orders of magnitude [31]. α-Conotoxin OmIA is the first that exhibits a ~20-fold higher affinity towards the α3β2 subtype over the α6β2 and may serve as a platform for synthetic creation of more selective ligands.

Extensive structural studies, surface comparisons as well as generation of mutants have been carried out for the α4/7 subfamily conotoxins [7,15–18,25–28,42–44], showing that the surface characteristics such as amphipathicity, hydrophobicity, surface charge, and steric factors determine the nAChR recognition profile of an α-conotoxin. For example, α-conotoxins MII and GIC exhibit the highest binding affinity to the α3β2 subtype with IC_{50} of 1–2 nM (Table 1) and α-conotoxins PnIA, OmIA, and PIA rank next with the α3β2 subtype IC_{50} of 10–75 nM. On the other hand, the binding affinity of α-conotoxin PnIB for the α3β2 subunit is the lowest ($IC_{50} \cong 2000$ nM). Surface representations for these toxins and for the newly determined α-conotoxin OmIA are shown in Fig. 3. The six toxins share similar surface characteristics that are probably suitable for recognition of the α3β2 subtype. The location of two disulfide bonds is invariant. The frontal side of these toxins (the left panel in Fig. 3) shows a prominent and contiguous hydrophobic region at the bottom (shown in purple) with varying sizes. The fact that these toxins have such a hydrophobic region is consistent with the observations that hydrophobic interaction is one of the major ligand-binding mechanisms of nAChRs [6,7,9,12].

On the contrary, hydrophilic areas are less contiguous and form dispersed patches (shown in cyan). The hydrophilic locus occupied by a serine in the upper left corner is ubiquitous in these toxins. The Asn^9, Thr^{11}, and His^9 in α-conotoxins OmIA, PIA, and MII, respectively, forms a central hydrophilic locus while the same spot is occupied

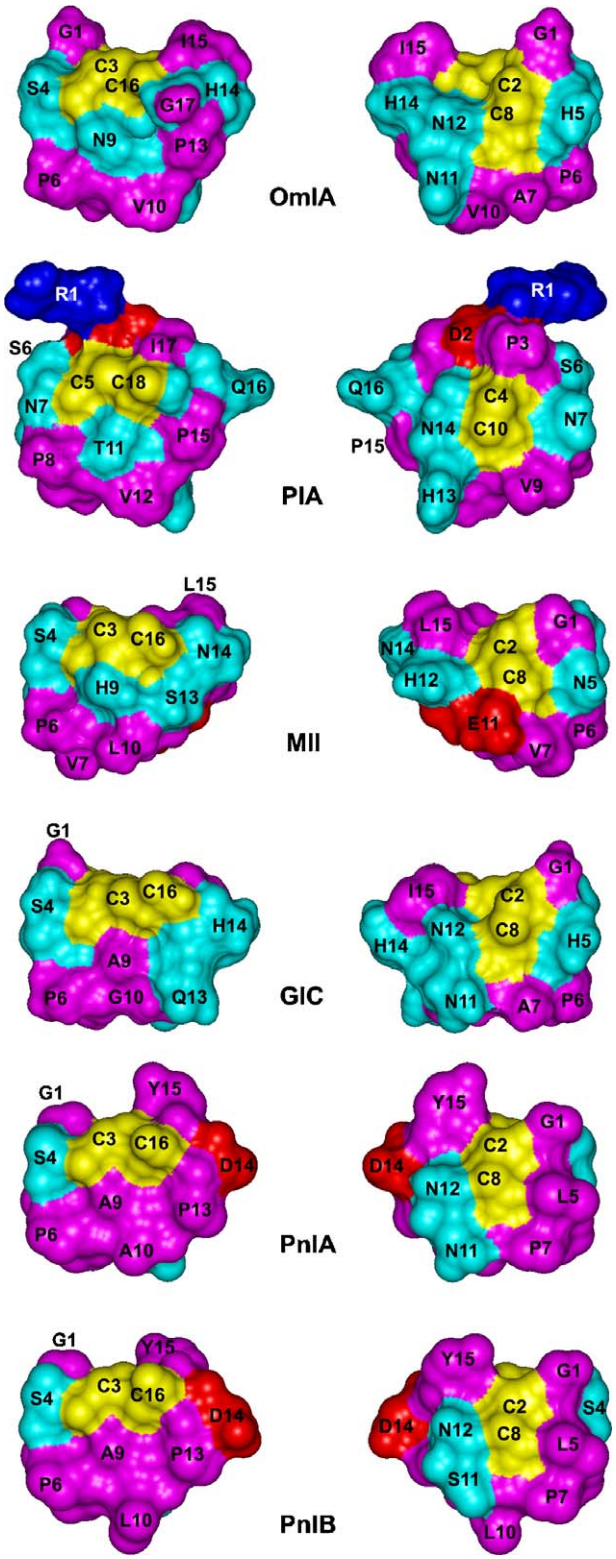

Fig. 3. Surface comparison of α-conotoxin OmIA with the other five α3β2 nAChR targeting α-conotoxins. Positively and negatively charged residues are shown in blue and red, respectively. Hydrophobic and hydrophilic residues are shown in purple and cyan, respectively. Cystein residues involved in disulfide bonds are shown in yellow. Left panel, a front view. Right panel, a view from the other side. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

by a hydrophobic alanine (Ala^{9}) in α-conotoxins GIC, PnIA, and PnIB. In the right side of the molecule α-conotoxins MII and GIC have relatively large hydrophilic domains that are much larger than those in the other four toxins. Introducing a proline (Pro^{13} or Pro^{15}) and thereby reducing the size of this hydrophilic domain to those found in α-conotoxins OmIA, PIA, PnIA, and PnIB may be responsible for reduced potency at the α3β2 in these toxins (Table 1). The back side of the toxins (the right panel in Fig. 3) shows a common hydrophilic region in the left formed by three hydrophilic (polar or negatively charged) residues. Thus, presence of such common hydrophobic and hydrophilic domains, albeit different in their exact sizes, is likely to represent a minimal structural basis for recognition of α3β2 and/or α6β2 nAChR subtypes.

Close examination of Table 1 reveals an interesting fact that the affinity of α-conotoxin OmIA to the α3β2 subtype is actually weaker than those of MII and GIC by a factor of 5–10. The α3 vs. α6 discriminatory capacity of α-conotoxin OmIA in fact stems from its lower affinity to the α6β2 subtype. When the surface property of α-conotoxin OmIA is compared in more details with those of other α4/7 members, one finds that the hydrophobic region at the frontal side of this toxin (left panel in Fig. 3) is somewhat more extended than in others due to its C-terminal Gly^{17}. This glycine is unique since no other α-conotoxins, α3/5, or α4/7-conotoxins, have been found to contain such a C-terminal extra residue. Another potential contributor to the lowered α6β2 affinity of α-conotoxin OmIA is its Pro^{13} that introduces a kink in the second loop. However, since a proline (Pro^{15}) is also present at the exactly same spatial location in α-conotoxin PIA that is highly α6β2-selective, it is not clear if the reduced α6β2 affinity of α-conotoxin OmIA can be attributed to its Pro^{13}. In α4/7 subfamily of conotoxins the residue at the 11th position has been shown to be particularly critical for the α3β2 potency [16,17]. For example, α-conotoxin [N11S]PnIA is 24-fold less active against the α3β2 subtype than a wild-type PnIA [17] (Table 1). Also, mutation of Glu^{11} to Ala in α-conotoxin MII reduces its activity by a factor of 4 at the α3β2 subtype, but slightly increases its potency for the α6β2 subtype [16]. Placing a histidine instead of Asn^{11} at the 11th position and adding an N-terminal Arg-Asp-Pro tail such as found in α-conotoxin PIA is expected to further decrease the α3β2 specificity. A similar replacement into α-conotoxin PnIA at the same position ($Asn^{11} \rightarrow Ser$) and enhancing the hydrophobicity at the 10th position by $Ala \rightarrow Leu^{10}$ substitution converts it into α-conotoxin PnIB, the worst α3β2 binder.

Acknowledgments

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