Structure-Function Elucidation of a New α-Conotoxin, Lo1a, from Conus longurionis

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Background: α-Conotoxins are small toxins produced by cone snails and antagonists of nicotinic acetylcholine receptors. They are peptidic toxins found in the venom of marine cone snails and potent antagonists of various subtypes of nicotinic acetylcholine receptors (nAChRs). nAChRs are cholinergic receptors forming ligand-gated ion channels in the plasma membranes of certain neurons and the neuromuscular junction. Because nAChRs have an important role in regulating transmitter release, cell excitability, and neuronal integration, nAChR dysfunctions have been implicated in a variety of severe pathologies such as epilepsy, myasthenic syndromes, schizophrenia, Parkinson disease, and Alzheimer disease. To expand the knowledge concerning cone snail toxins, we examined the venom of Conus longurionis. We isolated an 18-amino acid peptide named α-conotoxin Lo1a, which is active on nAChRs. To the best of our knowledge, this is the first characterization of a conotoxin from this species. The peptide was characterized by electrophysiological screening against several types of cloned nAChRs expressed in Xenopus laevis oocytes. The three-dimensional solution structure of the α-conotoxin Lo1a was determined by NMR spectroscopy. Lo1a, a member of the αδ/7 family, blocks the response to acetylcholine in oocytes expressing α7 nAChRs with an IC50 value of 3.24 ± 0.7 μM. Furthermore, Lo1a shows a high selectivity for neuronal versus muscle subtype nAChRs. Because Lo1a has an unusual C terminus, we designed two mutants, Lo1a-ΔD and Lo1a-RRR, to investigate the influence of the C-terminal residue. Lo1a-ΔD has a C-terminal Asp deletion, whereas in Lo1a-RRR, a triple-Arg tail replaces the Asp. They blocked the neuronal nAChR α7 with a lower IC50 value, but remarkably, both adopted affinity for the muscle subunit α2β3δε.

Nicotinic acetylcholine receptors (nAChRs) are expressed in the central and peripheral nervous systems where they are involved in many neuronal functions. These neuronal functions include differentiation and synaptic plasticity, which are the basis for learning and memory (1–3). The nicotinic acetylcholine receptor family is classified into two subtypes based on their primary sites of expression, namely neuronal and muscle subtype nAChRs. Both subtypes are pentameric integral membrane protein complexes classified as ligand-gated ion channels that open in response to binding of the neurotransmitter acetylcholine (ACh) (4, 5). The neuronal subtype nAChRs can include either exclusively α-subunits such as α7, α4, and α9, called homomeric ion channels or a combination of two or more different types of subunits, in a heteromeric assembly. These heteromeric channels are compiled of at least one α subunit (α7−α2) and one β subunit (β2−β4). The muscle subtype nAChRs are composed of four different types of subunits (α1, β1, δ, and ε/γ) (6–8).

One of the neuronal nAChRs, α2, has received much attention since its discovery thanks to the role of α2 in the central nervous system (CNS) (9). This is because α2 nAChRs are highly distributed in the brain, including regions involved in learning and memory, hippocampus, and cerebral cortex (10–12). Consequently, nAChR dysfunctions have been implicated in a variety of severe pathologies such as certain types of epilepsy, myasthenic syndromes, schizophrenia, Parkinson disease, and Alzheimer disease (13–15). Therefore, the discovery of new ligands binding with high affinity and selectivity to nAChR subtypes is of prime interest to study these receptors.

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3 Supported by Grants G.0433.12, G.0471.10N, and G.0257.08 from F.W.O. Vlaanderen; EU-FLP7-MAREX, IUAP 7/10, and IUAP 7/24 from the Inter-University Attraction Poles Program (Belgian State, Belgian Science Policy); OT/12/081 and GOA 12/016 from the University of Leuven; and 2013/146 of the Inter-University Attraction Poles Program (Belgian State, Belgian Science Policy).
4 The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; ACN, acetonitrile; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation.
and to potentially discover new drugs for the treatment of these pathologies (16).

New ligands may be found in cone snail species, from which the so-called family of α-conotoxins is a group of potent nAChRs antagonists (17, 18). These α-conotoxins are a series of structurally and functionally related peptides found in the venom of cone snail species. They are classified into subfamilies based on the number of residues in their two “loops” between conserved Cys residues, with 3/5 (CCX₃CCₓC), 4/3 (CCX₄CCₓC), and 4/7 (CCX₄CₓC) subfamilies the most common (19). The toxins of Conus sp. are usually potent, selective, and small (12–25 amino acids), which is an advantage for cost-effective synthesis (20). Moreover, they are shown to function as specific probes to investigate the structure-function relationship of nAChRs (17).

In this study, we report the isolation of a novel 18-amino acid α-conotoxin from the venom of the marine snail Conus longurionis and its electrophysiological screening against six different types of nAChRs. To the best of our knowledge, this is the first conotoxin to be characterized from this species found in the Indian Ocean near Tamil Nadu, India. The peptide, called Lo1a, has a W-shaped structural conformation with two loops that are reinforced by two disulfide bonds. The function of the peptide revealed that Lo1a was most active against neuronal homomeric α₁ nAChRs. To further determine the structure-function relationship of Lo1a and its target α₁, we engineered two synthetic analogues, namely Lo1a-ΔD and Lo1a-RRR, based on the protein sequence of Lo1a and its homology to other conotoxins from the α4/7 family. The first peptide, Lo1a-ΔD, has an Asp deletion at the C terminus, whereas in the second peptide, an Arg tail replaces this Asp. Both analogues were found to block the neuronal nAChR α₁ with a lower IC₅₀, but remarkably, they adopted affinity for the muscle subtype α₁β₁δε. These results revealed an unexpected role for the C terminus in determining subtype selectivity and efficacy. Consequently, our findings might be relevant in the context of designing novel therapeutic compounds with potential utility in diseases such as Alzheimer disease, schizophrenia, and attention deficit hyperactivity disorder because, as previously mentioned, α₁ nAChRs are thought to play important roles in the brain (21).

**EXPERIMENTAL PROCEDURES**

**Cone Snail Specimens and Venom Extraction**—Specimens of Conus longurionis (identified by Kiener in 1845 and classified by Tucker and Tenorio (22)) were collected from the Indian Ocean near Tamil Nadu, India. The venomous apparatuses (venom bulbs and venom ducts) were extracted from the specimens as previously described (23). The collected tissue was preserved in RNAlater solution (Ambion) and stored at −20 °C. The venomous apparatuses were used for total RNA extraction and peptide/protein extraction.

**Peptide and Purification**—Sample fractionation occurred by reversed phase HPLC (Gilson, Middleton, WI). Two steps were followed for the separation of the venom compounds. In the first step, the lyophilized crude venom powder was solubilized into 50% acetonitrile (ACN)/water, and aliquots were loaded on a gel filtration Superdex™ peptide 10/300 GL column with 50% ACN/water as mobile phase (flow rate, 0.5 ml/min) to separate the peptides and proteins based on their size. Two sample collections were made that were stored overnight at −80 °C, freeze-dried and finally solubilized in 5% ACN/water. For the second step, an analytical Vydac C₁₈ column (218MS54, 4.6 × 250 mm, 5-μm particle size; Grace, Deerfield, IL) with a two-solvent system was used: (A) 0.1% TFA/H₂O and (B) 0.085% TFA/ACN. The sample was eluted at a constant flow rate of 1 ml min⁻¹ with a 0–80% gradient of solvent B over 90 min (1% ACN per min after 10 min of solvent A). The HPLC column fractions were monitored by a UV/VIS-155 detector (Gilson) scanning both 214 and 280 nm.

**Peptide Sequencing**—Isolated Lo1a was collected and freeze-dried for direct peptide sequencing and molecular mass analysis (MALDI-TOF). A Protein Sequencer PPSQ-31A/33A (Shimadzu, Japan) was used to determine the amino acid sequence of the separated compound. In this Edman degradation method, the sample was loaded on a polybrene-pretreated, precycled glass fiber disk and Edman sequenced for 24 residue cycles.

**Peptide Synthesis and Folding**—Lo1a was synthesized using Fmoc (N-(9-fluorenlylmethoxycarbonyl) chemistry by GenCust (Luxemburg). Lo1a-ΔD and Lo1a-RRR were synthesized by GenicBio Limited (Shanghai, China). Formation of the two disulfide bridges was carried out by adopting the selective protection and deprotection strategy in vitro. The resulting bicyclic peptides were subsequently purified by HPLC and analyzed with ESI-MS, then freeze-dried, and stored at −20 °C until use.

**Functional Characterization**—Complementary DNA encoding the nAChR channels was subcloned into the corresponding vector: human α₁/pDNA3(Xbal), human α₁/pGEM-HE(Nhel), chick α₁/pBlueScript(NotI), human β₁/SP64(Pvull), human β₁/pDNA3(Xbal), rat α₁/pSP0oD(Sall), rat β₁/pSP0oD(Sall), rat γ/pSP0oD(Sall), rat δ/pSP0oD(Sall), and rat ε/pSP0oD(Sall). The linearized plasmids (restrictive restriction enzymes are indicated in parentheses) were transcribed using the T7 (α₁, α₂, α₃, or α₄) or the SP6 (β₂, α₂, α₁, γ, δ, or ε) mMESSAGE mMACHINE transcription kit (Ambion, Austin, TX).

The harvesting of stage V–VI oocytes from anesthetized female Xenopus laevis frogs was previously described (24). Oocytes were injected with 50–70 nl of cRNA at a concentration of 1–3 ng/ml using a micro-injector (Drummond Scientific, Broomall, PA). The oocytes were incubated in a ND-96 solution containing: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, and 5 mM HEPES (pH 7.4), supplemented with 1.25 ml/liter gentamicin and 90 mg/liter theophylline. The oocytes were stored for 1–5 days at 16 °C until sufficient expression of nAChRs was achieved.

Whole cell currents from oocytes were recorded at room temperature (18–22 °C) by the two-electrode voltage clamp technique using a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA) controlled by a pClamp data acquisition system (Molecular Devices). The oocytes were placed in a bath containing ND-96 solution. Voltage and current electrodes were filled with 3 M KCl, and the resistances of both electrodes were between 0.5 and 1.5 MΩ. The elicited currents were sampled at 100 Hz and filtered at 50 Hz using a four-pole, low pass Bessel filter. To eliminate the effect of the voltage drop across the bath grounding electrode, the bath potential was...
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Isolation of a Novel \( \alpha \)-Conotoxin from C. longurionis Venom—
N-terminal Edman degradation of the purified active peptide revealed a novel 18-residue \( \alpha \)-conotoxin, called Lo1a, with the sequence H-EGCCSNPACRTNHPEVCD-NH\(_2\) (bridges Cys\(_3\)–Cys\(_9\) and Cys\(_3\)–Cys\(_{17}\)) and a molecular mass of 1930.12 Da, determined by MALDI-TOF (4800 Analyzer; Applied Biosystems). To the best of our knowledge, Lo1a is the first conotoxin isolated and pharmacologically characterized from C. longurionis, a species of a (vermivorous) cone snail commonly found in the Indian Ocean in Tamil Nadu, India. It has highest sequence homology with Qc1.5 (81%; Fig. 1), which is isolated from Conus quercinus, another vermivorous cone snail (33).

RESULTS

Isolation of a Novel \( \alpha \)-Conotoxin from C. longurionis Venom—
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—Typically, α-conotoxins end C-terminally with a Cys (Fig. 1). Interestingly, Lo1a ends with an Asp following this Cys. Only two more conotoxins having an Asp at the C terminus are described, namely Lp1.4 (34) and Bt1.91 (35). To explore the influence of the C-terminal Asp18 to negatively charged Asp is replaced by a positively charged Arg tail.

Lo1a Inhibition of Recombinant nAChR Subtypes—The potency and selectivity of Lo1a at neuronal nAChRs was determined by examining its effect on ACh-evoked currents mediated by different nAChR subunit combinations expressed in Xenopus oocytes. Lo1a (10 μM) inhibited ACh-evoked current amplitude mediated by α7 (85%), α5β2 (40%), α6β2 (19%), and α5β4 (13%) (Fig. 2). No remarkable effect was seen at the muscle subtype nAChRs α2β, γδ and α1β, δε for concentrations up to 50 μM. Concentration-response curves for Lo1a inhibition of ACh-evoked currents at the α2 nAChR revealed an IC50 value of 3.24 ± 0.70 μM (Fig. 3).

Influence of C-terminal Truncation and Replacement of D by RRR—We synthesized a C-terminally truncated and a triple Arg-tailed analogue of Lo1a to examine its activity at different nAChR subtypes (Figs. 4 and 5). Interestingly, Lo1a-ΔD was more potent at the neuronal nAChR α7 (IC50 = 0.80 ± 0.01 nM) but surprisingly also adopted affinity for the muscle subtype α1β, δε (IC50 = 4.40 ± 0.18 μM) (Fig. 6). The same was noticed for Lo1a-RRR, where the IC50 for α5 was 1.06 μM, and an IC50 value of 1.47 ± 0.39 μM was determined for α1β, δε (Fig. 6). An overview of subtype selectivity for Lo1a and both mutants is given in Fig. 7.

NMR Spectroscopy—NMR spectral analysis shows the formation of a single set of resonances for the conopeptide, indicating that it adopts one type of structural form in solution. Resonance assignment was performed by following standard procedures as outlined by Wüthrich (36). Complete sequence specific proton assignments were achieved by analyzing homonuclear two-dimensional spectra (double quantum-filtered COSY, TOCSY, and NOESY). Initially the NH and δH resonances of the individual spin systems (except Pro) were identified by analyzing the “fingerprint” region of the double quantum-filtered COSY and TOCSY spectra, and the remaining resonances of the spin systems were identified by following the “TOCSY tower.” Sequence specific assignments were achieved by linking individual spin system via sequential inter-residue Hα-HN(n+1) (n+1) cross-peaks in the fingerprint region of the NOESY spectrum. Carbon assignments were also performed by using 1H-13C HSQC spectra, which further confirmed most of the homonuclear proton assignments and clarified the Hβ and Hy proton assignments of the two proline residues that were not resolved in the homonuclear two-dimensional spectra because of signal overlaps. The geminal methylene protons were not assigned stereo-specifically, and the NOE distance constraints involving these protons were used ambiguously during structure calculation in the Xplor-NIH program. Fig. 8 shows the observed short and medium range NOEs that were used for the resonance assignment, 3JHαHNα, and chemical shift index along the amino acid sequences of α-conotoxin Lo1a. The chemical shift index values indicate the presence of a β-helix in the middle of the peptide from residue Pro10 to Asn12.

Fig. 9 displays the structural representations of the final ensemble of 15 superimposed structures (Fig. 9A) and the minimum energy closest to average structure (Fig. 9B) of α-conotoxin Lo1a. The peptide structures are well defined with backbone and heavy atom root mean square deviations of 0.43 and
0.83, respectively, over residues 3–17. The structural evaluation using PROCHECK demonstrates that all the resulted structures have no bad nonbonded contacts, and all the backbone dihedral angles are within the allowed regions of the Ramachandran plot (63% residues fall in the most favored region). Detailed structure determination statistics are provided in Table 1. The coordinates for 15 structures, NMR restraints, and chemical shifts have been deposited in the RCSB Protein Data Bank with RCSB identifier RCSB103496 and Protein Data Bank code 2MD6.

A close look into the structure (Fig. 9) of the α-conotoxin Lo1a reveals that the peptide backbone adopts a compact W-shaped conformation having two loops that are reinforced by two disulfide bonds (Cys3–Cys9 and Cys4–Cys17). Residues from Pro7 to Asn12 at the bottom of the W shape formed a /H9251/helix involving two turns. In addition to this, in the N-terminal part of the peptide structure, two overlapping turn (type IV) exist between Gly2 and Ser5 and between Cys4 and Pro7. In the C-terminal part of the peptide, residues from Pro14 to Cys17 formed a type I/IV turn. This type of overall W-shaped molecular topology/fold was previously identified in the reported structures of other 4/7 subfamily α-conotoxins (37, 38).

**DISCUSSION**

α-Conotoxins are a family of cysteine-rich peptides that behave pharmacologically as competitive antagonists of the nicotinic acetylcholine receptor (39). In general, there are two main nAChR subtypes, the neuronal and muscle subtype nAChRs. Considering the neuronal subtype nAChRs, and particularly their ACh-binding site, a high percentage of sequence identity exists among the known neuronal nAChR subunits (40, 41). Because of the high sequence conservation, it has been difficult to obtain subtype selective ligands, principally agonists that take action deep within the conserved ACh-binding pocket. However, recently, selective peptide antagonists from cone snail venom have shown to be highly selective pharmacological tools displaying the ability to discriminate among many of the different nAChR subunit combinations (41). This high selectivity toward a particular mammalian nAChR subtype is often established through specific interactions with particular residues located outside the conserved ACh-binding site (42).
Nowadays, co-crystal structures of α-conotoxins binding to the ACh-binding protein (43), which is a protein model for the extracellular ligand-binding domain of nAChRs, can offer useful information about the molecular interactions of these small peptides (44, 45).

**Lo1a Has a Typical α-Conotoxin Structure**—In our study, we revealed the amino acid composition and the three-dimensional structure of a new α4/7-conotoxin from *C. longurionis*, called Lo1a. The peptide was investigated electrophysiologically, and it was found that Lo1a inhibits α7 nAChRs preferentially. Once the activity of Lo1a had been determined, we employed NMR-based techniques to elucidate the structure of this novel peptide. According to Marx *et al.* (46), NMR is the method of choice for determining conotoxin structures.
because conotoxins are generally difficult to crystallize and are not amenable to x-ray methods, with a few exceptions such as PnIA and PnIB (47, 48). α-Conotoxin Lo1a was shown to be highly soluble, and NMR spectra were readily assigned and used to generate a high resolution structure. This structure demonstrated that Lo1a shares many of the structural and biochemical properties that define α-conotoxins, including the characteristic I–III, II–IV disulfide connectivity and the size of the first and the second loop of the peptide, i.e. four and seven residues, respectively. Several α-conotoxins including AnIB (49), OmlA (50), GID (51), RegIIA (52), and LsIA (5) have an SXP motif similar to the first loop of Lo1a. The peptide also shares the common fold comprising a short disulfide bond stabilized helix and a conserved proline. This proline is shown to be the only highly conserved amino acid residue apart from the cysteines and is responsible for helix initiation by inducing the 3\textsubscript{10} helix turn in the peptide backbone (39). Dutertre and Lewis (39) revealed that this small α-helix structure appears to be a very important determinant for binding, even if the orientations and specific interactions differ significantly. Because the side chains in an α-helix protrude at 360°, this structure is likely suitable to allow multiple contacts on both sides of a binding pocket located at the interface of two subunits (39).

As indicated in the previous section, Lo1a was shown to be very selective for the neuronal versus the muscle nicotinic receptors. Other α-conotoxins having these characteristics are ImI, α4/3 conotoxin from Conus imperialis (54) and MII, α4/7 conotoxin from Conus magus (55). ImI targets α\textsubscript{7} and α\textsubscript{8} subunits with an IC\textsubscript{50} of 220 nM and 1.8 μM, respectively (56). Its amino acid sequence, GCCSDPRCAWRC, shares three non-Cys amino acid residues with the sequence of Lo1a. These similar residues are Gly at the N terminus, Ser after the second Cys, and Trp10 to influence the potency of ImI at the 2 nAChR. Consequently, it is expected that Lo1a also targets α\textsubscript{7} nACHRs.

Lo1a Has an Atypical C Terminus—The N-terminal amino acid residue of α-conotoxins is typically a glycine followed by a conserved proline. This proline is shown to be the only highly conserved amino acid residue apart from the cysteines and is responsible for helix initiation by inducing the 3\textsubscript{10} helix turn in the peptide backbone (39). Dutertre and Lewis (39) revealed that this small α-helix structure appears to be a very important determinant for binding, even if the orientations and specific interactions differ significantly. Because the side chains in an α-helix protrude at 360°, this structure is likely suitable to allow multiple contacts on both sides of a binding pocket located at the interface of two subunits (39).
unique loop 2 sequence, an atypical terminal amino acid sequence, because both N and C termini contain negatively charged amino acid residues, i.e. glutamic acid and aspartic acid, respectively. Few conotoxins have other residues preceding the first cysteine. From these conotoxins, ArIA (61), ArIB (61), GID (51), PIA (62), EI (63), and LsIA (5) are the most investigated (Fig. 1). On the other hand, a negative residue at the C terminus of a \( \alpha \)-conotoxin has only been observed in conotoxins Lp1.4 from \textit{Conus leopardus} (33) and Bt1.91 from \textit{Conus betulinus} (35). However, to the best of our knowledge, the influence of this negative C-terminal residue has never been investigated before.

C-terminal Charges Are Important for Neuronal/Muscle Subtype Selectivity and Efficacy—To explore the role of the C-terminal Asp of Lo1a, two analogues were synthesized: one C-terminally truncated peptide named Lo1a-\( \Delta \)D and another named Lo1a-RRR with an extremely positively charged Arg tail at the C terminus. Deleting the C-terminal amino acid resulted in a more positively charged peptide, whereas the rationale for the Arg tailing peptide can be found in the sequences of ArIA and ArIB from \textit{Conus arenatus} (33) and Bt.91 from \textit{Conus betulinus} (35). However, to the best of our knowledge, the influence of this negative C-terminal residue has never been investigated before.

\textit{Structure-Function Elucidation of a New \( \alpha \)-Conotoxin}

\[ \text{FIGURE 9. NMR solution structure of \( \alpha \)-conotoxin Lo1a. A, stereo views of a family of 15 final structures of \( \alpha \)-conotoxin Lo1a with superimposed backbone heavy atoms (N, CA, C). Disulfide bonds are shown in yellow as pseudo bonds. B, front and back ribbon views of the closest to average structure of \( \alpha \)-conotoxin Lo1a. Side chain heavy atoms are displayed along with residue label. The two views are related by a 180° rotation around the vertical axis.} \]

\( \alpha \)-Conotoxins that target selectively muscle subtype nAChRs typically have a 3/5 structure (64). An exception is the \( \alpha \)-conotoxin EI (\( \alpha \)4/7) from \textit{Conus ermineus} that selectively targets the \( \alpha /\delta \) interface of muscle subtype nAChRs (63). Conotoxins that distinguish between the adult and the fetal muscle subtype nAChRs are rare. One example is \( \psi \)-conotoxin PrIIIE from \textit{Conus parius}, characterized by Lluisma \textit{et al.} (65), which showed higher inhibition potency against the adult subtype (IC\textsubscript{50} of 245 nM) than the fetal subtype nAChR (IC\textsubscript{50} of 3.24 \( \mu \)M).
Another ψ-conotoxin PIIE from Conus purpurascens shows an IC\textsubscript{50} of 7.4 μM on the adult muscle subtype, but no inhibition on the fetal muscle subtype for concentrations up to 10 μM. Teichert et al. (66) reports αA-conotoxin OIVB from Conus obscursus, a unique selective inhibitor of the mammalian fetal muscle nAChR (IC\textsubscript{50} of 56 nM), whereas affinity for the adult muscle nAChR is more than 1800-fold lower. According to Groebe et al. (67), many of the α-conotoxins bind with 10,000-fold higher affinity to the mammalian α/δ interface than the α/γ interface. The peptides Lo1a-ΔΔ and Lo1a-RRR, which are described in this work, apparently demonstrate higher affinity to the α/ε interface.

Considering the terminal charges of Lo1a and its analogues, it can be expected that the positively charged triple-Arg tail in Lo1a-RRR is more likely to adopt a conformation extended away from the negatively charged N terminus (as derived by the structural model). In contrast, the structure from Lo1a has a more “compact” conformation, with an inward-facing C terminus (as derived by the structural model). Consequently, because the C and N terminus of Lo1a are in close proximity (less than 10 nm), the charges at both termini may interact, playing a role in making the distinction between neuronal versus muscle subtype nAChRs. Further structure-function studies combined with co-crystallization experiments are necessary to see whether this hypothesis is applicable for Lo1a-ΔΔ.

Muscle and Neuronal Subtype nAChR Binding Sites: Structural Receptor Elements for Binding of Lo1a and Its Homologous—The muscle subtype nAChR has a pentameric structure comprised of two α\textsubscript{1} subunits, one β\textsubscript{1}, one δ, and, depending on whether the receptor is in an embryonic or adult stage, one γ or ε subunit, respectively. Each α\textsubscript{1} subunit folds such that the primary binding site directly faces a neighboring subunit, which is either a γ/ε or a δ subunit. The γ subunit is believed to be the one that forms stable contacts being the lone subunit between the two α\textsubscript{1} subunits, whereas the δ subunit pairs with the β subunit to form stable contacts between the α\textsubscript{1} subunits on the opposite side. Because two α\textsubscript{1} subunits are separated by at least one non-α\textsubscript{1} subunit, correct coupling between these subunits is required for cooperative binding of agonists (68). Agonists of the muscle subtype nAChR initiate channel opening and desensitization by binding to a site on each of these two α\textsubscript{1} subunits (69). Moreover, Arias and Blanton (19) established that two adjacent cysteines (at positions 192 and 193 according to the sequence number of Torpedo AChR) in the α\textsubscript{1} subunits are involved in the recognition and binding of cholinergic agonists and competitive antagonists. Later on, Sine (70) demonstrated that not only do the two α\textsubscript{1} subunits form the binding sites of agonists and antagonists, but the γ/ε and δ subunits are also involved. Agonists and antagonists can specifically distinguish between the α\textsubscript{1} γ/(α\textsubscript{1} ε) and α\textsubscript{1} δ binding sites of the fetal/(adult) muscle acetylcholine receptor because of different contributions by the γ/ε and δ subunits where a minimum of four loops in both subunits is required to create the agonist binding site (71).

TABLE 1

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<th>NMR structure determination statistics of α-conotoxin Lo1a for an ensemble of 15 structures</th>
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<td>The values where applicable are the means ± S.D.</td>
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