Solution conformation of α -conotoxin GIC, a novel potent antagonist of $\alpha 3\beta 2$ nicotinic acetylcholine receptors

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α -Conotoxin GIC is a 16-residue peptide isolated from the venom of the cone snail *Conus geographus*. α -Conotoxin GIC potently blocks the $\alpha 3\beta 2$ subtype of human nicotinic acetylcholine receptor, showing a high selectivity for neuronal versus muscle subtype [McIntosh, Dowell, Watkins, Garrett, Yoshikami, and Olivera (2002) J. Biol. Chem. 277, 33610–33615]. We have now determined the three-dimensional solution structure of α -conotoxin GIC by NMR spectroscopy. The structure of α -conotoxin GIC is well defined with backbone and heavy atom root mean square deviations (residues 2–16) of 0.53 Å and 0.96 Å respectively. Structure and surface comparison of α -conotoxin GIC

with the other $\alpha 4/7$ subfamily conotoxins reveals unique structural aspects of α -conotoxin GIC. In particular, the structural comparison between α -conotoxins GIC and MII indicates molecular features that may confer their similar receptor specificity profile, as well as those that provide the unique binding characteristics of α -conotoxin GIC.

Key words: α -conotoxin, *Conus geographus* (cone snail), nicotinic acetylcholine receptor (nAChR), NMR, solution structure, venom.

INTRODUCTION

α -Conotoxins are small peptide toxins that are found in the venom of marine snails, *Conus*, and typically comprise 13–19 amino acid residues [1–3] (Table 1). α -Conotoxins that contain two disulphide bridges, and their homologues, αA -conotoxins, which are somewhat larger than α -conotoxins, having three disulphide bridges, are all extremely potent antagonists against various subtypes of nAChRs (nicotinic acetylcholine receptors). These *Conus* peptides, hence, have been extensively used for studying the mechanism of ligand–nAChR interactions [4–8]. The $\alpha 3/5$ subfamily of conotoxins, such as GI, MI, and SI, as well as αA subfamily conotoxins PIVA and EIVA, act on neuromuscular nAChRs. (The α -conotoxins are grouped according to the number of amino acid residues enclosed within each disulphide loop. Loop sizes of three residues in the first loop and five in the second are denoted as $\alpha 3/5$. Other sub-families include $\alpha 4/7$ and $\alpha 4/3$.) Another subfamily of α -conotoxins (MII, PnIA, AuIB, and GID) known as the $\alpha 4/7$ subfamily, has no or low activity against neuromuscular receptors, but potently blocks various subtypes of neuronal nAChRs. Since there are more than 500 different species of cone snails, each containing many neuropeptides, more novel peptide toxins from *Conus* venoms are yet to be discovered [3,9].

In order to fully understand the receptor subtype specificity of α -conotoxins, much effort has gone into delineating key residues potentially involved in receptor binding using mutant nAChRs, as well as ligand analogues [4,10–12]. High-resolution structures of several α - αA -conotoxins, determined mainly by NMR methods, have also been published [5–8,13–18]. Such efforts may eventually lead to development of novel neuromodulators useful for treatment of Alzheimer's disease, epilepsy, Parkinson's disease or other nAChR-related diseases. Nevertheless, a clear picture of ligand–receptor binding is not likely to

be available without high-resolution three-dimensional structures of free and ligand-bound nAChRs, since nAChRs are complex heteropentameric proteins and the ligands need to bind to the interfaces of different subunits [19–21].

In the absence of suitable three-dimensional structures of nAChRs, structural comparison among various nAChR ligands has provided useful information regarding important pharmacophores involved in ligand–receptor interactions [18,22–26]. Our laboratory has been applying a 'reverse-mapping strategy' to delineate key receptor contact residues within α -conotoxins by comparing the structures of several α -conotoxins with a similar molecular folding pattern, but which exhibit different receptor selectivities. Following this strategy, we were able to suggest the presence of a putative $\alpha 1$ subunit binding face within neuromuscular α - αA -conotoxins [6], and to delineate potential receptor subtype-specific residues in neuronal α -conotoxins [17]. The size of the ligand-binding pocket in nAChR was also inferred from the reverse mapping [6]. In the present study, we report the three-dimensional solution structure of α -conotoxin GIC, a novel peptide from *Conus geographus* that potently blocks $\alpha 3\beta 2$ nAChRs. The high selectivity for the $\alpha 3\beta 2$ nAChR subtype makes the α -conotoxin GIC structure valuable for integrating structure and activity studies of all the α -conotoxins that discriminate between $\alpha 3\beta 2$ versus other neuronal subtypes.

EXPERIMENTAL

Peptide preparation

α -Conotoxin GIC, originally cloned from *C. geographus*, was synthesized and purified as described previously [27]. A two-step oxidation protocol was used to selectively fold the peptide.

Abbreviations used: CSI, chemical shift index; nAChR, nicotinic acetylcholine receptor; NOE, nuclear Overhauser effect; RMSD, root mean square deviation; TOCSY, total COSY.

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The atomic coordinates for α -conotoxin GIC have been deposited in the Protein Data Bank (accession code 1UL2).

Table 1 Three sub-families of nAChR-targeting conotoxinsDisulphide bridges are indicated. γ , γ -carboxyglutamic acid; O, γ -hydroxyproline.

Name†	Sequence	Specificity‡
$\alpha 3/5$ sub-family		
GI	ECC NPAC GRHYS C*	$\alpha 1/\gamma$
MI	GRCC HPAC GKNYS C*	$\alpha 1/\gamma$
SI	ICC NPAC GPKYS C*	None
SIA	YCC HPAC GKNFD C*	$\alpha 1/\gamma$
CnIA	GRCC HPAC GKYS C*	$\alpha 1/\gamma, \alpha 1/\delta$
CnIB	CC HPAC GKYS C*	$\alpha 1/\gamma, \alpha 1/\delta$
$\alpha 4/7$ sub-family		
EI	RDOCCYHPTCNMSNPQIC*	$\alpha 1/\delta$
MII	GCCSNPVCHEHSNLC*	$\alpha 6/\beta 2 \approx \alpha 3/\beta 2, \alpha 4/\beta 2, \alpha 3/\beta 4$
PnIA	GCCSLFPACANNPDYC*	$\alpha 3/\beta 2, \alpha 7, \alpha 3/\beta 4$
PnIB	GCCSLFPACALSNPDYC*	$\alpha 7, \alpha 3/\beta 2$
AuIA	GCCSYFPFCFATNSDYC*	$\alpha 3/\beta 4$
AuIB	GCCSYFPFCFATNPD C*	$\alpha 3/\beta 4$
AuIC	GCCSYFPFCFATNSGYC*	$\alpha 3/\beta 4$
EpI	GCCSDPRCNMNNPDYC*	$\alpha 3/\beta 2, \alpha 3/\beta 4$
GID	IRDYCCSNPACRVNNOHVC	$\alpha 3/\beta 2, \alpha 7, \alpha 4/\beta 2$
GIC	GCCSHPACAGNNQHIC*	$\alpha 3/\beta 2 \approx \alpha 6/\beta 2, \alpha 4/\beta 2, \alpha 3/\beta 4$
αA sub-family		
PIVA	GCCGSYPNAACHPCSK DROSYCGQ*	$\alpha 1/\gamma, \alpha 1/\delta$
EIVA	GCCGPYONAACHOCCKVGRGOYCDROSGG*	$\alpha 1/\gamma, \alpha 1/\delta$

* Amidated C-terminus.

† The first capital letter indicates the species origin.

‡ For the $\alpha 3/5$ subfamily and α -conotoxin EI, the specificity is for *Torpedo* receptor.

NMR spectroscopy

Samples for the NMR acquisition were prepared in 90 % H_2O /10 % 2H_2O or in 100 % 2H_2O , pH 3.5, with a final concentration of 11.5 mM. All NMR experiments were performed using a Varian UNITY INOVA 600 spectrometer at 20 °C and 10 °C in order to obtain unambiguous resonance assignment. Mixing times of 150–400 ms for NOESY and 87 ms for TOCSY experiments [28] were used. $^3J_{HNH\alpha}$ coupling constants for backbone torsion angles were measured using phase-sensitive double-quantum-filtered COSY experiments [29]. Spectral widths were 6 kHz in both dimensions. Typical two-dimensional NMR data consist of 2048 complex points in the t_2 dimension with 256 complex t_1 increments.

Structure calculations

A total of 181 NOE (nuclear Overhauser effect) cross-peaks were manually assigned in the NOESY spectrum and seven dihedral angle restraints were derived from $^3J_{HNH\alpha}$ coupling constants. Backbone torsion angle, Φ , was set to $-120^\circ (\pm 20^\circ)$ when $^3J_{HNH\alpha} > 8$ Hz and to $-65^\circ (\pm 20^\circ)$ if $^3J_{HNH\alpha} < 6$ Hz. Using these data set, structures were calculated using ARIA (version 1.1) as described previously [30]. Floating chirality assignment [31,32] was employed for all methylene and isopropyl groups. A final set of 50 structures was calculated using the simulated annealing protocol from CNS-SOLVE 1.1 [33]. In the last round of calculation, the NMR-derived experimental restraints included 314 unambiguous NOE restraints [155 intra-residue, 98 sequential, 45 medium-range ($|i - j| < 4$) and 16 long-range ($|i - j| \geq 4$) inter-proton restraints], and 7 dihedral angle restraints. The 20 low-energy structures of α -conotoxin GIC were selected and a quality analysis of the final superimposed ensemble was performed using the program PROCHECK [34].

Figure 1 Amino acid sequence of α -conotoxin GIC and a summary of short- and medium-range NOEs, $^3J_{HNH\alpha}$, and CSI for H_α protons

Thickness of bar represents relative strength (strong, medium and weak) of NOEs. ●, $^3J_{HNH\alpha} < 6$ Hz; ○, $^3J_{HNH\alpha} > 8$ Hz. The filled squares above and below the horizontal line represent CSI values of +1 and -1 respectively. Note the presence of the α -helix from His⁵–Asn¹² which is common to all $\alpha 4/7$ subfamily.

RESULTS

NMR assignments

The complete 1H resonance assignment for α -conotoxin GIC was achieved using double-quantum-filtered COSY, TOCSY and NOESY spectra, following the standard sequential assignment procedure [35]. Firstly, Ser⁴, Pro⁶, Gln¹³ and Ile¹⁵ were unambiguously assigned based on the COSY and TOCSY spectra according to their characteristic coherence transfer patterns. Due to their unique 'back-transfer' cross-peaks, His⁵ and His¹⁴ were assigned using a TOCSY spectrum without relying on the NOE connectivity. For the other residues, classification of spin systems in a TOCSY spectrum, along amide NH resonances, preceded the sequential assignment procedure. Strong sequential $d_{\alpha\delta}$ -type NOE cross-peaks were clearly observed for Pro⁶, indicating that Pro⁶ has a *trans* conformation [36]. Due to *trans*–*cis* isomerization of proline, minor peaks originating from a *cis* conformer were observed. Detailed analyses of NMR data and structure calculation have been performed for the *trans* isomer. Figure 1 is a summary of short- and medium-range NOEs used for the resonance assignment, $^3J_{HNH\alpha}$, and CSI (chemical shift index) [37], together with the amino acid sequence of α -conotoxin GIC.

Three-dimensional structure of α -conotoxin GIC

Figure 2 is the superposition of a final ensemble of 20 structures for α -conotoxin GIC. The structure is well defined with backbone and heavy atom RMSDs (root mean square deviations) over residues 2–16 of 0.53 Å and 0.96 Å respectively. Further details on structure calculation is provided in Table 2. When subject to quality evaluation by PROCHECK, 87.9% and 12.1% of backbone dihedral angles of all non-glycine and non-proline residues were found to reside within the most favoured region and additional allowed region of the Ramachandran plot respectively.

α -Conotoxin GIC has an ω -shaped molecular backbone (Figure 2), a common structural feature of all $\alpha 4/7$ subfamily conotoxins. The secondary structure analysis with PROMOTIF [38] reveals that the toxin consists of two β -turns and one amphipathic α -helix. The N-terminal Cys²–His⁵ residues constitute a type I β -turn, whereas the C-terminal arm of the ω fold contains a type IV β -turn formed by residues Gln¹³–Cys¹⁶. The helix is found at

Figure 2 Stereo view of the final 20 structures for α -conotoxin GIC

The ribbon traces the molecular backbone. The N-terminus of the molecule is placed at the upper left-hand corner. Top: backbone, Bottom: heavy atoms.

Table 2 MR structural determination statistics of α -conotoxin GIC for an ensemble of 20 structures

Values, where applicable, are expressed as the means $\pm$ S.D.

Parameter	Value
RMSDs from experimental restraints	
NOE distance restraints ($\text{\AA}$)	0.008 ± 0.001
Dihedral angle restraints ($^\circ$)	0.097 ± 0.029
Number of NOE distance restraints	314
Intra-residue	155
Sequential	98
Medium range (< 4)	45
Long range (≥ 4)	16
Number of backbone dihedral angle restraints	7
Ramachandran plot regions (%)	
Residues in most favoured region	87.9
Residues in additional allowed region	12.1
Residues in generously allowed region	0.0
Residues in disallowed region	0.0
Angular order parameters	
Phi	0.9864 ± 0.0192
Psi	0.9124 ± 0.2091
Chi 1	0.7630 ± 0.2721
RMSDs from the average structure (residues 2–16) ($\text{\AA}$)	
Backbone atoms*	0.53 ± 0.17
Heavy atoms	0.96 ± 0.27

* Backbone atoms are N, C $^\alpha$, C' and O.

the bottom portion of the ω -shape and runs from His⁵ to Asn¹². One face of this amphipathic helix comprises surface-exposed hydrophobic residues [Pro⁶, Ala⁷, Ala⁹ and Gly¹⁰], which hence are likely to be involved with receptor binding via hydrophobic interactions. The other face comprises hydrophilic residues, such as Ser⁴, Asn¹¹, Asn¹² and Gln¹³.

Structural comparison of α -conotoxin GIC with other α 3 β 2-blocking α -conotoxins

Other α -conotoxins, such as MII, PnIA, EpI and GID, are also capable of antagonizing the α 3 β 2 nAChR subtype. These toxins

along with α -conotoxin GIC, in fact, constitute one of the major groups of the α 4/7 subfamily (Table 1). High-resolution structures of these α -conotoxins [7,14–16,18] are superimposed in Figure 3. All, but α -conotoxin GID, have the common ω -backbone fold. The backbone RMSDs over residues 2–16 of α -conotoxin GIC with the other toxins are 0.97 $\text{\AA}$ with MII (PDB accession no. 1M2C), 0.77 $\text{\AA}$ with PnIA (PDB accession no. 1PEN), 0.64 $\text{\AA}$ with PnIB (PDB accession no. 1AKG), 0.71 $\text{\AA}$ with [Tyr15]EpI (PDB accession no. 1A0M), and 1.18 $\text{\AA}$ with GID (PDB accession no. 1MTQ). The similar backbone topology of these α -conotoxins suggests that differences in the nAChR rank order of potency among these toxins should originate from differences in the surface charge and size of the side chains [17,39,40]. The N-terminal disulphide loop of all of these α -conotoxins contains a reasonably hydrophobic SvP ψ motif (shown in grey in Figure 3), where ν is a variable residue and ψ is a hydrophobic residue, except for α -conotoxin EpI. The binding of α -conotoxins to nAChRs occurs at the interface where an α -subunit is facing its partner non- α -subunit [19,41,42]. Hence, one may see that the N-terminal loop of α -conotoxins mainly provides a motif that interacts with α -subunits, whereas the C-terminal loop contains residues contacting non- α -subunits of nAChRs [6,13].

DISCUSSION

Six members of the α 4/7 subfamily conotoxins, including α -conotoxin GIC, exhibit antagonistic activity against the α 3 β 2 nAChR subtype [7,27,43–46]. It should be noted that although each peptide blocks the α 3 β 2 subtype, not only their individual IC₅₀ values, but also their nAChR subtype-binding profiles differ to a significant degree. The observation that most of the α 3 β 2 subtype-targeting α -conotoxins also show activity against other subtypes, such as α 7, α 4 β 2 and α 3 β 4 subtypes, is associated with the fact that these subunits all have originated from one ancestral gene and hence are capable of providing a similar ligand-binding environment. For example, α -conotoxins GIC and MII show very high apparent binding affinities both for the α 3 β 2 subtype and α 6-containing subtypes, as indicated by their low nanomolar IC₅₀ values, as the α 3 and α 6 subunits of nAChR are most homologous. Even though α -conotoxins EpI and GID have similarly high apparent affinities to the α 3 β 2 subtype they are also active against other receptor subtypes. Since α -conotoxin PnIB binds to the α 3 β 2 subtype with a weak apparent affinity, it is generally viewed as an α 7 binder.

One way of explaining diverse receptor subtype specificities and profiles of the α 4/7 subfamily conotoxins, which have virtually the same backbone topology, is to examine differences in surface charge distribution. On one side of the α 4/7 conotoxins GIC, MII, PnIA/B, EpI, and GID that antagonize the α 3 β 2 nAChR subtype there is a rather large hydrophobic surface within the N-terminal loop region. Hydrophobic interaction is known as one of the important binding mechanisms for ligand–nAChR binding [4,47]. On the other side of these toxins one can find two sequential asparagines [Asn¹¹/Asn¹² or Asn¹⁴/Asn¹⁵] clustered in the middle, except for MII where the two are replaced by Glu¹¹ and His¹², and for PnIB where Asn¹¹ is replaced by a serine residue. This finding suggests that presence of such a hydrophobic surface on one side of the toxin in combination with two contiguous hydrophilic residues on the other side, such as asparagine, histidine or glutamic acid, is necessary for an α -conotoxin to interact with the α 3 β 2 subtype. Actual binding strength and full receptor specificity profile would, of course, be governed by the precise nature of such hydrophobic and hydrophilic areas, as well as by actual sequence differences in other parts of the

Figure 3 Ribbon diagrams of six superimposed structures of α -conotoxins in stereo view

The structures of α -conotoxins GIC (green), MII (blue), PnIA (yellow), PnIB (cyan), [Tyr15]Epl (red), and GID (violet) are superimposed. The putative α -subunit binding face (see the text for details) is shown in a space-filling mode (grey).

toxin. The fact that α -conotoxin [N11S]PnIA is 24-fold less active against the $\alpha 3\beta 2$ subtype than the wild-type [43] supports the notion that Asn¹¹ is important for the $\alpha 3\beta 2$ selectivity. Large contribution by one asparagine residue is not unreasonable, because a subtle difference in the orientation and length of a side chain can be crucial for hydrogen bonding in many protein–protein interactions. Importance of residue 11 for receptor selectivity has been suggested in another $\alpha 4/7$ subfamily member, α -conotoxin MII [24]. More recent results suggest that mutation of Glu¹¹ to Ala in α -conotoxin MII significantly decreases its activity at $\alpha 3\beta 2$ without decreasing its activity at $\alpha 6\beta 2$ -containing nAChRs (J. M. McIntosh, unpublished work).

Two members, in particular, of the $\alpha 4/7$ subfamily, α -conotoxins GIC and MII, have a very similar receptor specificity profile, as well as high potency against the $\alpha 3\beta 2$ subtype [27,45]. Thus a question arises as to how seven out of eight C-terminal residues in these toxins could be different (Table 1) when their biological properties are so similar. The surface charge analysis of the two toxins reveals that, despite the sequence variation, the two have a similar surface charge distribution. One should note that the electronic and steric states of a histidine are not much different from those of an asparagine at neutral pH. In fact, these two polar residues, which are capable of forming hydrogen bonds, are interchangeably found in α -conotoxins (Table 1), as well as in conotoxins that target other ion channels [48,49]. Thus the remarkably similar pharmacological activity profile must arise from a similar surface charge distribution pattern found in α -conotoxins GIC and MII. Such a conclusion is further supported by the fact that other $\alpha 3\beta 2$ subtype targeting α -conotoxins, such as PnIA, Epl, GID and PnIB, which have different receptor selectivity profiles from those of α -conotoxins GIC and MII, indeed have substantially different surface charge distribution patterns from those of α -conotoxins GIC and MII.

Despite similarities, α -conotoxins GIC and MII differ in a few aspects. For example, α -conotoxin GIC is more than 100 000-fold selective for the $\alpha 3\beta 2$ subtype versus the muscle receptor of nAChR. In contrast, α -conotoxin MII is only approx. 1000-fold selective against the muscle receptor. In addition, high-affinity block of nAChRs by α -conotoxin GIC can be reversed relatively rapidly upon toxin washout in contrast with the much slower reversibility of α -conotoxin MII [27].

Figure 4 Connolly surface comparison between α -conotoxins GIC and MII in stereo view

In α -conotoxins GIC (light grey) and MII (dark grey), some residues exposed out of the surface of the other molecule are labelled. Upper panels: a front view. Lower panels: a view from the other side.

These differences may be associated with the following differences between the two toxins. First, α -conotoxin MII has the negatively charged Glu¹¹ at the important position 11, whereas an Asn¹¹ is found in α -conotoxin GIC. Secondly, α -conotoxin GIC is more hydrophilic than α -conotoxin MII, as was demonstrated by the HPLC elution profile [27]; Ala⁷ and Gly¹⁰ that form the hydrophobic core of α -conotoxin GIC are certainly less hydrophobic than Val⁷ and Leu¹⁰ in α -conotoxin MII. Also, Gln¹³ in α -conotoxin GIC is slightly more hydrophilic than Ser¹³ in α -conotoxin MII.

When a comparative surface exposure approach is applied to compare the α -conotoxins GIC and MII, His⁵, Gln¹³ and His¹⁴ of α -conotoxin GIC become exposed out of the MII surface,

whereas Val⁷, His⁹, Leu¹⁰, Glu¹¹ and His¹² of α -conotoxin MII are exposed outside of the α -conotoxin GIC surface (Figure 4). Note that Gln¹³ is the most solvent exposed residue within α -conotoxin GIC. This Gln¹³ in α -conotoxin GIC is particularly unique in that no other α 4/7 subfamily members have a residue with such a long hydrophilic side chain. Other α 4/7 members have either a smaller residue (a serine in MII) or hydrophobic residues, such as proline or hydroxyproline, at the corresponding position. The longer aliphatic side chain of Gln¹³ may allow α -conotoxin GIC to interact better with a particular receptor subtype.

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REFERENCES

- McIntosh, J. M., Santos, A. D. and Olivera, B. M. (1999) Conus peptides targeted to specific nicotinic acetylcholine receptor subtypes. *Annu. Rev. Biochem.* **68**, 59–88
- Dutton, J. L. and Craik, D. J. (2001) α -Conotoxins: nicotinic acetylcholine receptor antagonists as pharmacological tools and potential drug leads. *Curr. Med. Chem.* **8**, 327–344
- Arias, H. R. and Blanton, M. P. (2000) Leu¹⁰ of α -conotoxin PnIB confers potency for neuronal nicotinic responses in bovine chromaffin cells. *Int. J. Biochem. Cell Biol.* **32**, 1017–1028
- Quiram, P. A., McIntosh, J. M. and Sine, S. M. (2000) Pairwise interactions between neuronal α (7) acetylcholine receptors and α -conotoxin PnIB. *J. Biol. Chem.* **275**, 4889–4896
- Han, K.-H., Hwang, K.-J., Kim, S.-M., Kim, S.-K., Gray, W. R., Olivera, B. M., Rivier, J. and Shon, K.-J. (1997) NMR structure determination of a novel conotoxin, [Pro⁷,13] α A-conotoxin PIVA. *Biochemistry* **36**, 1669–1677
- Park, K.-H., Suk, J.-E., Jacobsen, R., Gray, W. R., McIntosh, J. M. and Han, K.-H. (2001) Solution conformation of α -conotoxin EI, a neuromuscular toxin specific for the α 1/ δ subunit interface of Torpedo nicotinic acetylcholine receptor. *J. Biol. Chem.* **276**, 49028–49033
- Nicke, A., Loughnan, M. L., Millard, E. L., Alewood, P. F., Adams, D. J., Daly, N. L., Craik, D. J. and Lewis, R. J. (2003) Isolation, structure, and activity of GID, a novel α 4/7-conotoxin with an extended N-terminal sequence. *J. Biol. Chem.* **278**, 3137–3144
- Chi, S.-W., Park, K.-H., Suk, J.-E., Olivera, B. M., McIntosh, J. M. and Han, K.-H. (2003) Solution conformation of α A-conotoxin EIVA, a potent neuromuscular nicotinic acetylcholine receptor antagonist from *Conus ermineus*. *J. Biol. Chem.* **278**, 42208–42213
- Myers, R. A., Cruz, L. J., Rivier, J. E. and Olivera, B. A. (1993) *Conus* peptides as chemical probes for receptors and ion channels. *Chem. Rev.* **93**, 1923–1936
- Chiara, D. C. and Cohen, J. B. (1997) Identification of amino acids contributing to high and low affinity δ -tubocurarine sites in the Torpedo nicotinic acetylcholine receptor. *J. Biol. Chem.* **272**, 32940–32950
- Sugiyama, N., Marchot, P., Kawanishi, C., Osaka, H., Molles, B., Sine, S. M. and Taylor, P. (1998) Residues at the subunit interfaces of the nicotinic acetylcholine receptor that contribute to α -conotoxin M1 binding. *Mol. Pharmacol.* **53**, 787–794
- Chiara, D. C., Xie, Y. and Cohen, J. B. (1999) Structure of the agonist-binding sites of the Torpedo nicotinic acetylcholine receptor: affinity-labeling and mutational analyses identify γ -Tyr-111/ δ Arg-113 as antagonist affinity determinants. *Biochemistry* **38**, 6689–6698
- Gehrmann, J., Alewood, P. F. and Craik, D. J. (1998) Structure determination of the three disulfide bond isomers of α -conotoxin GI: a model for the role of disulfide bonds in structural stability. *J. Mol. Biol.* **278**, 401–415
- Shon, K.-J., Koerber, S. C., Rivier, J. E., Olivera, B. M. and McIntosh, J. M. (1997) Three-dimensional solution structure of α -conotoxin MII, an α 3/ β 2 neuronal nicotinic acetylcholine receptor-targeted ligand. *Biochemistry* **36**, 15693–15700
- Hu, S.-H., Gehrmann, J., Alewood, P. F., Craik, D. J. and Martin, J. L. (1997) Crystal structure at 1.1 Å resolution of α -conotoxin PnIB: comparison with α -conotoxins PnIA and GI. *Biochemistry* **36**, 11323–11330
- Hu, S.-H., Loughnan, M. L., Miller, R., Weeks, C. M., Blessing, R. H., Alewood, P. F., Lewis, R. J. and Martin, J. L. (1998) The 1.1 Å resolution crystal structure of [Tyr¹⁵]Epl, a novel α -conotoxin from *Conus episcopatulus*, solved by direct methods. *Biochemistry* **37**, 11425–11433
- Cho, J.-H., Mok, K.-H., Olivera, B. M., McIntosh, J. M., Park, K.-H. and Han, K.-H. (2000) Nuclear magnetic resonance solution conformation of α -conotoxin AulB, an α 3/ β 4 subtype-selective neuronal nicotinic acetylcholine receptor antagonist. *J. Biol. Chem.* **275**, 8680–8685
- Hu, S.-H., Gehrmann, J., Guddat, L. W., Alewood, P. F., Craik, D. J. and Martin, J. L. (1996) The 1.1 Å crystal structure of the neuronal acetylcholine receptor antagonist, α -conotoxin PnIA from *Conus pennaceus*. *Structure* **4**, 417–423
- Karlin, A. and Akabas, M. H. (1995) Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* **15**, 1231–1244
- Sargent, P. B. (1993) The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* **16**, 403–443
- McGehee, D. S. and Role, L. W. (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu. Rev. Physiol.* **57**, 521–546
- Walkinshaw, M. D., Saenger, W. and Maelicke, A. (1980) Three-dimensional structure of the 'long' neurotoxin from cobra venom. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2400–2404
- Love, R. A. and Stroud, R. M. (1986) The crystal structure of α -bungarotoxin at 2.5 Å resolution: relation to solution structure and binding to acetylcholine receptor. *Protein Eng.* **1**, 37–46
- Basus, V. J., Song, G. and Hawrot, E. (1993) NMR solution structure of an α -bungarotoxin/nicotinic receptor peptide complex. *Biochemistry* **32**, 12290–12298
- Fraenkel, Y., Gershoni, J. M. and Navon, G. (1994) NMR analysis reveals a positively charged hydrophobic domain as a common motif to bound acetylcholine and δ -tubocurarine. *Biochemistry* **33**, 644–650
- Behling, R. W., Yamane, T., Navon, G. and Jelinek, L. W. (1988) Conformation of acetylcholine bound to the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6721–6725
- McIntosh, J. M., Dowell, C., Watkins, M., Garrett, J. E., Yoshikami, D. and Olivera, V. M. (2002) α -Conotoxin GIC from *Conus geographus*, a novel peptide antagonist of nicotinic acetylcholine receptors. *J. Biol. Chem.* **277**, 33610–33615
- Griesinger, C., Otting, G., Wüthrich, K. and Ernst, P. R. (1988) Clean TOCSY for proton spin system identification in macromolecules. *J. Am. Chem. Soc.* **110**, 7870–7872
- Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R. and Wüthrich, K. (1987) Improved spectral resolution in COSY ¹H NMR spectra of proteins via double quantum filtering. *Biochem. Biophys. Res. Commun.* **117**, 479–485
- Nilges, M., Macias, M., O'nohgue, S. and Oshkinat, H. (1997) Automated NOESY interpretation with ambiguous distance restraints: the refined NMR solution structure of the pleckstrin homology domain from β -spectrin. *J. Mol. Biol.* **269**, 408–422
- Wever, P., Morrison, A. and Hare, D. (1998) Determining stereo-specific ¹H nuclear magnetic resonance assignments from distance geometry calculations. *J. Mol. Biol.* **204**, 483–487
- Flomer, R., Hibers, C., Konings, R. and Nilges, M. (1997) Floating stereospecific assignment revisited: application to an 18 kDa protein and comparison with J-coupling data. *J. Biomol. NMR* **9**, 245–258
- Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S. et al. (1998) Crystallography & NMR system: a new software suite for macro-molecular structure determination. *Acta Crystallogr. Sec. D: Biol. Crystallogr.* **54**, 905–921
- Laskowski, R. A., Rullman, J. A., MacArthur, M. W., Kaptein, R. and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* **8**, 477–486
- Billeter, M., Braun, W. and Wüthrich, K. (1982) Sequential resonance assignments in protein ¹H nuclear magnetic resonance spectra. Computation of sterically allowed proton-proton distances and statistical analysis of proton-proton distances in single crystal protein conformations. *J. Mol. Biol.* **155**, 321–346
- Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley and Sons, New York
- Wishart, D. S. and Sykes, B. D. (1994) Chemical shifts as a tool for structure determination. *Methods Enzymol.* **239**, 363–392
- Hutchinson, E. G. and Thornton, J. M. (1996) PROMOTIF: a program to identify and analyze structural motifs in proteins. *Prot. Sci.* **5**, 212–220
- Franco, A. and Mari, F. (1999) Three-dimensional structure of α -conotoxin EI determined by ¹H NMR spectroscopy. *Lett. Pept. Sci.* **6**, 199–207
- Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I. (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science* **253**, 872–879
- Hucho, F., Tsetlin, V. I. and Machold, J. (1996) The emerging three-dimensional structure of a receptor. The nicotinic acetylcholine receptor. *Eur. J. Biochem.* **239**, 539–557

- 42 Quiram, P. A., Jones, J. J. and Sine, S. M. (1999) Pairwise interactions between neuronal $\alpha 7$ acetylcholine receptors and α -conotoxin Iml. *J. Biol. Chem.* **274**, 19517–19524
- 43 Luo, S., Nguyen, T. A., Cartier, G. E., Olivera, B. M., Yoshikami, D. and McIntosh, J. M. (1999) Single-residue alteration in α -conotoxin PnIA switches its nAChR subtype selectivity. *Biochemistry* **38**, 14542–14548
- 44 Dowell, C., Olivera, B. M., Garrett, J. E., Staheli, S. T., Watkins, M., Kuryatov, A., Yoshikami, D., Lindstrom, J. M. and McIntosh, J. M. (2003) α -Conotoxin PIA is selective for $\alpha 6$ subunit-containing nicotinic acetylcholine receptors. *J. Neurosci.* **23**, 8445–8452
- 45 Cartier, G. E. (2002) α -Conotoxin MII: a new tool for the study of neuronal nicotinic acetylcholine receptors. Ph.D. thesis, University of Utah
- 46 Loughnan, M., Bond, T., Atkins, A., Cuevas, J., Adams, D. J., Broxton, N. M., Livett, B. G., Down, J. G., Jones, A., Alewood, P. F. and Lewis, R. J. (1998) α -Conotoxin Epl, a novel sulfated peptide from *Conus episcopatus* that selectively targets neuronal nicotinic acetylcholine receptors. *J. Biol. Chem.* **273**, 15667–15674
- 47 Bren, N. and Sine, S. M. (2000) Hydrophobic pairwise interactions stabilize α -conotoxin MI in the muscle acetylcholine receptor binding site. *J. Biol. Chem.* **275**, 12692–12700
- 48 Pallaghy, P. K. and Norton, R. S. (1999) Refined solution structure of ω -conotoxin GVIA: implications for calcium channel binding. *J. Pept. Res.* **53**, 343–351
- 49 Bulaj, G., DeLaCruz, R., Azimi-Zonooz, A., West, P., Watkins, M., Yoshikami, D. and Olivera, B. M. (2001) δ -Conotoxin structure/function through a cladistic analysis. *Biochemistry* **40**, 13201–13208

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