Published in final edited form as:

J Mol Biol. 2008 April 4; 377(4): 1216–1227.

α -RgIA, a Novel Conotoxin that Blocks the $\alpha 9\alpha 10$ nAChR: Structure and Identification of Key Receptor Binding Residues

Michael Ellison^{1,^}, Zhi-Ping Feng^{2,^}, Anthony J. Park¹, Xuecheng Zhang², Baldomero M. Olivera¹, J. Michael McIntosh¹, and Raymond S. Norton^{2,*}

¹ Department of Biology, University of Utah, Salt Lake City, Utah, 84112, USA

² The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville 3050 Australia

Abstract

α -Conotoxins are small disulfide-constrained peptides from cone snails which act as antagonists at specific subtypes of nicotinic acetylcholine receptors (nAChRs). The 13-residue peptide α -RgIA is a member of the α -4,3 family of α -conotoxins and selectively blocks the $\alpha 9\alpha 10$ nAChR subtype, in contrast to another well characterized member of this family, α -ImI, which is a potent inhibitor of the $\alpha 7$ and $\alpha 3\beta 2$ nAChR subtypes. In this study, we have altered side chains in both the 4-residue and 3-residue loops of α -RgIA, and have modified its C-terminus. The effects of these changes on activity against $\alpha 9\alpha 10$ and $\alpha 7$ nAChRs were measured, the solution structures of α -RgIA and its Y10W, D5E and P6V analogues were determined from NMR data, and resonance assignments made for α -RgIA[R9A]. The structures for α -RgIA and its three analogues were well-defined except at the chain termini. Comparison of these structures with reported structures of α -ImI reveals a common two-loop backbone architecture within the α -4,3 family, but with variations in side chain solvent accessibility and orientation. Asp5, Pro6 and Arg7 in loop 1 are critical for blockade of both the $\alpha 9\alpha 10$ and $\alpha 7$ subtypes. In loop 2, α -RgIA[Y10W] had activity near that of wild-type α -RgIA, with high potency for $\alpha 9\alpha 10$ and low potency for $\alpha 7$, and had a similar structure to wild-type. By contrast, Arg9, in loop 2, is critical for specific binding to the $\alpha 9\alpha 10$ subtype, probably because it is larger and more solvent accessible than Ala9 in α -ImI. Our findings contribute to a better understanding of the molecular basis for antagonism of the $\alpha 9\alpha 10$ nAChR subtype, which is a target for the development of analgesics for treatment of chronic neuropathic pain.

Keywords

conotoxin; structure; peptide; NMR; nicotinic acetylcholine receptor; pain

Introduction

Nicotinic acetylcholine receptors (nAChRs) are acetylcholine (ACh) gated ion channels.¹ They are composed of pentamers of transmembrane proteins organized so as to form a central ion-conducting pore. Many different nAChR subunits exist, with δ , γ , ϵ , $\alpha 1$ - $\alpha 10$ and $\beta 1$ - $\beta 4$ subunits

*Corresponding author Raymond S. Norton, The Walter & Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, 3050, Australia. Phone: +61 3 9345 2306. Fax: +61 3 9345 2686. Email: ray.norton@wehi.edu.au.

[^]These authors contributed equally to this work.

ACCESSION CODES:

The structures have been deposited in the Protein Data Bank⁴³ with accession codes 2JUT, 2JUS, 2JUR and 2JUQ.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

having been described in vertebrates. Different arrangements of these subunits can be incorporated into functional receptor pentamers, giving rise to diverse nAChR subtypes. For example, at the neuromuscular junction, receptors composed of $(\alpha 1)_2\beta 1, \delta$ and γ mediate synaptic transmission at the motor end plate. Heteromeric receptors composed of only α and β subunits also occur, e.g. the $\alpha 3\beta 2$ nAChR is formed from $\alpha 3$ and $\beta 2$ subunits. Certain nAChR subtypes contain only α subunits, of which there are two types; the $\alpha 7$ and $\alpha 9$ subunits form homopentamers, whereas the $\alpha 9$ and $\alpha 10$ subunits combine in the heteropentameric $\alpha 9\alpha 10$ subtype.

Acetylcholine, competitive antagonists and other competitive agonists bind nicotinic receptors at a site in the interface between the N-terminal extracellular domains of adjacent subunits. At these interfaces one side of the inter-subunit cleft is the principal (+) face which is characterized by a vicinal disulfide bond in a backbone loop, the C-loop, which projects from it and partially buries bound competitive ligands.^{2,3} Only α subunits are thought to form the principal side of the subunit interfaces and only they have a vicinal disulfide bridge in their C-loop. The complementary (–) side of the ACh site is opposite the (+) face on the other side of the inter-subunit cleft; in heteromeric receptors it is provided by non- α subunits whereas in $\alpha 7$, $\alpha 9$ and $\alpha 9\alpha 10$ receptors it is provided by α subunits.

Subtype-specific inhibitors of nAChRs are potentially valuable neurochemical tools for dissecting the roles of the different nAChRs and may also be important therapeutic agents. One particularly rich source of subtype-specific nAChR antagonists is the α -conotoxin family.^{4,5} α -conotoxins are venom peptides from snail species in the genus *Conus*.^{6,7} They have the invariant disulfide scaffold shown in Figure 1 but are also characterized by high sequence diversity in their inter-cysteine loops. α -conotoxins can be further subdivided based on the number of amino acids in their inter-cysteine loops; one subclass, found almost exclusively in *Conus* species that hunt marine worms (unpublished observation), are the α -4,3 toxins which have four amino acid residues in the first loop and three in the second. Two peptides in the α -4,3 family are α -ImI (from *Conus imperialis*) and α -RgIA (from *Conus regius*). α -ImI is a potent inhibitor of the $\alpha 7$ and $\alpha 3\beta 2$ nAChR subtypes,⁸ whereas α -RgIA selectively blocks the $\alpha 9\alpha 10$ subtype.⁹ The ability of α -RgIA to block $\alpha 9\alpha 10$ receptors appears to account for its potent analgesic properties in animal models of neuropathic pain.¹⁰

Here we describe the solution structures of α -RgIA and three analogues, Y10W, D5E and P6V, sequence-specific resonance assignments for the R9A analogue, and structure/function studies carried out using analogues of α -RgIA. This has enabled the identification and visualization of residues critical for $\alpha 9\alpha 10$ potency and specificity. Comparison with previous structures of α -ImI supports the model that different peptides in the α -4,3 subfamily have very similar two-loop backbone structures and their diverse functional properties appear to result from the presentation of different side chains by fundamentally the same scaffold. Our mutational studies indicated that Asp5, Pro6 and Arg7 in loop 1 are critical for $\alpha 9\alpha 10$ potency and that Arg9 in loop 2 is critical for high potency for $\alpha 9\alpha 10$ and low potency for $\alpha 7$, i.e. for the subtype specificity of α -RgIA. Based on our findings we speculate on how α -RgIA might interact with the $\alpha 9\alpha 10$ nAChR.

Results

NMR spectroscopy

α -RgIA and its four analogues gave good quality spectra at pH 3 – 6 and 298 K. The spectra collected at lower pH or lower temperature showed greater peak overlap. At higher pH, cross-peaks from the amide of Cys2 disappeared in NOESY spectra because of exchange with solvent water, but other peaks did not shift appreciably, reflecting the fact that the Asp5 and C-terminal carboxyl groups would be ionized above pH 4 (the amide and aromatic regions of 1D spectra

of wild-type α -RgIA at different temperatures and different pH are shown in Figure S1A and B, respectively, of the Supplementary Material). Peaks for Asp5, Arg9, Tyr10 and Cys12 of wild type α -RgIA were broadened in NOESY spectra, but no other spectral inhomogeneities were observed under the experimental conditions, indicating the presence of a single predominant conformation for each peptide in solution. One-dimensional spectra of the four analogues, Y10W, D5E, P6V and R9A, are shown in Figure S1C.

Spin system assignments in TOCSY spectra and sequential connectivities in the fingerprint region of corresponding NOESY spectra acquired on a Bruker DRX-600 spectrometer at 298 K for all five peptides are shown in Figure S2A to E. Chemical shift assignments are presented in Tables S1A to E, and have been deposited in the BioMagResBank.¹¹

Self diffusion coefficients of α -RgIA and its analogues are $(1.71 \pm 0.08) \times 10^{-10}$ m²/s, $(2.00 \pm 0.05) \times 10^{-10}$ m²/s and $(3.26 \pm 0.14) \times 10^{-10}$ m²/s at 5, 10 and 25 °C, respectively. The values at 5 °C are 10% faster than J1cc, a 19-residue peptide with a less well-defined structure,¹² and the values at 10 °C are comparable with extrapolated values for similar size peptides with well-defined structures,¹³ indicating that α -RgIA and its analogues are monomeric in solution.

The *trans* orientation of the peptide bond preceding Pro6 was established by the intense H ^{α} -H ^{δ} NOEs between residues 5 and 6 for wild-type α -RgIA and its Y10W, D5E and R9A analogues. For wild-type α -RgIA, the backbone amide temperature coefficient for Tyr10 was smaller than 3 ppk/K in magnitude, and those for Cys2, Cys3, Arg7 and Cys12 were around 5 ppk/K, indicating that these amides were partially protected from solvent. However, no slowly exchanging amide protons were observed in 1D spectra recorded after dissolution of α -RgIA in 100% ²H₂O at pH 5 and 5 °C.

Deviations of the backbone NH and H ^{α} chemical shifts of α -RgIA and the four analogues from random coil values¹⁴ are shown in Figures S3A to E in the Supplementary Material. The significant deviations across the sequence indicate that α -RgIA is structured (Figure S3A), as expected from the presence of two disulfide bridges and the structures of other α -conotoxins. The similarities in the patterns of these deviations (Figures S3B to E) with α -ImI (Figures S3F and G) confirm that all three analogues adopt similar backbone structures.

The changes in NH shifts for the R9A analogue relative to wild-type (Figure 2A) were mostly < 0.1 ppm. Larger shifts were observed for Ser4 and Arg 7 of α -RgIA[D5E], Val6 of α -RgIA [P6V] and the C-terminal residues of α -RgIA[Y10W]. Differences in H ^{α} shifts for the four analogues relative to wild-type (Figure 2B) were mostly < 0.1 ppm, except for Cys2 and Arg11 in α -RgIA[Y10W], Glu5 and Cys8 in α -RgIA[D5E], and Asp5, Val6 and Cys8 in α -RgIA [P6V].

Solution structures

A summary of experimental constraints and structural statistics for α -RgIA and the three analogues, Y10W, D5E and P6V, is given in Table 1. Values of the angular order parameters $S > 0.8$ generally indicate that an angle is well defined within the family of structures; for wild type α -RgIA, the angular order parameters for ϕ and ψ angles in the final ensemble of 20 structures were both > 0.8 over residues 2-12 (Figure S4A). The mean pairwise RMSD over the backbone heavy atoms of residues 2-12 in this family of structures was 0.43 Å. The closest-to-average structure of α -RgIA (Figure 3) is characterized by two compact loops of four and three residues, defined by the two disulfide bonds. The first loop is better defined than the second. Residues 2-5 approximate a type-I β -turn formed, which is supported by the observation of hydrogen bond between the Asp5 NH and Cys2 O in 13 of the 20 structures, as well as the backbone ϕ and ψ angles of Ser4 and Asp5. Side chains for residues in the first loop are mostly well defined except for Ser4 (Figures 4 and S5). Although the second loop is less

well defined, Tyr10-Cys12 form an inverse γ -turn, with most (18) of the final structures showing a Cys12 NH to Tyr10 O hydrogen bond.

In order to ensure that changes in activity associated with side chain substitutions (see below) were not associated with changes in structure, we have also determined the structure of α -RgIA [Y10W] and sequence-specific resonance assignments for α -RgIA[R9A], both of which have substitutions in loop 2, as well as low resolution structures for α -RgIA[D5E] and α -RgIA[P6V], with substitutions in loop 1. The backbone angular order parameters (S) in the final ensembles of structures for the three analogs are plotted as a function of residue number in Figure S4B-D. For α -RgIA[Y10W], ϕ and ψ angles were well defined over residues 2-11, with a pairwise RMSD of 0.64 Å over the backbone heavy atoms (N, C $^\alpha$, C'), while for α -RgIA[D5E] and α -RgIA[P6V], ϕ and ψ angles were well defined over residues 2-10 and 3-10 and corresponding RMSD values were 0.77 and 0.67 Å, respectively. These structures are not as well-defined as those for α -RgIA because the samples were more dilute and some of the spectra were acquired on lower field spectrometers. Nonetheless, they are all characterized by two compact loops of four and three residues, respectively, and the similar secondary shifts of α -RgIA[R9A] to wild-type α -RgIA indicate these two peptides have similar backbone structures. Cys12 NH to Arg9 O hydrogen bonds were present in 8 out of 20 of the α -RgIA[Y10W] structures, as in α -ImI (see below), and support a sharp bend about Trp10. Hydrogen bonds were also observed from HN of Glu5 or Asp5 to O of Cys2 in 11 of the 20 structures for α -RgIA[D5E] and α -RgIA [P6V], and 7 of 20 for α -RgIA[Y10W]. The second loop was less well defined in all of these peptides than the first loop, as indicated by the reduction in pairwise RMSD values from 0.43, 0.64, 0.77 and 0.67 Å when superimposed over all well-defined residues (Table 1) to 0.20, 0.34, 0.53 and 0.52 Å when superimposed over Cys2 to Cys8, for wild-type α -RgIA, α -RgIA [Y10W], α -RgIA[D5E] and α -RgIA[P6V], respectively.

The other 3,4 α -conotoxin for which three-dimensional structures have been determined is α -ImI.^{2,3,15-19} Considering wild-type α -RgIA and the three analogues for which solution structures were determined in this study, α -RgIA[Y10W] is the most similar chemically to α -ImI, so we have compared its structure to α -ImI. Superposition of the backbone heavy atoms (N, C $^\alpha$ and C') of the final ensembles of 20 structures with α -ImI structures over residues 1-12 (Figure 5) gave average RMSD values of 0.77, 0.74, 1.00, and 0.68 Å for α -ImI solution structures solved by (A) Rogers et al. (PDB code 1IM1, 20 structures),¹⁸ (B) Maslennikov et al. (PDB code 1IMI, 20 structures),¹⁷ (C) Gehrmann et al. (PDB code 1CNL, 10 structures),¹⁵ and (D) Lamthanh et al. (PDB code 1G2G, 20 structures),¹⁶ respectively. Although the 1IMI, 1CNL and 1G2G structures are for C-terminally amidated peptides (unlike α -RgIA and 1IM1) and are apparently very well-defined structures, significant differences are evident in the backbone conformation around Cys3 and Cys8 and in the orientation of the Arg7 side chain and Trp10 ring, as shown in Figure 5B-D. An average RMSD value of 0.72 Å was obtained to the mean crystal structures of α -ImI in complex with *Aplysia californica* AChBP solved by (E) Hansen et al. (PDB code 2BPY, non C-terminally amidated)² and (F) Ulens et al (PDB code 2C9T, C-terminally amidated).¹⁹

α -RgIA binds at the ACh binding site on the $\alpha 9\alpha 10$ nAChR

Three conserved aromatic residues on the principal side of the subunit interfaces in nAChR pentamers are highly conserved and interact with competitive agonists and antagonists. Initially these residues were identified due to cross-linking with reactive competitive ligands of nAChRs²⁰ and their presence at the ACh site was confirmed in structures of ACh binding proteins from *Lymnaea stagnalis*^{3,21} and *A. californica*.^{2,3,19} as well as the *Torpedo* nAChR.²² Subsequent co-crystal structures with the ACh binding protein from *Aplysia* revealed interactions between the conserved aromatic residues and competitive nicotinic ligands,^{2,23} and receptor mutagenesis indicated that these residues in the $\alpha 7$ nAChR were crucial for α -ImI

binding.²⁴ To determine whether α -RgIA was a competitive inhibitor of the $\alpha 9\alpha 10$ nAChR, Trp176 of the $\alpha 9$ subunit (Figure 6) was mutated to threonine and this mutant subunit was co-expressed with wild type $\alpha 10$ subunits in *Xenopus* oocytes. The mutant $\alpha 9[W176T]\alpha 10$ receptor was less potently gated by ACh and less potently inhibited by α -RgIA (Figure 7 and Table S2), showing that the toxin is a competitive inhibitor of the receptor and binds at the ACh binding site, i.e. at the interface between adjacent subunits.

The DPR motif in loop 1 is crucial for high potency block of $\alpha 9\alpha 10$ nAChRs by α -RgIA

It has been shown that the Asp-Pro-Arg (DPR) motif in the first loop of α -ImI is crucial for its high affinity binding to the $\alpha 7$ nAChR.^{25,26} We therefore made mutations in the first loop of α -RgIA that had been made previously in α -ImI (S4A, D5E, P6V, R7K) and evaluated the potency of the analogues on oocyte-expressed $\alpha 7$ and $\alpha 9\alpha 10$ nAChRs. As shown in Figure 8, the changes D5E, P6V and R7K, but not S4A, caused large reductions in the potency of α -RgIA on both $\alpha 9\alpha 10$ and $\alpha 7$ receptors, although the effects on $\alpha 9\alpha 10$ (Figure 8A) are larger than on $\alpha 7$ (Figure 8B). Since D5E, P6V and R7K mutations in α -ImI were found previously to reduce its affinity for the $\alpha 7$ receptor our data imply that the DPR motifs in α -RgIA and α -ImI play similar roles in anchoring these toxins to both the $\alpha 9\alpha 10$ and $\alpha 7$ nAChRs.

Residues in the second half of α -RgIA are crucial for the $\alpha 9\alpha 10$ subtype selectivity of α -RgIA

α -RgIA is a much more potent inhibitor of the $\alpha 9\alpha 10$ nAChR than the $\alpha 7$ nAChR⁹ (Table S3 in Supplementary Material). The only sequence differences between α -RgIA and α -ImI are in the C terminal halves of the peptides at positions 9 (Arg in α -RgIA, Ala in α -ImI) and 10 (Tyr in α -RgIA, Trp in α -ImI) and the C terminal (α -RgIA ends at Arg13, and α -ImI Cys12, with C-terminal amidation). The preference of α -RgIA for the $\alpha 9\alpha 10$ subtype must therefore be due to these amino acid differences. To explore the role of these residues we made three point mutations in α -RgIA that replaced native amino acids with the corresponding residues in α -ImI i.e. $\Delta R13, \#C12$, (Arg13 removed and Cys12 amidated), R9A and Y10W. Testing on $\alpha 7$ and $\alpha 9\alpha 10$ receptors expressed in oocytes revealed that the R9A and $\Delta R13, \#C12$ analogues had mildly increased potency on the $\alpha 7$ receptor whereas the R9A analogue had a dramatically reduced potency on the $\alpha 9\alpha 10$ receptor (Figure 9). $\Delta R13, \#C12$ had affinity very similar to wild-type α -RgIA on the $\alpha 9\alpha 10$ receptor. Surprisingly, Y10W had affinity very similar to wild-type α -RgIA on both receptor types.

Discussion

The mutant $\alpha 9[W176T]\alpha 10$ receptor was found to be less potently gated by ACh and less potently inhibited by α -RgIA, as shown in Figure 7. This indicates that the toxin is a competitive inhibitor of the receptor and binds at the ACh binding site; i.e. at the interface between adjacent subunits. Although the competitive inhibition of the $\alpha 9\alpha 10$ receptor by α -RgIA might be expected in view of its similarity to the competitive $\alpha 7$ nAChR antagonist α -ImI, another closely related α -conotoxin, α -ImII was shown to block the $\alpha 7$ receptor at a site other than the ACh binding site.²⁷ Therefore, *competitive* antagonism is not an invariant feature of even similar α -4,3 toxins. The demonstration of competitive block of $\alpha 9\alpha 10$ receptors by α -RgIA is thus an important finding. Position 6 of α -ImI (Pro) and α -ImII (Arg) was found to be critical for determining competitive versus non-competitive block.²⁷ Both α -RgIA and α -ImI have a Pro at this position, indicating that Pro6 in α -4,3 toxins may be a general determinant of competitive nAChR antagonism.

The mutations D5E, P6V and R7K in the first loop of α -RgIA caused large reductions in the potency of α -RgIA on $\alpha 9\alpha 10$ nAChRs (Figure 8). The same mutations were previously shown to reduce the affinity of α -ImI for the $\alpha 7$ nAChR,^{24,25} suggesting that the Asp-Pro-Arg triads

in α -RgIA and α -ImI play the same roles in anchoring the peptides to nAChRs. This is supported by the similar structures of the first loops of α -RgIA and α -ImI (Figure S5).

Several lines of evidence identify the regions of nAChRs that interact with the Asp-Pro-Arg motif of α -ImI. Mutation of the $\alpha 7$ nAChR indicated that three (+) face aromatic residues, Y115, W171 and Y217 (which align with Y120, W176 and Y224 of $\alpha 9$, Figure 6), contribute to high affinity α -ImI binding, while double mutant cycle analyses that defined pairwise interactions between toxin and receptor indicated a dominant interaction between α -ImI Arg7 and Y217 on the receptor^{8,26} (in earlier work^{8,24–26} Y115, W171 and Y217 were identified as Y93, W149 and Y195 corresponding to their position in the mature protein in which an N-terminal signal sequence has been removed). *In silico* docking of a solution structure of α -ImI¹⁷ to a model of the $\alpha 7$ nAChR based on the structure of an AChBP³ indicated that Arg7 of α -ImI interacts with three aromatic residues (Y115, W171 and Y217) on the principal side of the subunit interfaces.^{8,26} Also, the crystal structure of α -ImI bound to the *A. californica* AChBP supported the interaction of the α -ImI Asp-Pro-Arg motif with (+) face aromatic residues in nAChRs. In this AChBP, Y93, W147 and Y195 align with Y115, W171 and Y217, respectively, of the $\alpha 7$ nAChR (Figure 6), which correspond to Y93, W149 and Y195, respectively, of the mature $\alpha 7$ nAChR. Both AChBP Y93 and Y195 are close to Arg7 of bound α -ImI (the centroid of Y93 is 5.1 Å from Arg7 C ζ , and Y195 is 5.4 and 5.6 Å from C α and C ζ , respectively, of Arg7). The distances from α -ImI Pro6 C β and C γ to the centroids of Y93 and W147 are < 5 Å, and Asp5 is close to Y195 of the AChBP (the Y195 ring centroid is 4.7 Å from Asp5 C γ). In addition, the AChBP (+) face aromatic Y188 is close to Asp5 of bound α -ImI (its centroid is 5.0 Å from Asp5 C β and Asp5 O δ is only 2.5 Å from Y188 OH (Figure S7)). Clearly, Asp5, Pro6 and Arg7 in the first loop of α -ImI interact strongly with the conserved aromatic residues on the principal interface of the $\alpha 7$ nAChR and *A. californica* AChBP. Because these aromatic residues are conserved throughout the nAChR subunits (Figure 6), Asp5, Pro6 and Arg7 of α -RgIA are expected to bind to a similar aromatic pocket on the $\alpha 9\alpha 10$ receptor.

A model of α -ImI bound to the $\alpha 7$ nAChR⁸ indicates that Ala9 and Trp10 of α -ImI project towards the complementary side of the subunit interfaces; in this model, N133, S135 and Q139 closely approach Ala9 or Trp10 of the receptor-bound toxin⁸ (in the published model these residues were N111, S113 and Q117, corresponding to their positions in the mature protein in which an N-terminal signal sequence has been removed). This is supported by mutagenesis of these three residues, which altered the potency of the toxin for the receptor,²⁶ and by double mutant cycles analysis showing coupling between Trp10 of the toxin and N133 and S135 of the complementary face.²⁶ Since Ala9 in α -ImI is close to the $\alpha 7$ complementary face (Figure S7),² the much larger and more solvent exposed Arg9 of α -RgIA (Figure S6) would be difficult to accommodate when α -RgIA binds to this receptor subtype. This might explain the lower potency of α -RgIA, relative to α -ImI, on $\alpha 7$ receptors and the increased potency of α -RgIA [R9A], relative to α -RgIA, on these receptors (Figure 9). By contrast, Arg9 of α -RgIA is necessary for stabilizing the α -RgIA- $\alpha 9\alpha 10$ interaction and its replacement by Ala greatly reduces α -RgIA potency for $\alpha 9\alpha 10$ receptors (Figure 9). Thus, differences between the complementary faces of the $\alpha 7$ and $\alpha 9\alpha 10$ receptors make Arg9 of α -RgIA stabilizing in the case of $\alpha 9\alpha 10$ binding but destabilizing in the case of $\alpha 7$ binding. In fact, the sequence of the $\alpha 7$ subunit in the vicinity of the α -ImI interacting residues N133, S135 and Q139 is quite different from those of both the $\alpha 9$ and $\alpha 10$ subunits (Figure 6B). Clearly, there are distinct binding surfaces at the complementary faces of $\alpha 9\alpha 10$ and $\alpha 7$ nAChRs.

In conclusion, the Asp-Pro-Arg motif in the first loop of α -RgIA appears to be crucial for interaction of the toxin with $\alpha 9\alpha 10$ nAChRs (Figure 8), just as the same motif in α -ImI appears to be crucial for stabilizing its interactions with the $\alpha 7$ nAChR. Since this motif of α -ImI binds to aromatic residues on the (+) face of the $\alpha 7$ nAChR and since these aromatic residues are

conserved throughout α nicotinic subunits (Figure 6), the Asp-Pro-Arg motif in α -RgIA most likely interacts with the (+) face of the $\alpha 9\alpha 10$ receptor in the same way. In contrast, Ala9 of α -ImI appears to interact with the complementary face of the $\alpha 7$ receptor, suggesting that Arg9 of α -RgIA might interact with this face of the $\alpha 9\alpha 10$ receptor. Since there is greater $\alpha 7$ -to- $\alpha 9\alpha 10$ sequence variability on the complementary face than in the principal face aromatic residues (Figure 6B), Arg9 of α -RgIA presumably has charge and or shape compatibility only with the unique molecular surface at the complementary face of the $\alpha 9\alpha 10$ subtype. Thus, while the first loops of α -ImI and α -RgIA anchor the peptides to $\alpha 7$ and $\alpha 9\alpha 10$ receptors in similar fashion, the different residues at position 9 of the toxins account for subtype specificity by allowing unique interactions with the complementary surfaces. The nature of the aromatic side chain at position 10 and of the C-terminus appears to have a negligible effect on affinity for the $\alpha 9\alpha 10$ subtype.

The peptide backbone of the structure of α -RgIA determined in this study is similar to that of α -ImI determined in numerous earlier studies (Figure S5). The dramatic differences in subtype specificity among α -4,3 toxins (as exemplified by α -RgIA and α -ImI) must thus arise from the presentation of different side chains from the same, disulfide-constrained framework. This indicates that α -4,3 toxins, and analogues thereof, are a valuable set of tools with which to define pharmacophores that block nAChRs in a subtype-selective manner.

Our findings contribute to a better understanding of the molecular basis for antagonism of the $\alpha 9\alpha 10$ nAChR subtype. This is of potential value pharmacologically given the recent recognition¹⁰ that blockade of the $\alpha 9\alpha 10$ nAChR may be valuable in treatment of neuropathic pain. $\alpha 9\alpha 10$ nAChRs are found in discrete tissues, including immune cells that mediate inflammation.²⁸ α -RgIA and α -Vc1.1, another conotoxin that also blocks $\alpha 9\alpha 10$ nAChRs, have potent antinociceptive activity in rat animal models of nerve injury induced pain,^{10,29} and both α -RgIA and Vc1.1 reduce the immune response to peripheral nerve damage.¹⁰ Understanding the key residues of α -RgIA involved in $\alpha 9\alpha 10$ block thus helps to define a pharmacophore useful in certain pain and inflammatory conditions.³⁰

Materials and Methods

Peptide synthesis

Linear α -RgIA and α -RgIA analogues were synthesized by standard F-moc [N-(9-fluorenyl) methoxycarbonyl] chemistry at a University of Utah core facility. Linear peptides were cleaved from solid phase synthesis resin and were oxidized to give the correct disulfide connectivity using an orthogonal cysteine protection strategy that has been described previously.³¹

NMR spectroscopy

Samples of wild-type α -RgIA (1.0 mg) and the α -RgIA analogues Y10W (1.6 mg), D5E (0.8 mg), P6V (0.8 mg) and R9A (0.5mg) were each dissolved in 380 μ l H₂O containing 5% 2H₂O for NMR studies. Two-dimensional homonuclear total correlation (TOCSY) spectra with a spin-lock time of 70 ms, nuclear Overhauser enhancement (NOESY) spectra with a mixing time of 250 ms, and double quantum filtered correlation (DQF-COSY) spectra were acquired at 600 MHz on a Bruker DRX-600 spectrometer for wild-type α -RgIA and its four analogues. For wild-type α -RgIA a NOESY spectrum with a mixing time of 250 ms was also acquired at 800 MHz on a Bruker Avance 800 equipped with a TCI-cryoprobe. All spectra were collected at 25 °C unless otherwise stated and were referenced to the water resonance. Water was suppressed using the WATERGATE pulse sequence.³² A series of 1D spectra over the temperature range 5–25 °C, at 5 °C intervals, was collected for each peptide. Amide exchange rates in wild-type α -RgIA were monitored by dissolving freeze-dried material

in $^2\text{H}_2\text{O}$ then recording a series of 1D spectra, followed by 70 ms TOCSY and 50 ms NOESY spectra. In addition, ^1H - ^{13}C HSQC spectra for the assignment of ^{13}C chemical shifts and a ^1H - ^{15}N HSQC spectrum for the assignment of ^{15}N chemical shifts^{33,34} were collected at 5 and 25 °C for wild-type α -RgIA and α -RgIA[Y10W] on a Bruker Avance 500 spectrometer equipped with a TXI-cryoprobe.

Diffusion measurements were performed using a pulsed field gradient longitudinal eddy-current delay pulse sequence^{35,36} as implemented by Yao et al.¹³ at 5, 10 and 25 °C for wild-type α -RgIA and at 25 °C for its four analogues.

Spectra were processed using TOPSPIN (Version 1.3, Bruker Biospin) and analyzed using XEASY (Version 1.3.13).³⁷

Structural constraints

$^3J_{\text{HNHA}}$ coupling constants were measured from DQF-COSY spectra at 600 MHz, and then converted to dihedral restraints as follows: $^3J_{\text{HNH}\alpha} > 8$ Hz, $\phi = -120 \pm 40^\circ$; $^3J_{\text{HNH}\alpha} < 6$ Hz, $\phi = -60 \pm 30^\circ$. χ^1 angles for some residues were determined based on analysis of a short mixing time (50ms) NOESY spectrum. In addition, PREDITOR³⁸ (<http://wishart.biology.ualberta.ca/predictor>) was used to predict torsion angle (ϕ , ψ , χ^1 and ω) restraints based on chemical shifts. The predicted ϕ and χ^1 angles were compared with the values measured from the NMR experiments mentioned above. Residues with consistent ϕ angles (range $\pm 20^\circ$) were constrained in preliminary structural calculations in CYANA (version 1.0.6);³⁹ this included seven ϕ angle restraints for wild-type α -RgIA (Cys2, Cys3, Arg7, Arg9, Tyr10, Arg11 and Cys12), four ϕ angle restraints for α -RgIA[Y10W] (Cys3, Arg9, Trp10 and Arg11) and the same seven ϕ angle restraints as in wild-type α -RgIA for α -RgIA [D5E] and α -RgIA[P6V]. Residues with consistent χ^1 angles (Cys3 and Asp5 for α -RgIA; Cys8, Asp5 and Trp10 for α -RgIA[Y10W]) were constrained in later energy minimization in Xplor-NIH.⁴⁰ Stereo-specific assignments were not made for other non-degenerate protons in the peptides. Some ϕ and χ^1 angles with predicted confidence scores > 0.75 could not be confirmed experimentally because of overlapping or weak cross-peaks in spectra; these were constrained in the structure refinement before energy minimization in water provided they did not increase total or NOE energy or cause significant distance restraints violations (> 0.2 Å); there were two such χ^1 angle restraints for α -RgIA (Cys2 and Tyr10 both with -60 ± 30) and three loose ϕ angle restraints for α -RgIA[Y10W] (-118 ± 50 for Cys2, -90 ± 40 for Asp5 and -60 ± 40 for Arg7). Other non-degenerate protons in the four peptides were assigned only by *in silico* means without further intervention. The final numbers of dihedral angle constraints for these peptides are listed in Table 1 and details have been deposited along with distance constraints in BioMagResBank¹¹ as entries 15435, 15368, 15367, 15436 and 15586 for wild-type α -RgIA, α -RgIA[Y10W], α -RgIA[D5E], α -RgIA[P6V] and α -RgIA[R9A], respectively. Wild-type α -RgIA and its analogues contain two disulfide bonds, Cys2-Cys8 and Cys3-Cys12, which were included as structural restraints. No hydrogen bond restraints were included for any of the peptides.

Structure calculations

Intensities of NOE cross peaks were measured in XEASY and calibrated using the CALIBA macro of the program CYANA (version 1.0.6).³⁹ NOEs providing no restraint or representing fixed distances were removed. The constraint list resulting from the CALIBA macro of CYANA was used in Xplor-NIH to calculate a family of 200 structures using the simulated annealing script.⁴⁰ The 40 lowest energy structures for each peptide were then subjected to energy minimization in water; during this process, a box of water with a periodic boundary of 18.856 Å was built around the peptide structure and the ensemble was energy minimized based on NOE and dihedral restraints and the geometry of the bonds, angles and impropers. From

this set of structures, final families of 20 lowest energy structures were chosen for analysis using PROCHECK-NMR⁴¹ and MOLMOL.⁴² In all cases, the final structures had no experimental distance violations greater than 0.2 Å or dihedral angle violations greater than 5°. The structures have been deposited in the Protein Data Bank⁴³ with accession codes 2JUT, 2JUS, 2JUR and 2JUQ for wild-type α -RgIA, α -RgIA[Y10W], α -RgIA[D5E] and α -RgIA[P6V], respectively. Structural figures were prepared using the programs MOLMOL⁴² and PyMOL (Delano, W.L. The PyMOL Molecular Graphics System (2002) Delano Scientific, San Carlos, CA, USA. <http://www.pymol.org>).

nAChR mutagenesis

An α 9 nAChR subunit clone (see below) was mutated such that the codon for Trp176 was replaced by a codon for threonine using the QuickChange system (Stratagene).

Electrophysiology

A plasmid carrying a cDNA clone encoding the rat α 7 nAChR subunit was provided by S. Heinemann and D. Johnson (Salk Institute, San Diego, CA, USA). Plasmids carrying cDNA clones encoding the rat α 9 and α 10 nAChR subunits were provided by Belen Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). For all nAChR subunits, plasmids were linearized with NheI and used to prepare cRNA by in vitro transcription using the T7 mMESSAGE mMACHINE system (Ambion TX, USA). The RNA was injected into oocytes that had been isolated and maintained as before.⁴⁴ 50 nl of water containing 5 ng of the α 7 RNA, 5 ng each of the α 9 and α 10 RNAs or 5 ng each of the α 9[W176T] and α 10 RNAs were injected per oocyte as described earlier.⁴⁴

The oocytes were maintained in a 30 μ l bath made from Sylgard and were gravity perfused with ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH ~7.3). The perfusion solution could be changed to one composed of ND96 and ACh using a series of three way solenoid valves. The oocytes were held at -70 mV using a two-electrode voltage clamp (model OC-725B, Warner Instrument, Hamden, CT). The fluidics and voltage clamp apparatus have been described in detail previously.⁴⁴ The oocytes were pulsed for 1 s by 200 μ M ACh (α 7 receptor), 50 μ M ACh (α 9 α 10 receptor) or 1 mM (α 9[Y195T] α 10 receptor) at 1-min intervals. These concentrations of ACh are close to the EC₅₀ values for the corresponding receptors in this system.

The concentration dependence of block by conotoxins was measured using a static bath method; the ACh pulses and ND96 flow were halted and toxin was applied to various final concentrations in the bath. After 5 min the flow of ND96 was resumed at the same time as an ACh pulse was applied. The peak current elicited by the first ACh pulse following toxin exposure was converted to a percentage (percent response) of the peak current elicited in controls where ND96 alone, instead of toxin, was applied. In all cases where block was seen, the recovery from block was complete within 1 min (i.e. by the second ACh pulse after toxin application).

The concentration dependence of channel gating by ACh for the nAChRs used in this study was measured by pulsing oocytes expressing the receptors with different concentrations of ACh. In these experiments the electrophysiology system was the same as described above except that the oocytes were maintained in a stream of ND96 flowing along the bottom of a 300 μ l pipette tip from the narrow end to the wide end; ND96 flowing out of the tip was removed under vacuum. Two holes were cut in the top of the pipette tip, the larger downstream one held the oocyte and allowed access of the recording electrodes to the ND96, the smaller upstream hole allowed ACh to be added. To pulse with ACh the flow of ND96 was stopped and a 10 μ l bolus of ACh was added upstream of the oocyte via the smaller hole; following resumption

of the ND96 flow the ACh washed over the oocyte giving an agonist pulse of about 1 s. ACh pulses were applied no more frequently than once every 2 min and to ensure that the higher ACh concentrations were not causing receptor desensitization the rate of pulsing by these concentrations was increased to once per min and no reduction in peak currents were seen.

Data were analyzed and plotted using Prism Software (GraphPad Software, San Diego, CA). Inhibition data were fit to the equation: % response = $100/[1+([toxin]/IC_{50})^{nH}]$ where nH is the Hill slope. Data for the concentration dependence of channel gating by ACh were fitted to: normalized response = $1/[1+(EC_{50}/[ACh])^{nH}]$ where the best fit upper plateau of each curve was defined as 1 and all responses to ACh were normalized to it.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Andrew Low for valuable assistance with MS analyses. This work was supported in part by N.I.H. grants GM 48677 (to B.M.O.) and MH 53631 (to J.M.M.). R.S.N. acknowledges support from the NHMRC.

Abbreviations used

ACh	acetylcholine
AChBP	acetylcholine binding protein
α-ImI	α -conotoxin ImI
α-RgIA	α -conotoxin RgIA
nAChR	nicotinic acetylcholine receptor
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect

References

1. Changeux, JP.; Edelstein, SJ. Nicotinic Acetylcholine Receptors. In: Odile, J., editor. From Molecular Biology to Cognition. Johns Hopkins University Press; New York: 2005.
2. Hansen SB, Sulzenbacher G, Huxford T, Marchot P, Taylor P, Bourne Y. Structures of *Aplysia* AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. EMBO J 2005;24:3635–46. [PubMed: 16193063]
3. Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, Sixma TK. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 2001;411:269–76. [PubMed: 11357122]
4. Nicke A, Wonnacott S, Lewis RJ. α -conotoxins as tools for the elucidation of structure and function of neuronal nicotinic acetylcholine receptor subtypes. Eur J Biochem 2004;271:2305–19. [PubMed: 15182346]

5. Janes RW. α -Conotoxins as selective probes for nicotinic acetylcholine receptor subclasses. *Curr Opin Pharmacol* 2005;5:280–92. [PubMed: 15907916]
6. Norton RS, Olivera BM. Conotoxins down under. *Toxicon* 2006;48:780–98. [PubMed: 16952384]
7. Terlau H, Olivera BM. *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiol Rev* 2004;84:41–68. [PubMed: 14715910]
8. Ellison M, Gao F, Wang HL, Sine SM, McIntosh JM, Olivera BM. α -conotoxins ImI and ImII target distinct regions of the human $\alpha 7$ nicotinic acetylcholine receptor and distinguish human nicotinic receptor subtypes. *Biochemistry* 2004;43:16019–26. [PubMed: 15609996]
9. Ellison M, Haberlandt C, Gomez-Casati ME, Watkins M, Elgoyhen AB, McIntosh JM, Olivera BM. α -RgIA: a novel conotoxin that specifically and potently blocks the $\alpha 9\alpha 10$ nAChR. *Biochemistry* 2006;45:1511–7. [PubMed: 16445293]
10. Vincler M, Wittenauer S, Parker R, Ellison M, Olivera BM, McIntosh JM. Molecular mechanism for analgesia involving specific antagonism of $\alpha 9\alpha 10$ nicotinic acetylcholine receptors. *Proc Natl Acad Sci U S A* 2006;103:17880–4. [PubMed: 17101979]
11. Seavey BR, Farr EA, Westler WM, Markley JL. A relational database for sequence-specific protein NMR data. *Journal of Biomolecular NMR* 1991;1:217–36. [PubMed: 1841696]
12. Sabo JK, Keizer DW, Feng ZP, Casey JL, Parisi K, Coley AM, Foley M, Norton RS. Mimotopes of apical membrane antigen 1: Structures of phage-derived peptides recognized by the inhibitory monoclonal antibody 4G2dc1 and design of a more active analogue. *Infect Immun* 2007;75:61–73. [PubMed: 17060469]
13. Yao S, Howlett GJ, Norton RS. Peptide self-association in aqueous trifluoroethanol monitored by pulsed field gradient NMR diffusion measurements. *J Biomol NMR* 2000;16:109–19. [PubMed: 10723990]
14. Merutka G, Dyson HJ, Wright PE. ‘Random coil’ ^1H chemical shifts obtained as a function of temperature and trifluoroethanol concentration for the peptide series GGXGG. *Journal of Biomolecular NMR* 1995;5:14–24. [PubMed: 7881270]
15. Gehrman J, Daly NL, Alewood PF, Craik DJ. Solution structure of α -conotoxin ImI by ^1H nuclear magnetic resonance. *J Med Chem* 1999;42:2364–72. [PubMed: 10395477]
16. Lamthanh H, Jegou-Matheron C, Servent D, Menez A, Lancelin JM. Minimal conformation of the α -conotoxin ImI for the $\alpha 7$ neuronal nicotinic acetylcholine receptor recognition: correlated CD, NMR and binding studies. *FEBS Lett* 1999;454:293–8. [PubMed: 10431825]
17. Maslennikov IV, Shenkarev ZO, Zhmak MN, Ivanov VT, Methfessel C, Tsetlin VI, Arseniev AS. NMR spatial structure of α -conotoxin ImI reveals a common scaffold in snail and snake toxins recognizing neuronal nicotinic acetylcholine receptors. *FEBS Lett* 1999;444:275–80. [PubMed: 10050774]
18. Rogers JP, Luginbuhl P, Shen GS, McCabe RT, Stevens RC, Wemmer DE. NMR solution structure of α -conotoxin ImI and comparison to other conotoxins specific for neuronal nicotinic acetylcholine receptors. *Biochemistry* 1999;38:3874–82. [PubMed: 10194298]
19. Ulens C, Hogg RC, Celie PH, Bertrand D, Tsetlin V, Smit AB, Sixma TK. Structural determinants of selective α -conotoxin binding to a nicotinic acetylcholine receptor homolog AChBP. *Proc Natl Acad Sci U S A* 2006;103:3615–20. [PubMed: 16505382]
20. Galzi JL, Changeux JP. Neuronal nicotinic receptors: molecular organization and regulations. *Neuropharmacology* 1995;34:563–82. [PubMed: 7566492]
21. Smit AB, Syed NI, Schaap D, van Minnen J, Klumperman J, Kits KS, Lodder H, van der Schors RC, van Elk R, Sorgedraeger B, Brejc K, Sixma TK, Geraerts WP. A glia-derived acetylcholine-binding protein that modulates synaptic transmission. *Nature* 2001;411:261–8. [PubMed: 11357121]
22. Miyazawa A, Fujiyoshi Y, Unwin N. Structure and gating mechanism of the acetylcholine receptor pore. *Nature* 2003;423:949–55. [PubMed: 12827192]
23. Celie PH, van Rossum-Fikkert SE, van Dijk WJ, Brejc K, Smit AB, Sixma TK. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron* 2004;41:907–14. [PubMed: 15046723]
24. Quiram PA, Sine SM. Structural elements in α -conotoxin ImI essential for binding to neuronal $\alpha 7$ receptors. *J Biol Chem* 1998;273:11007–11. [PubMed: 9556581]

25. Quiram PA, Sine SM. Identification of residues in the neuronal $\alpha 7$ acetylcholine receptor that confer selectivity for conotoxin ImI. *J Biol Chem* 1998;273:11001–6. [PubMed: 9556580]
26. Quiram PA, Jones JJ, Sine SM. Pairwise interactions between neuronal $\alpha 7$ acetylcholine receptors and α -conotoxin ImI. *J Biol Chem* 1999;274:19517–24. [PubMed: 10391883]
27. Ellison M, McIntosh JM, Olivera BM. Alpha-conotoxins ImI and ImII. Similar alpha 7 nicotinic receptor antagonists act at different sites. *J Biol Chem* 2003;278:757–64. [PubMed: 12384509]
28. Peng H, Ferris RL, Matthews T, Hiel H, Lopez-Albaitero A, Lustig LR. Characterization of the human nicotinic acetylcholine receptor subunit α (9) (CHRNA9) and α (10) (CHRNA10) in lymphocytes. *Life Sci* 2004;76:263–80. [PubMed: 15531379]
29. Livett BG, Sandall DW, Keays D, Down J, Gayler KR, Satkunathan N, Khalil Z. Therapeutic applications of conotoxins that target the neuronal nicotinic acetylcholine receptor. *Toxicon* 2006;48:810–29. [PubMed: 16979678]
30. Vincler M, McIntosh JM. Targeting the $\alpha 9\alpha 10$ nicotinic acetylcholine receptor to treat severe pain. *Expert Opin Ther Targets* 2007;11:891–7. [PubMed: 17614758]
31. Luo S, Nguyen TA, Cartier GE, Olivera BM, Yoshikami D, McIntosh JM. Single-residue alteration in α -conotoxin PnIA switches its nAChR subtype selectivity. *Biochemistry* 1999;38:14542–8. [PubMed: 10545176]
32. Piotto M, Saudek V, Sklenar V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *Journal of Biomolecular NMR* 1992;2:661–5. [PubMed: 1490109]
33. Palmer AG, Cavanagh J, Wright PE, Rance M. Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR spectroscopy. *J Magn Reson* 1991;93:151–170.
34. Kay LE, Keifer P, Saarinen T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J Am Chem Soc* 1992;114:10663–5.
35. Gibbs SJ, Johnson CS. A PFG-NMR experiment for accurate diffusion and flow studies in the presence of eddy currents. *J Magn Reson* 1991;93:395–402.
36. Dingley AJ, Mackay JP, Chapman BE, Morris MB, Kuchel PW, Hambly BD, King GF. Measuring protein self-association using pulsed-field-gradient NMR spectroscopy: application to myosin light chain 2. *J Biomol NMR* 1995;6:321–8. [PubMed: 8520223]
37. Bartels C, Xia TH, Billeter M, Güntert P, Wüthrich K. The program XEASY for computer-supported NMR spectral-analysis of biological macromolecules. *J Biomol NMR* 1995;6:1–10.
38. Berjanskii MV, Neal S, Wishart DS. PREDITOR: a web server for predicting protein torsion angle restraints. *Nucleic Acids Res* 2006;34:W63–9. [PubMed: 16845087]
39. Herrmann T, Güntert P, Wüthrich K. Protein NMR structure determination with automated NOE-identification in the NOESY spectra using the new software ATNOS. *J Biomol NMR* 2002;24:171–89. [PubMed: 12522306]
40. Schwieters CD, Kuszewski JJ, Tjandra N, Clore GM. The Xplor-NIH NMR molecular structure determination package. *J Magn Reson* 2003;160:65–73. [PubMed: 12565051]
41. Laskowski RA, Rullmann JA, MacArthur MW, Kaptein R, Thornton JM. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J Biomol NMR* 1996;8:477–86. [PubMed: 9008363]
42. Koradi R, Billeter M, Wüthrich K. MOLMOL: a program for display and analysis of macromolecular structures. *J Mol Graph* 1996;14:51–5. 29–32. [PubMed: 8744573]
43. Berman HM, Battistuz T, Bhat TN, Bluhm WF, Bourne PE, Burkhardt K, Feng Z, Gilliland GL, Iype L, Jain S, Fagan P, Marvin J, Padilla D, Ravichandran V, Schneider B, Thanki N, Weissig H, Westbrook JD, Zardecki C. The Protein Data Bank. *Acta Crystallogr D Biol Crystallogr* 2002;58:899–907. [PubMed: 12037327]
44. Cartier GE, Yoshikami D, Gray WR, Luo S, Olivera BM, McIntosh JM. A new α -conotoxin which targets $\alpha 3\beta 2$ nicotinic acetylcholine receptors. *J Biol Chem* 1996;271:7522–8. [PubMed: 8631783]

Figure 1.

The conserved disulfide scaffold of the α -conotoxin family and the sequences of α -RgIA (top) and α -ImI (bottom) are shown. The three differences between the two peptides are in bold and underlined. # represents C-terminal amidation.

Figure 2. Deviations of the chemical shifts of (A) NH and (B) H α for α -RgIA[D5E], α -RgIA[P6V], RgIA [R9A] and α -RgIA[Y10W] from those of wild type α -RgIA.

Figure 3.

Translucent surface view of the closest-to-average structure of α -RgIA. The side chains of Asp, Pro and Arg in the first loop point to the same direction and those of Arg and Tyr in the second loop point in a direction that is roughly perpendicular. This figure was prepared using PyMol (Delano, W.L. The PyMOL Molecular Graphics System (2002) Delano Scientific, San Carlos, CA, USA. <http://www.pymol.org>).

Figure 4.

Ensembles of 20 structures of (A) α -RgIA, (B) α -RgIA[Y10W], (C) α -RgIA[D5E], and (D) α -RgIA[P6V]. The backbones are shown in black and heavy atoms of the Asp-Pro-Arg motif in the first loop and residues 9-10 in the second loop, are shown. The 2–8 and 3–12 disulfide bonds are shown in orange.

Figure 5.

Ensembles of 20 solution structures of α -RgIA[Y10W] (in red) superimposed with α -ImI structures solved by (A) Rogers et al. (PDB code 1IM1, in black),¹⁸ (B) Maslennikov et al. (PDB code 1IMI, in blue),¹⁷ (C) Gehrmann et al. (PDB code 1CNL, in green),¹⁵ (D) Lamthanh et al. (PDB code 1G2G, in navy),¹⁶ (E) Five crystal structures in *Aplysia* AchBP complexes solved by Hansen et al. (PDB code 2BYP, in royal blue)² and (F) Eight crystal structures in *Aplysia* AchBP complexes solved by Ulens et al (PDB code 2C9T, in black).¹⁹

A

```

rat  α10  ...116IVLYNK...173GSWTH...215TYGCCSEPYPD225...
rat  α9    ...117IVLYNK...174GSWTY...216SYGCCSEPYPD226...
rat  α7    ...112ILLYNS...169GSWSY...209FYECCSEPYPD219...
rat  α6    ...120IVLYNN...177GSWTY...219KYNCCEEIYPD229...
rat  α5    ...118IVLFDN...174GSWTY...216TDSCC--WYPY224...
rat  α4    ...125IVLYNN...182GSWTY...224KYECCAEIYPD234...
rat  α3    ...115IVLYNN...172GSWSY...214KYNCCEEIYQD224...
rat  α2    ...122IVLYNN...179GSWTY...221KYDCCAEIYPD231...
human α10 ...116IVLYNK...173GSWTH...215TYGCCSEPYPD225...
human α9  ...117IVLYNK...174GSWTY...216SYGCCSEPYPD226...
human α7  ...112ILLYNS...169GSWSY...209FYECCKEPYPD219...
Aplysia ...90ITAYSS...145GSWVY...187HYSCCPEPYID197...

```

B

```

rat  α7    ...125TFHTNVLVNASGHCQYLPP143...
rat  α9    ...119PVNTNVVLRYDGLITWDSP137...
rat  α10   ...129SASTNVVVRHDGAVRWDAP147...
Aplysia ...102LSPQIAVVTHDGSVMFIPA120...

```

Figure 6.

Amino acid sequence alignments. (A) Regions of nAChR α subunits and *Aplysia californica* AChBP that form part of the principal face. Four conserved principal face aromatic residues are in bold and underlined; the residues Y93, W147, Y188 and Y195 in *Aplysia californica* AChBP align with Y115, W171, Y210 and Y217, respectively, in the rat $\alpha 7$ subunit. (B) Region of nAChR α subunits that constitutes part of the complementary subunit interface. Discrete loops of nAChR subunits which converge to form the ACh binding site in nAChRs have been identified and the sequences above correspond to loop E according to one nomenclature scheme. Mutation of the bolded residues affects the affinity of α -ImI for the $\alpha 7$ nAChR²⁶ and they closely approach Ala9 or Trp10 of α -ImI in a model of α -ImI bound to the $\alpha 7$ nAChR⁸. The bolded Asn and Ser interact with Trp10 of α -ImI in double mutant cycles analysis²⁶. The $\alpha 7$ sequence is very distinct from the $\alpha 9$ and $\alpha 10$ sequences, and even more distinct from the *Aplysia* sequence. The spatial disposition of these residues in the crystal structure of *A. californica* AChBP in complex with α -ImI is illustrated in Figure S7.

Figure 7. Mutation of the principal face residue W176 in the $\alpha 9$ subunit reduces the potency of both ACh gating of $\alpha 9\alpha 10$ nAChRs and blockade by α -RgIA. (A) Concentration dependence of ACh gating of $\alpha 9\alpha 10$ nAChRs (black circles) and $\alpha 9[W176T]\alpha 10$ nAChRs (open squares). (B) Concentration dependence of α -RgIA block of $\alpha 9\alpha 10$ nAChRs (black circles) and $\alpha 9[W176T]\alpha 10$ nAChRs (open squares). The error bars are the standard error of the mean for at least three repetitions. The IC_{50} and Hill slopes for all inhibition curves are in Table S2.

Figure 8.

Effects of loop 1 mutations in α -RgIA. Single amino acid substitutions were made in the first loop of RgIA and the resulting analogues were tested on $\alpha 7$ and $\alpha 9\alpha 10$ nAChRs expressed in oocytes. (A) The left panel shows the concentration dependence of block of $\alpha 9\alpha 10$ nAChRs by wild type α -RgIA and analogues. Error bars are the standard error of the mean for at least three repetitions. The right panel shows IC_{50} values of wild-type α -RgIA and analogues against $\alpha 9\alpha 10$ nAChRs. The error bars are the 95% confidence interval. (B) As for (A) except that block of $\alpha 7$ nAChRs is shown. The IC_{50} and Hill slopes for all inhibition curves are in Table S3.

Figure 9.

Effects of loop 2 mutations in α -RgIA. Single amino acid substitutions were made in the second loop of α -RgIA and the resulting analogues were tested on $\alpha 7$ and $\alpha 9 \alpha 10$ nAChRs expressed in oocytes. (A) The left panel shows the concentration dependence of block of $\alpha 9 \alpha 10$ nAChRs by wild type α -RgIA and analogues. The error bars are the standard error of the mean for at least three repetitions. The right panel shows IC_{50} values of wild-type α -RgIA and analogues against $\alpha 9 \alpha 10$ nAChRs. The error bars are the 95% confidence interval. $\Delta R13, \#C12$ refers to an α -RgIA analogue in which Arg13 was removed and C-terminal amidation was added to Cys12. (B) As for (A) except block of $\alpha 7$ nAChRs is shown. The IC_{50} and Hill slopes for all inhibition curves are in Table S3.

Table 1Summary of experimental constraints and structural statistics for α -RgIA and three analogues.

	α -RgIA(wt)	α -RgIA[Y10W]	α -RgIA[D5E]	α -RgIA[P6V]
No. of distance restraints	161	160	134	141
intraresidue ($i=j$)	82	90	80	82
sequential ($ i-j =1$)	46	44	42	46
medium-range ($1 < i-j < 5$)	25	17	5	8
long-range ($ i-j > 4$)	8	9	7	5
hydrogen bond restraints	0	0	0	0
No. of dihedral restraints	11	10	7	7
Energies ^a				
$E_{\text{NOE}}(\text{kcal mol}^{-1})$	1.7 $\pm$ 1.1	1.2 $\pm$ 0.5	1.6 $\pm$ 0.8	1.6 $\pm$ 0.8
RMS deviations from experimental data				
NOEs ($\text{\AA}$)	0.014 $\pm$ 0.004	0.012 $\pm$ 0.003	0.015 $\pm$ 0.004	0.015 $\pm$ 0.004
Dihedrals (deg)	0.50 $\pm$ 0.22	0.23 $\pm$ 0.26	0.25 $\pm$ 0.23	0.14 $\pm$ 0.19
Deviations from ideal ^b				
angles (deg)	0.58 $\pm$ 0.02	0.55 $\pm$ 0.02	0.57 $\pm$ 0.03	0.50 $\pm$ 0.02
bonds ($\text{\AA}$)	0.0023 $\pm$ 0.0002	0.0018 $\pm$ 0.0003	0.0020 $\pm$ 0.0003	0.0017 $\pm$ 0.0002
impropers (deg)	0.37 $\pm$ 0.02	0.35 $\pm$ 0.01	0.37 $\pm$ 0.02	0.35 $\pm$ 0.02
RMS deviations ($\text{\AA}$) ^c				
Backbone (bb) atoms (global)	0.71	1.24	1.48	1.49
bb atoms [$S(\varphi)$ and $S(\phi) > 0.8$]	(2–12) 0.43	(2–11) 0.64	(2–10) 0.77	(3–10) 0.67
heavy atoms [$S(\varphi)$ and $S(\phi) > 0.8$]	(2–11) 1.38	(2–11) 1.66	(2–10) 2.10	(3–10) 1.90
Ramachandran plot ^d				
Most favoured (%)	69.5	70.5	65.0	62.7
Allowed (%)	30.5	29.5	34.0	36.5
Additionally Allowed (%)	0	0	1.0	0.9
Disallowed (%)	0	0	0	0

^aThe values for E_{NOE} are calculated from a square well potential with force constants of 50 kcal mol⁻¹ $\text{\AA}^2$.^bThe values for the bonds, angles, and impropers show the deviations from ideal values based on perfect stereochemistry.^cThe average RMSD over the backbone heavy atoms (N, C $^{\alpha}$, C') over the indicated residues calculated in MOLMOL.^dAs determined by the program PROCHECK-NMR for all residues.