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# RegIIA: An α4/7-conotoxin from the venom of *Conus regius* that potently blocks α3β4 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 α4/7-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 α3β4 nAChRs found to date and is also active at α3β2 and α7 nAChRs. The 3D structure of RegIIA reveals the typical folding of most α4/7-conotoxins. Thus, while structurally related to other α4/7 conotoxins, RegIIA has an exquisite balance of shape, charge, and polarity exposed in its structure to potently block the α3β4 nAChRs.



## 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 hetero-pentamers of α2-6 and β2-4 subunits combined in different stoichiometries [2]. There are homomeric nAChRs, notable among these is the α7 nAChR, and heteromeric receptors that are composed of only alpha subunits such as α9α10 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. α4β2 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 α4 or β2 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 α3β4, can also be involved in addiction of nicotine and other drugs of abuse [4]. The α3β4 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 β4 null mice, thus emphasizing the importance of α3β4 in the nicotine related effects in the CNS [5]. While the involvement of α3β4 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 α4/7-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 α4/7-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 α3β4 nAChRs to date, and it does not inhibit the α4β2 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 potently block the α3β4 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 × *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 cm × 100 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 mm × 300 mm) equilibrated and eluted with 0.1 M $NH_4HCO_3$. Chromatographic fractions were monitored at λ = 220, 250, and 280 nm. Additional purification of peptide-containing peaks was achieved by RP-HPLC on a C18 semipreparative column (Vydac, 218TP510, 10 mm × 250 mm; 5 μm particle diameter; 300 Å pore size). Further peptide purification was carried out by re-chromatographing fractions on an analytical C18 column (Vydac, 238TP54, 4.6 mm × 250 mm; 5 μm particle diameter; 300 Å 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 (~1 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 μl with 20 mM IAM (Sigma–Aldrich, MO) and 2 μl 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 μM) 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