



RegIIA: An $\alpha 4/7$ -conotoxin from the venom of *Conus regius* that potently blocks $\alpha 3\beta 4$ nAChRs

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) play pivotal roles in the central and peripheral nervous systems. They are implicated in disease states such as Parkinson's disease and schizophrenia, as well as addictive processes for nicotine and other drugs of abuse. Modulation of specific nAChRs is essential to understand their role in the CNS. α -Conotoxins, disulfide-constrained peptides isolated from the venom of cone snails, potently inhibit nAChRs. Their selectivity varies markedly depending upon the specific nAChR subtype/ α -conotoxin pair under consideration. Thus, α -conotoxins are excellent probes to evaluate the functional roles of nAChRs subtypes.

We isolated an $\alpha 4/7$ -conotoxin (RegIIA) from the venom of *Conus regius*. Its sequence was determined by Edman degradation and confirmed by sequencing the cDNA of the protein precursor. RegIIA was synthesized using solid phase methods and native and synthetic RegIIA were functionally tested using two-electrode voltage clamp recording on nAChRs expressed in *Xenopus laevis* oocytes. RegIIA is among the most potent antagonist of the $\alpha 3\beta 4$ nAChRs found to date and is also active at $\alpha 3\beta 2$ and $\alpha 7$ nAChRs. The 3D structure of RegIIA reveals the typical folding of most $\alpha 4/7$ -conotoxins. Thus, while structurally related to other $\alpha 4/7$ conotoxins, RegIIA has an exquisite balance of shape, charge, and polarity exposed in its structure to potently block the $\alpha 3\beta 4$ nAChRs.

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1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) play an important role in the central and peripheral nervous system and are implicated in certain disease states including Parkinson's disease, schizophrenia, depression, Alzheimer's disease, and nicotine addiction [1]. These receptors are nicotine-sensitive ligand gated ion channels that are endogenously activated by acetylcholine. Structurally, most neuronal nAChRs are heteropentamers of $\alpha 2-6$ and $\beta 2-4$ subunits combined in different

stoichiometries [2]. There are homomeric nAChRs, notable among these is the $\alpha 7$ nAChR, and heteromeric receptors that are composed of only alpha subunits such as $\alpha 9\alpha 10$ nAChRs.

The specific combinations of α and β subunits mediate the diverse population of neuronal nAChRs subtypes with different subtypes having a specific function and distribution in the central and peripheral nervous system. $\alpha 4\beta 2$ nAChRs are the predominant subtype in the brain, they have the highest affinity for nicotine ($K_i = 0.6-10$ nM) and account for >90% of binding of nicotine in brain tissues [3]. Transgenic knockout of $\alpha 4$ or $\beta 2$ subunits eliminate nicotine self-administration in mice. Re-instatement of these subunits in the knockout restores nicotine self-administration, implicating this receptor in nicotine addiction [4].

Other nAChR subtypes, particularly the $\alpha 3\beta 4$, can also be involved in addiction of nicotine and other drugs of abuse [4]. The $\alpha 3\beta 4$ is the predominant nAChR in the sensory and autonomic ganglia and in subpopulations of CNS neurons, such as medial habenula and dorsal medulla [2]. This receptor is involved in the mesolimbic dopamine pathway and is thought to be important in certain feedback rewarding effects under a substance abuse

Abbreviations: AChBP, acetylcholine-binding protein; CNS, central nervous system; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EST, expressed sequence tag; GABA_BR, γ -aminobutyric acid type B receptor; IAM, iodoacetamide; nAChR, nicotinic acetylcholine receptor; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SCUBA, self-contained underwater breathing apparatus; SE-HPLC, size exclusion HPLC; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid.

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regimen. Furthermore, nicotine-induced hypolocomotion is reduced in $\beta 4$ null mice, thus emphasizing the importance of $\alpha 3\beta 4$ in the nicotine related effects in the CNS [5]. While the involvement of $\alpha 3\beta 4$ receptors in psychostimulant and drug-abusive behavior has been established [6], the lack of adequate molecular probes that allow exploring the neurophysiology of these receptors is a limiting factor for establishing their precise role in addiction.

α -Conotoxins, ubiquitous compounds found in the venoms of cone snails [7], are short disulfide-constrained peptides that target various nAChR subtypes. α -Conotoxin sequences have four cysteines arranged in CC- X_n -C- Y_m -C pattern, where X_n is a loop of amino acids with $n = 3$ –4 and Y_m is a loop of amino acids with $m = 3$ –7. The sizes of the loops are used for α -conotoxin classification (i.e., an $\alpha 4/7$ -conotoxin has 4 residues in the X loop and 7 residues in the Y loop). The number and nature of the amino acids in these loops are defining for the binding and selectivity of α -conotoxins towards nAChRs subtypes. The sequences of α -conotoxins are species-specific, therefore, the discovery of new α -conotoxins can provide new tools for the functional exploration of nAChR subtypes. In general, α -conotoxins target more than one nAChR subtype; however, their selectivity and potency can vary widely. Here we describe the discovery, together with the biochemical, biophysical and functional characterization of RegIIA, an $\alpha 4/7$ -conotoxin isolated from the venom of *Conus regius*, a worm-hunting cone snail species that inhabits the Western Atlantic Ocean. RegIIA is among the most potent antagonist of $\alpha 3\beta 4$ nAChRs to date, and it does not inhibit the $\alpha 4\beta 2$ subtype. This selectivity profile makes RegIIA a prospective probe for studying nicotine addiction processes. RegIIA has a classical α -conotoxin globular structure (ω -shaped fold) indicating that it has an exquisite balance of shape, charges, and polarity exposed on its surface to enable it to potentially block the $\alpha 3\beta 4$ nAChR.

2. Methods and materials

2.1. Specimen collection, RegIIA isolation and characterization

Specimens of *C. regius* (35–70 mm in length) were collected off the Florida Keys (Plantation Key), USA, using SCUBA at depths ranging from 2 to 10 m. Venom ducts dissected from specimens of *C. regius* were homogenized in 0.1% TFA (Fisher Scientific, PA) at 4 °C. Whole extracts were centrifuged at $10,000 \times g$ for 20 min, at 4 °C, and the resulting pellets were washed three times with 0.1% TFA and re-centrifuged under identical conditions. The supernatants containing the soluble peptides were pooled, lyophilized, and stored at -80 °C until further use. Batches of 50 mg of crude venom were separated by SE-HPLC on a Pharmacia Superdex-30 column ($2.5 \text{ cm} \times 100 \text{ cm}$) equilibrated and eluted with 0.1 M NH_4HCO_3 (Fisher Scientific, PA). Further separation of fractions obtained from the Superdex-30 column was performed on a Superdex Peptide (Amersham Biosciences, MA) column ($10 \text{ mm} \times 300 \text{ mm}$) equilibrated and eluted with 0.1 M NH_4HCO_3 . Chromatographic fractions were monitored at $\lambda = 220, 250$, and 280 nm . Additional purification of peptide-containing peaks was achieved by RP-HPLC on a C18 semipreparative column (Vydac, 218TP510, $10 \text{ mm} \times 250 \text{ mm}$; $5 \mu\text{m}$ particle diameter; 300 \AA pore size). Further peptide purification was carried out by re-chromatographing fractions on an analytical C18 column (Vydac, 238TP54, $4.6 \text{ mm} \times 250 \text{ mm}$; $5 \mu\text{m}$ particle diameter; 300 \AA pore size). For semipreparative and analytical RP-HPLC separation, the buffers were 0.1% TFA (buffer A) and 0.1% TFA in 60% acetonitrile (Fisher Scientific, PA) (buffer B). Peptides were eluted with an incremental linear gradient of 1% B/min. All HPLC fractions were manually collected, lyophilized and kept at -40 °C prior to further use.

Reduction and alkylation of cystine groups were carried out as previously described [8] with slight modifications. An aliquot of

each peptide ($\sim 1 \text{ pmol}$) was dried, re-dissolved in 0.1 M Tris-HCl (Fisher Scientific, PA) (pH 8.2), 5 mM EDTA (Fisher Scientific, PA), 0.1% sodium azide and reduced with 6 mM DTT (Fisher Scientific, PA). Following incubation at 60 °C for 30 min, peptides were alkylated in a final volume of $15 \mu\text{l}$ with 20 mM IAM (Sigma-Aldrich, MO) and $2 \mu\text{l}$ of NH_4OH (Fisher Scientific, PA) (pH 10.5) at room temperature for 1 h in the dark. The reduced and alkylated peptides were purified using a Zip Tip (C18, size P10, Millipore, MA). Alkylated peptides were adsorbed onto Biobrene-treated glass fiber filters and amino acid sequencing was carried out by Edman degradation using an Applied Biosystems Procise model 491A Sequencer. Positive ion MALDI-TOF mass spectrometry was carried out on an Applied Biosystems Voyager-DE STR spectrometer. Samples were dissolved in 0.1% TFA, 50% acetonitrile, and applied on α -cyano-4-hydroxycinnamic acid matrix. Amidation of the C-terminus was determined by the difference between the calculated and experimental molecular weight and confirmed by nanoNMR spectroscopy.

2.2. Cloning and sequencing of the precursor of RegIIA

One venom duct from *C. regius* was removed from the freshly sacrificed animal on dry ice and stored at -70 °C. Approximately 1 cm of the duct was used for mRNA isolation with the Dynabeads[®] mRNA DIRECT[™] Kit (Invitrogen, CA). First strand cDNA synthesis was performed as described previously [9]. 3'-RACE PCR was performed using a 5' forward primer (5'-ATG GGC ATG CGG ATG ATG TTC-3') binding on the conserved signal sequence of the A-superfamily [10], the reverse primer was a shorter version of the adapter primer without the poly dT tail as reverse primer. The PCR conditions consisted of an initial denaturation of 94 °C for 2 min followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. 3'-RACE PCR products were ligated into the T-tailed plasmid vector pGEM[®]-T Easy (Promega, WI). The ligation products were transformed into NEB 5-alpha competent *E. coli*. Transformed colonies were screened using blue white selection. Plasmid DNA of 20 clones, containing an insert of the expected size, was isolated using the QIAprep Spin Miniprep kit (Qiagen, MD) and both strands of the insert were sequenced. Nucleotide sequences were analyzed using Bioedit and MEGA 4 [11]. The cDNA sequence of the here described conotoxin precursor has been deposited in the DDBJ/EMBL/GenBank Nucleotide Sequence Database under the accession number FR871900.

2.3. Peptide synthesis

The peptide was assembled by manual Boc-SPPS using HBTU-mediated (Buch, Switzerland) *in situ* neutralization protocol with *N,N'*-dimethylformamide (Auspep, Melbourne, Australia) as solvent [12]. HF (Matheson, TX) deprotection and cleavage was performed by treatment of the dried peptide resin (300 mg) with 10 mL HF/*p*-cresol/*p*-thio-cresol (Aldrich-Sigma, MO) (10:0.5:0.5, v/v/v) for 2 h at 0 °C. Following evaporation of the HF, the peptide was precipitated and washed with cold ether, filtered, and re-dissolved in 30 mL of 50% ACN/0.05% TFA and lyophilized. Oxidative folding was carried out in 0.1 M NH_4HCO_3 (pH 8.2, $c = 0.1 \mu\text{M}$) resulting in formation of the globular isomer of RegIIA. The oxidation was monitored by RP-HPLC, LC-MS and MS, and the peptide isomers were isolated using preparative C18 RP-HPLC.

2.4. Electrophysiological recordings from exogenously expressed nAChRs in *Xenopus* oocytes

RNA preparation, oocyte preparation, and expression of nAChR subunits in *Xenopus* oocytes were performed as described

previously [13]. Briefly, plasmids with cDNA encoding the rat $\alpha 3$, $\alpha 4$, $\alpha 10$, $\beta 2$, $\beta 4$, $\alpha 1$, $\beta 1$, γ , δ nAChR and human $\alpha 7$, $\alpha 9$ subunits cloned into the oocyte expression vector pNKS2 were used for mRNA preparation using mMESSAGE mMACHINE Kit (Ambion Inc., USA). All oocytes were injected with 5 ng of cRNA, and then kept at 18 °C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, at pH 7.4) supplemented with 50 µg/liter gentamycin (Sigma–Aldrich, MO) and 5 mM pyruvic acid (Sigma–Aldrich, MO) 2–5 days before recording. Membrane currents were recorded from *Xenopus* oocytes using either a standard electrophysiology voltage clamp setup [14] for native RegIIA or an automated workstation with eight channels in parallel for synthetic RegIIA, which included a compound delivery system with on-line analysis (OpusXpress™ 6000A workstation, Molecular Devices, Sunnyvale, CA) and a Gene-Clamp 500B amplifier (Molecular Devices) used in single channel voltage-clamp setup. The voltage-recording and current-injecting electrodes were pulled from borosilicate glass (GC150T-15, Harvard Apparatus Ltd.) and had resistances of 0.3–1.5 MΩ when filled with 3 M KCl. All recordings were conducted at room temperature (20–23 °C) using a bath solution of ND96 as described above. During recordings, the oocytes were perfused

continuously at a rate of 1.5 ml/min, with 300 s incubation time for the conotoxin. Acetylcholine (Sigma–Aldrich, MO) (200 µM for $\alpha 7$, 30 µM for all other nAChR subtypes) was applied for 2 s at 5 ml/min, with 360 s washout periods between applications. Cells were voltage-clamped at a holding potential of −80 mV. Data were sampled at 500 Hz and filtered at 5 Hz. Peak current amplitude was measured before and following incubation of the toxin peptide [15]. Concentration–response curves for antagonists were fitted by unweighted nonlinear regression to the logistic equation

$$E_x = \frac{E_{\max} X^{nH}}{X^{nH} + IC_{50}^{nH}}$$

where E_x is the response, X is the antagonist concentration, E_{\max} is the maximal response, nH is the slope factor, and IC_{50} is the concentration of antagonist that gives 50% inhibition of the agonist response. All electrophysiological data were pooled ($n = 4–8$ for each data point) and represent arithmetic means \pm standard error of the fit. The data was processed using SigmaPlot 11.0 (Systat Software, Point Richmond, CA).

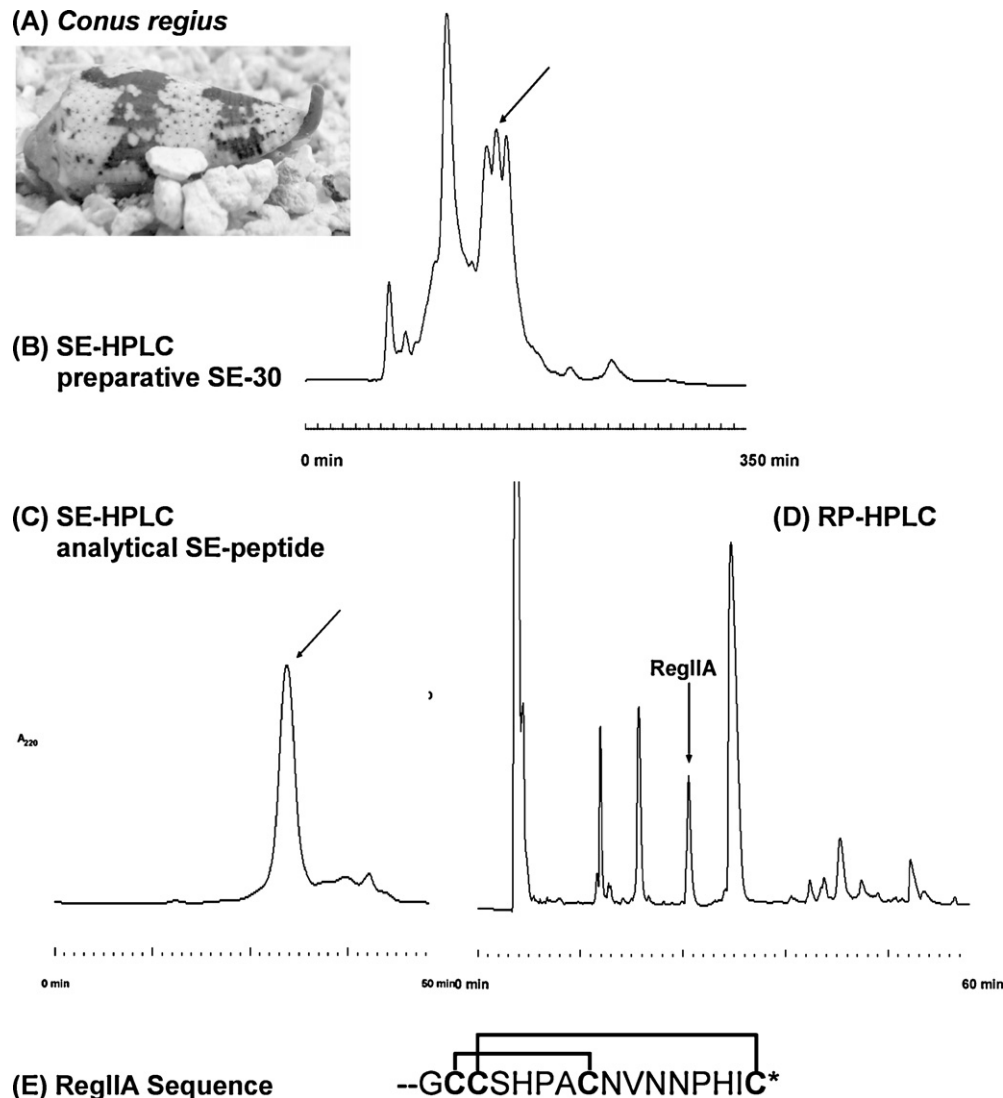


Fig. 1. Isolation of RegIIA from the venom of *C. regius*. (A) Live specimen of *C. regius*. (B) Venom separation by SE HPLC (Superdex 30) showing the absorbance profile at $\lambda = 220$ nm. The fraction containing RegIIA is indicated with an arrow and it was further purified using analytical SE HPLC (Superdex Peptide) (C). (D) UV trace of the analytical RP-HPLC separation. The RegIIA fraction is labeled with an arrow. (E) Amino acid sequence of RegIIA obtained by Edman degradation.

2.5. NMR spectroscopy and structure determination of RegIIA

NMR spectra of native RegIIA were acquired on a Varian Inova 500 MHz instrument as previously described [16]. Spectra of the native conotoxin were recorded at pH 3.60 and recorded at different temperatures (0, 10 and 25 °C). Spectra of synthetic RegIIA were recorded at 600 MHz (Bruker Avance NMR spectrometer) on a 0.5 mM solution of RegIIA in 10% D₂O/90% H₂O. The two-dimensional spectra were recorded and three-dimensional structures calculated as previously described [17]. Distance restraints were obtained from a NOESY spectrum recorded with a 200 ms mixing time at 17 °C.

3. Results

3.1. Discovery, characterization and synthesis of RegIIA

Fractionation of the venom of *C. regius* (SE and RP-HPLC) produced a pure peptide with a monoisotopic molecular mass of 1664.9 Da (Fig. 1). The amino acid sequence of the peptide (RegIIA) was obtained by Edman degradation (Fig. 1, Table 1), and corresponds to a 16-residue/2-disulfide bond hydrophilic conopeptide without aromatic residues, which has a sequence homology to other α 4/7-conotoxins (Table 1). RegIIA was synthesized with all L-amino acids. NMR comparison of the native material with the synthetic one confirmed the identity of RegIIA, including its amidated C-terminus.

3.2. Cloning and sequence analysis of the cDNA of the precursor of RegIIA

The cDNA sequence precursor of RegIIA is shown in Table 2. This precursor encodes 65 amino acids and has a 22-residue signal sequence. A sequence similarity search [18] detected homologies with the precursors of A-superfamily of conotoxins [19] (Table 2). As found in the isolated peptide, the precursor of RegIIA predicts the mature peptide to be C-terminally amidated,

since Gly is present at the loci preceding the C-terminal cleavage site [20].

3.3. Selective inhibition of α -conotoxin RegIIA of recombinant nAChR subtypes

The selectivity of RegIIA was examined by the inhibition of ACh-evoked currents mediated by various nAChRs subtypes expressed in *Xenopus* oocytes. ACh was applied at an interval of 5 min and the corresponding membrane currents were assessed. The peptide was applied to the bath 5 min prior to co-application of ACh plus peptide. Since RegIIA is homologous to several α 3 β 2 blocking conotoxins, we initially used native RegIIA against that nAChR subtype (Fig. 2A) resulting in >80% inhibition with a 100 nM solution of the native peptide. The limited amounts of peptide isolated precluded a selectivity profile of nAChR subtypes. Synthetic RegIIA (1 μ M) exhibited complete inhibition of ACh-evoked current amplitude produced by α 3 β 4, α 3 β 2, and α 7 nAChRs. However, RegIIA did not inhibit either muscle (α 3 β 2) or α 4 β 2 nAChRs ($n = 4-5$) (Fig. 3). RegIIA (1 μ M) inhibited only 20 \pm 5% of ACh-evoked current amplitude of α 9 α 10 nAChR. Concentration–response curves of RegIIA display the order of selectivity and their corresponding IC₅₀ values for α 3 β 2 (33 \pm 4 nM) > α 3 β 4 (97 \pm 20 nM) > α 7 (103 \pm 23 nM) (Fig. 3 and Table 3).

3.4. NMR spectroscopy of native and synthetic RegIIA

We were able to obtain ¹H NMR (1D and 2D-TOCSY) of nanomolar quantities (nanoNMR) of the RegIIA conotoxin directly isolated from the venom of the cone snails. Comparison of the 2D-TOCSY spectra of native RegIIA with its synthetic counterpart, confirmed the proper synthesis and folding of synthetic material. The 2D NOESY of synthetic RegIIA showed spectral quality suitable for structural determination. ¹H resonance assignments of RegIIA were achieved with the combined use of the TOCSY and NOESY spectra following the procedure outlined by Wuthrich [21]. Several unique spin systems within the sequence of RegIIA, Ser-4, Ala-7,

Table 1
Sequences of α -conotoxins related to RegIIA.

α -conotoxin	Sequence	Species	Prey	Target ⁺	Ref
RegIIA	GCCSHPA CNVNNPHI C*	<i>C. regius</i>	(v)	nAChRs	
OmIA	GCCSHPA CNVNNPHI CG*	<i>C. omaria</i>	(m)	nAChRs, AChBP	[28]
GIC	GCCSHPA CAGNNQHI C*	<i>C. geographus</i>	(p)	nAChRs	[29]
Mr1.1	GCCSHPA CSVNNPDI C*	<i>C. marmoreus</i>	(m)	nAChRs	[31]
PeIA	GCCSHPA CSVNHPDL C*	<i>C. pergrandis</i>	(ND)	nAChRs, GABA _B R	[45]
AnIB	GGCCSHPA CAANNQDY C*	<i>C. anemone</i>	(v)	nAChRs	[32]
GID	IRDYCCSNPA CRVNNOHVC	<i>C. geographus</i>	(p)	nAChRs	[30]
MII	GCCSNPV CHLEHSNL C*	<i>C. magus</i>	(p)	nAChRs	[14]
PIA	RDPCCSNPV CTVHNPDQI C*	<i>C. purpurascens</i>	(p)	nAChRs	[41]
Vc1.1	GCCSDPR CNYDHPEI C*	<i>C. victoriae</i>	(m)	nAChRs, GABA _B R	[45]
EpI	GCCSDPR CNMNNPDY C*	<i>C. episcopatus</i>	(m)	nAChRs	[33]
AuIB	GCCSYPP CFATNPD -C*	<i>C. aulicus</i>	(m)	GABA _B R	[39]
PnIA	GCCSLPP CAANNPDY C*	<i>C. pennaceus</i>	(m)	nAChRs, AChBP	[34]

Amino acid conservations are denoted by light grey shade. Dark grey shaded amino acids are homologous within that loci of the set. The framework I scaffold formed by disulfide bonded cysteins are bold faced and boxed. ⁺ Target criteria: IC₅₀ or K_i < 100 nM. See Table 3 for selectivity towards nAChRs subtypes. γ , γ -carboxyglutamate; O, 4-hydroxyproline; Y, sulfotyrosine; *, amidated C-terminal; ND, not determined.

Table 2

Sequence of the signal and prepro regions of the precursor of RegIIA and related A-superfamily α -conotoxins precursors. Alignment was performed using Clustal 2.1 by means of local pairwise alignment information [50].

Precursor to	Signal sequence	Prepro region	Ref.
RegIIA	MGMRMMFTVFLLVVLTTTVVS TS —VRASDGRNAAADNRASDLIAQIV RR —		
Mr1.1	MGMRMMFTVF—LVVLATTVV TS D—RASDGRKAAAKDKASDLVALTV K —		[31]
MII	MGMRMMFTVFLLVVLATTVV S FPS—DRASDGRNAAANDKASDVITLAL K —		[19]
Vc1.1	MGMRMMFTVFLLVVLATTVV S TSGRREFRGRNAAAK—ASDLVSLTD KKR		[37]
PnIA	MGMRMMFTVFLLVVLATTVV S TS—DRASDDGNAAAS—DLIALTI K —		[60]
LtIA	MGMRMMFIMFLVLVLATTVV T FTS—DRALDAMNAAASNKASRLIALAV R —		[54]
	***** : * ***** : ***** .. * : *** . : : :		

Dashes denote gaps. Amino acid conservations are denoted by light grey shade segments and an asterisk (*), whereas colons (:) and periods (.) represent a high and low degree of similarity respectively. Dark grey shaded amino acids are homologous within loci of the set. Bold-faced amino acids are points of cleavage for the release of the signal peptide or the mature α -conotoxin. See Table 1 for the sequences of corresponding mature α -conotoxins.

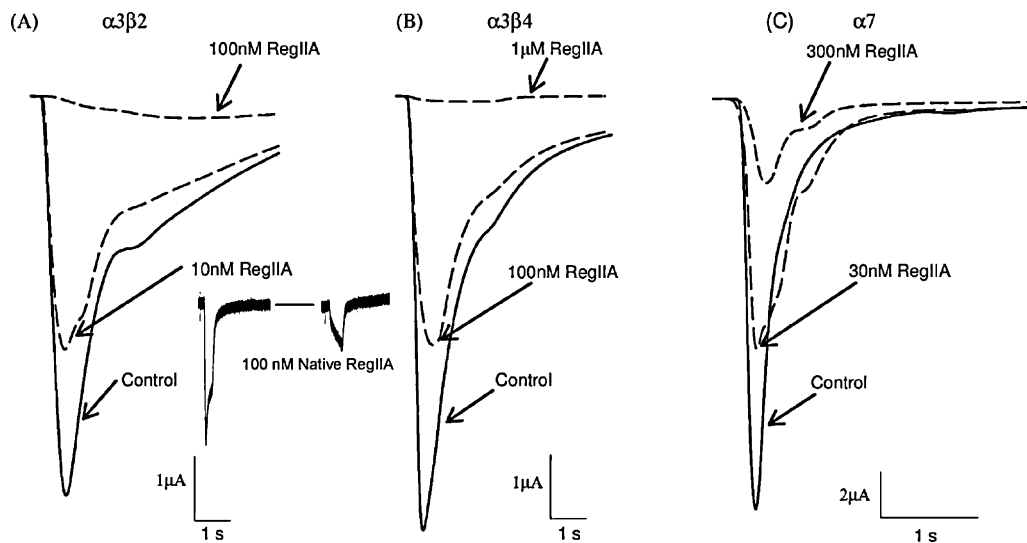


Fig. 2. Concentration-dependent inhibition of ACh-evoked current amplitude by RegIIA (synthetic). Representative superimposed ACh-evoked currents recorded in the absence and presence of RegIIA at $\alpha 3\beta 2$ (A), $\alpha 3\beta 4$ (B), and $\alpha 7$ (C) nAChR subunits expressed in *Xenopus* oocytes. Inset in (A) are current responses of *Xenopus* oocytes expressing $\alpha 3\beta 2$ nAChRs to 70 μ M ACh before and after 5 min incubation with 100 nM native RegIIA (scale: $y = 100$ nA, $x = 20$ s).

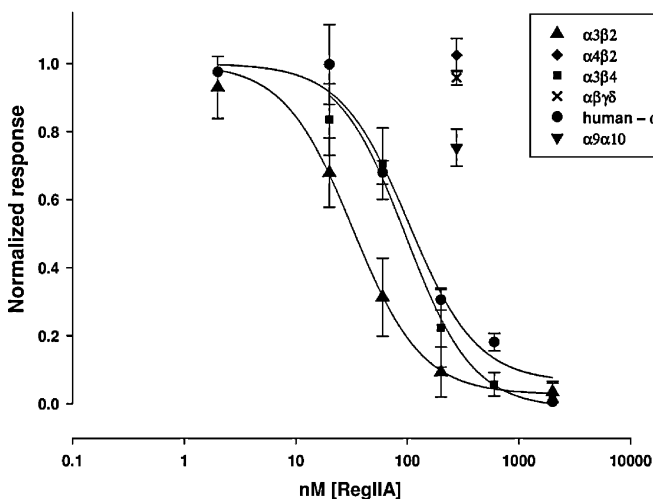


Fig. 3. Selectivity of RegIIA inhibition of nAChR subtypes. 1 μ M RegIIA completely inhibited $\alpha 3\beta 2$, $\alpha 3\beta 4$ and human $\alpha 7$ receptors and inhibited $\alpha 9\alpha 10$ receptors by 22%. Concentration–response curves obtained for RegIIA inhibition gave IC_{50} 's of 33 nM for $\alpha 3\beta 2$ (▲; $n = 5-8$), 97 nM for $\alpha 3\beta 4$ (■; $n = 5$) and 103 nM for human $\alpha 7$ (●; $n = 5-8$).

Val-10, and Ile-15, were readily identified in the 2D-TOCSY, thus facilitating the sequence-specific assignment process. Several HN_i to HN_{i+1} correlations along with αH_i to HN_{i+1} in the fingerprint region of the 2D-NOESY completed the assignments.

The secondary shift plot [22] of RegIIA (Fig. 4A) has negative values for the Pro⁶ to Val¹⁰ segment, indicative of a helical structure. A comparison of the secondary shifts with Vc1.1 [23] and MII [24] indicate similar folds for all three peptides.

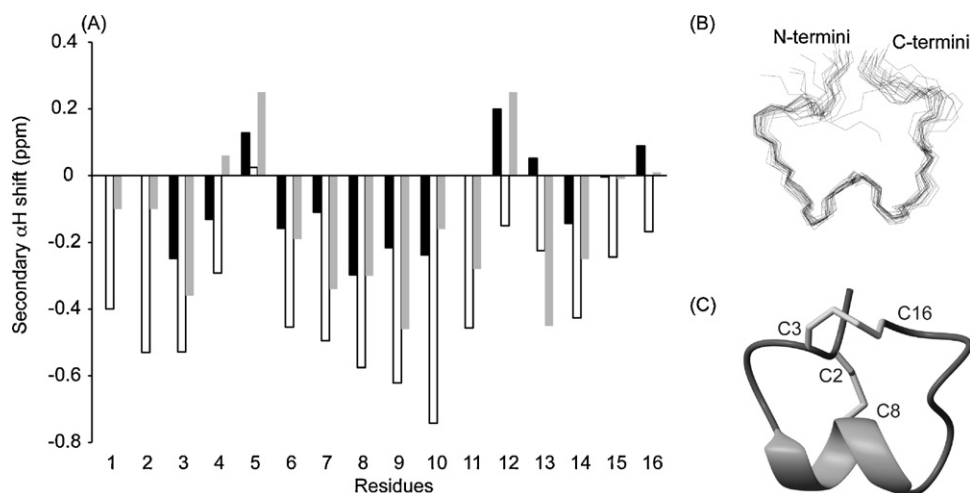
3.5. 3D structure of RegIIA

The three-dimensional structures of RegIIA were calculated with 60 NOE-derived distance restraints and 19 dihedral-derived constraints. A set of 50 structures was calculated using CNS [25] and the 20 lowest energy structures chosen to represent the structure of RegIIA. These structures were overlaid over residues Cys³–Pro¹³, as the N- and C-terminal ends are more disordered, with a backbone RMSD of 0.6 ± 0.2 Å (Fig. 4B). A Promotif evaluation of the lowest energy structures indicated that the major element of secondary structure is a helix between residues 6–9 consistent with the chemical shift analysis. The overall fold of RegIIA is highly compact; the location of the disulfide bonds keeps the structure together in a way that the N and C termini are restricted to close

Table 3IC₅₀ values (nM) of RegIIA ($n \geq 5$) and related α -conotoxins for nAChR subtypes.

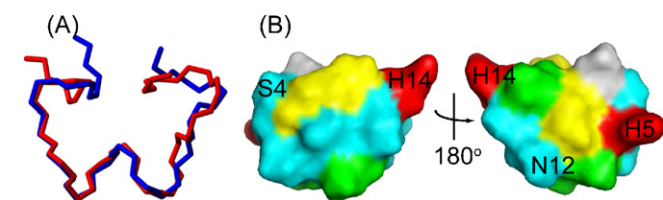
nAChR/ α -conotoxin	$\alpha 3\beta 4$	$\alpha 3\beta 2$	$\alpha 7$	$\alpha 4\beta 2$	$\alpha 9\alpha 10$	Ref.
RegIIA	97 \pm 20	33 \pm 4	103 \pm 23	>1000	>1000	This work
OmlA	>10,000	11	27	>10,000	ND	[28]
GIC	755	1.1	ND	309	ND	[29]
PelA	480	23	1800	>10,000	6.9	[45]
AnIB	~1000	0.3	76	~1000	ND	[32]
GID	>10,000	3.1	4.5	152	ND	[30]
MII	>1000	0.5	~200	>1000	>1000	[14]
PIA	518	74.2	ND	>10,000	ND	[41]
Vc1.1	4200	7300	>30,000	>30,000	109	[45]
AulB	770	>10,000	>10,000	>10,000	>1000	[39]
PnlA	>1000	9.6	252	>1000	ND	[34]

ND, not determined.

**Fig. 4.** Structure determination of RegIIA. (A) Secondary shift analysis of RegIIA (black bars), Vc1.1 (white bars) and MII (grey bars). (B) Overlay of the 20-lowest energy structures of RegIIA. (C) Ribbon representation of the lowest energy structure of RegIIA with the helical structure shown as a thickened ribbon and the disulfide bonds shown in stick format.

proximity, whereas the Cys³–Cys⁸ bridge forces the middle of the main chain into close proximity with the C-terminus. The rigidity of the system is completed with Cys²–Cys¹⁶, which keeps the compactness of the scaffold. The compact fold of the structure forced the main chain into several turns to fit the covalent structure requirements imposed by the disulfide bridges.

Fig. 5 shows an overlay of the average structures of RegIIA and OmlA [26]. There are striking similarities between the NMR structures of these two conotoxins. It is apparent from this comparison that these two conotoxins share the same fold of the main chain of most α -conotoxins, along with the covalent Cys framework. Therefore, the distribution of charged, polar, and hydrophobic residues in the surface of RegIIA may account for their differences in selectivity. Two views of the electrostatic surfaces of RegIIA are given in Fig. 5, highlighting the different faces of the molecule.

**Fig. 5.** Structure of RegIIA. (A) comparison of RegIIA (red) and OmlA (blue; PDB code 2GCZ). (B) Surface representation of the lowest energy structure of RegIIA. Cysteine residues are shown yellow, histidine in red, hydrophobic in green and polar residues in cyan.

4. Discussion

Here we describe the isolation and characterization of RegIIA, an $\alpha 4/7$ -conotoxin from the venom of a *C. regius*, a Western Atlantic worm-hunting cone snail species. This conotoxin, initially described as reg2a [27], has a sequence remarkably similar to OmlA [28], an α -conotoxin from *Conus omaria*, an Indo-Pacific mollusk-hunting species. These two conotoxins only differ in presence of an additional Gly residue at the C-terminal in OmlA. RegIIA has also significant sequence homology with GIC [29] and GID [30], which are α -conotoxins isolated from the venom of *Conus geographus*, an Indo-Pacific fish-hunting species. Given the homology to these α -conotoxins, we initially investigated the activity of native RegIIA on $\alpha 3\beta 2$ nAChRs, resulting in an estimated IC₅₀ of 50 nM, a common trait among the α -conotoxins shown in Table 1. However, RegIIA is a potent antagonist of the $\alpha 3\beta 4$ nAChR subtype.

RegIIA has a –SHPA– sequence in its first loop, which is shared with a selected group of α -conotoxins (Table 1). In the second loop RegIIA has a –NNP– motif, which is also shared by OmlA, Mr1.1 [31], AnIB [32], GID, Epl [33] and PnlA [34]. This Pro in the second loop is noteworthy, as cis/trans isomerism at this residue may play a role in the overall conformation of these peptides.

The sequence of the precursor of RegIIA contains a signal sequence that unequivocally places it in the A-superfamily of conotoxins. This signal sequence is essentially identical to other α -conotoxin precursors (Table 2). However, the prepro region of RegIIA shows sequence divergence among the panel of conotoxin precursors shown in Table 2. The prepro region has been

implicated in the secretory pathway of hydrophobic O-superfamily conotoxins [35]; however, its role in the folding and the posttranslational processing within the A-superfamily of conotoxins has not been studied extensively [36]. Other α -conotoxins isolated from *C. regius* show hydroxylation at the conserved Pro in loop one [27], a rare finding among A-superfamily conotoxins. Likewise, vc1a, the native α -conotoxin found the venom duct of *Conus victoriae* is also hydroxylated at that Pro [37]. Given that the most sequence divergence among these conotoxin precursors is at the prepro region, it is likely that this segment of the precursor is implicated in posttranslational processing. The prepro region binds to protein disulfide isomerase (PDI), which is a subunit of prolyl 4-hydroxylase (P4H), the enzyme catalyzing hydroxylation of proline residues [36]. The involvement of other proteins in the *in vivo* oxidative folding pathway of conotoxins precursors, such as PPI, can be expected [38]. In all these events, the prepro region appears to have a central role in directing the final conformation and level of modification of the mature toxin.

The ability of RegIIA to block $\alpha 3\beta 4$ nAChRs at low nanomolar levels sets it apart from other $\alpha 4/7$ -conotoxins. Weak inhibition of $\alpha 3\beta 4$ is observed for AulB [39], PeIA [40], GIC [29] and PIA [41] (Table 3). BulA, an unusual $\alpha 4/4$ -conotoxin discovered from the venom of *Conus bullatus*, also shows potent inhibition of $\alpha 3\beta 4$ nAChRs [42]. With the exception of AulB, these conotoxins inhibit other nAChR subtypes more potently. Despite its weak inhibition of $\alpha 3\beta 4$ nAChRs, AulB appears to be selective; however, it has been found that this α -conotoxin potently activates GABA_BR, and has been shown to produce analgesia by inhibiting Ca_v2.2 channels [43]. PeIA [44], Vc1.1 [45] and Vc1.2 [46] also share this trait, but not RegIIA (Table 1), which exhibited no effect on this GABA_BR mediated pathway (data not shown).

The selectivity profiles of α -conotoxins are known to change with single amino acid replacements [13,47–50]. This is the case with RegIIA, as its sequence differs only by one amino acid with OmIA, and three for PeIA. These differences translate into marked differences in their selectivity profiles. In addition to the unusual low nanomolar $\alpha 3\beta 4$ inhibition by RegIIA, this conotoxin can block $\alpha 7$ nAChRs, whereas PeIA does not. Conversely, PeIA potently inhibits $\alpha 9\alpha 10$ nAChRs and modulates Ca_v2.2 via GABA_BR, whereas RegIIA does not. Several scenarios can be envisioned to explain these changes in selectivity profiles with subtle differences in α -conotoxin sequences. These differences can have profound implications in the recognition of α -conotoxins by the receptor binding site. The delicate complementary shapes of the ligand and receptor can be easily upset by the presence of an additional amino acid or the lack of appropriate interactions between ligand and receptor, whether this occurs by fitting of the native structure of the ligand or receptor-induced conformation of the ligand to induce binding. The structures of α -conotoxins bound to AChBPs suggest the former as a more likely scenario [51]. However, there are some differences between the AChBP-bound structure of α -conotoxins and their unbound counterparts [52]. Furthermore, α -conotoxins such as lml can bind to nAChRs in their desensitized conformation, whereas others, such as PnIA, bind to nAChRs in their resting state [52]. All these considerations can contribute to the complex and delicate scenario that plays into the selectivity profiles of α -conotoxins towards nAChRs.

Conotoxin interactions with nAChRs can be further complicated by the existence of multiple microsites for conotoxins binding, i.e., α -conotoxins lml and lmlI bind to different microsites of $\alpha 7$ nAChRs, a switch that is caused by a three amino acid difference in the sequence of these related peptides [53]. Some nAChRs present a more complicated scenario, as multiple microsites for α -conotoxin binding to $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs have been proposed and rationalized using molecular models [54,55]. Further experiments on the binding of RegIIA to these nAChRs and their analogs

will be necessary to assess the precise binding mode of this peptide.

RegIIA is among the most potent inhibitor of $\alpha 3\beta 4$ nAChRs to date. This is of particular significance, as it does not inhibit the $\alpha 4\beta 2$ subtype (Table 3). This feature makes RegIIA a suitable probe to evaluate nicotine addiction processes. It has been shown that $\alpha 4\beta 2$ nAChRs are required for nicotine addiction and dependence [3], therefore, these receptors are actively pursued as targets for tobacco smoking cessation therapeutics [56]. However, other nAChR subtypes are also implicated in addiction to nicotine and other drugs of abuse. Particularly, antagonism of $\alpha 3\beta 4$ nAChRs has been proposed as a strategy to reduce self-administration of opiates and other stimulants [57]. The involvement of $\alpha 3\beta 4$ receptors in nicotine addiction processes has been demonstrated by a reduced nicotine-induced hypolocomotion in $\beta 4$ null mice [5]. However, further exploration of these findings has been hampered by the lack of tools to explore the role of $\alpha 3\beta 4$ nAChRs. The selectivity profile of RegIIA can provide advancement towards this end. However, as is the case for other related α -conotoxins, RegIIA also blocks $\alpha 7$ and $\alpha 3\beta 2$ nAChRs (Table 3). While structurally related to other α -conotoxins, RegIIA has an exquisite balance of shape, charges, and polarity exposed on its structure to potently block the $\alpha 3\beta 4$ nAChR subtype. Improving the selectivity of RegIIA towards $\alpha 3\beta 4$ nAChRs can be achieved by judicious design of RegIIA analogs (currently in progress). These studies can be valuable tools for further exploring ligand–receptor interactions and in the design of novel α -conotoxins with improved selectivity.

In summary, we have discovered an α -conotoxin with a unique selectivity profile, which includes low nanomolar blockage of $\alpha 3\beta 4$ nAChRs. This feature may lead to a better understanding of structural features for selectivity and function of nAChRs. Such selective profiles and potent blockage of $\alpha 3\beta 4$ nAChRs will be useful for testing the hypothesis that $\alpha 3\beta 4$ receptor antagonists can attenuate rewarding properties of nicotine and other drugs of abuse [57,58].

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