αS-Conotoxin RVIIIA: A Structurally Unique Conotoxin That Broadly Targets Nicotinic Acetylcholine Receptors†

Russell W. Teichert,*,‡,§ Elsie C. Jimenez,‡,§,|| and Baldomero M. Olivera‡

Department of Biology, University of Utah, Salt Lake City, Utah 84112, Department of Physical Sciences, College of Science, University of the Philippines Baguio, Baguio City, Philippines

Received December 29, 2004; Revised Manuscript Received March 1, 2005

ABSTRACT: We report the purification and characterization of a new conotoxin from the venom of *Conus radiatus*. The peptide, αS-conotoxin RVIIIA (αS-RVIIIA), is biochemically unique with respect to its amino acid sequence, post-translational modification, and molecular targets. In comparison to other nicotinic antagonists from *Conus* venoms, αS-RVIIIA exhibits an unusually broad targeting specificity for nicotinic acetylcholine receptor (nAChR) subtypes, as assayed by electrophysiology. The toxin is paralytic to mice and fish, consistent with its nearly irreversible block of the neuromuscular nAChR. Similar to other antagonists of certain neuronal nAChRs, the toxin also elicits seizures in mice upon intracranial injection. The only previously characterized conotoxin from the S superfamily, *σ*-conotoxin GVIIIA, is a specific competitive antagonist of the 5-HT_3 receptor; thus, αS-RVIIIA defines a novel family of nicotinic antagonists within the S superfamily. All previously characterized competitive conotoxin nAChR antagonists have been members of the A superfamily of conotoxins. Our working hypothesis is that the particular group of fish-hunting *Conus* species that includes *Conus radiatus* uses the αS-conotoxin family to target the muscle nAChR and paralyze prey.

The characterization of conotoxins has shown that the majority are small peptides typically 10−30 amino acids in length with multiple disulfide bonds that target ion channels (*1*). Most of the >50 000 different conotoxins belong to only a few gene superfamilies; conotoxin superfamilies are defined by a conserved signal sequence and a characteristic Cys pattern. The largest conotoxin characterized so far for which both the sequence and biological mechanism have been elucidated is *σ*-conotoxin GVIIIA (*σ*-GVIIIA) from *Conus geographus* venom (*2*), a 41-amino acid peptide that is an antagonist of the 5-HT_3 receptor; this peptide has a structural scaffold comprising 10 cysteine residues. *σ*-GVIIIA is the defining member of the S superfamily of *Conus* peptides; to date, no other peptide in the same superfamily has been characterized from any *Conus* venom.

In this report, we describe the purification and characterization of a novel peptide from the venom of *Conus radiatus* with the same Cys pattern as *σ*-GVIIIA but which diverges in its primary amino acid sequence, post-translational modification, and molecular targets. This peptide defines a new family of conotoxins within the S superfamily that we have named the αS family of conotoxins; the "α" designates a targeting selectivity for nicotinic acetylcholine receptors (nAChRs),[1] while the "S" indicates that the peptide belongs to the S-conotoxin gene superfamily. This peptide, designated αS-conotoxin RVIIIA (αS-RVIIIA) (R = *Conus radiatus*, VIII = class VIII Cys pattern) is a nearly irreversible inhibitor of the muscle nAChR. However, it also targets various neuronal nAChRs. This targeting specificity is broader than any polypeptide toxin previously characterized that is generally targeted to the nicotinic acetylcholine receptor family. The broad-spectrum targeting property of αS-RVIIIA was particularly surprising because the nicotinic antagonists previously characterized from *Conus* venoms were notable for their unprecedented subtype selectivity.

EXPERIMENTAL PROCEDURES

Purification of αS-RVIIIA by Reverse-Phase HPLC. A crude venom extract was prepared from *Conus radiatus* as described previously (*3*). The extract was applied into a Vydac C_{18} semipreparative column (10 × 250 mm, 5 μm particle size) and eluted at 5 mL/min with a linear gradient of solvent B_{90} [0.085% trifluoroacetic acid (TFA) in 90% acetonitrile]. Further purification was done on a Vydac C_{18} analytical column (4.6 × 250 mm, 5 μm particle size). The effluents were monitored at 220 nm, and aliquots of fractions were assayed for biological activity.

Mass Spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained at the Mass

† This work was supported by NIH Grant GM 48677, from the National Institute of General Medical Sciences.

* To whom correspondence should be addressed: Department of Biology, University of Utah, 257 South, 1400 East, Salt Lake City, UT 84112. Telephone: (801) 581-8370. Fax: (801) 585-5010. E-mail: teichert@biology.utah.edu.

‡ University of Utah.

§ These authors contributed equally to this work.

|| University of the Philippines Baguio.

[1] Abbreviations: α, α-conotoxin; ACh, acetylcholine; BSA, bovine serum albumin; CNS, central nervous system; Gla, γ-carboxyglutamate; GSH, reduced glutathione; GSSG, oxidized glutathione; i.c., intracranial; i.m., intramuscular; i.p., intraperitoneal; MALDI, matrix-assisted laser desorption ionization; nAChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi047274+ CCC: $30.25
Published on Web 05/07/2005

Spectrometry and Proteomic Core Facility of the University of Utah using a Voyager DE STR mass spectrometer.

Peptide Sequencing. The purified peptide was reduced and alkylated as described previously (*3*). The pH of the solution was adjusted to 7.5 by adding 0.5 M tris(hydroxymethyl)-aminomethane (Tris) base, and then dithiothreitol was added to a final concentration of 10 mM. The solution was flushed with argon, incubated at 65 °C for 20 min, and cooled to room temperature. 4-Vinylpyridine (2 μL) was added to the solution. The tube was wrapped in aluminum foil and allowed to sit for 25 min at room temperature. The solution was diluted with 0.1% TFA prior to application onto a Vydac C_{18} analytical column. The alkylated peptide was sequenced by automated Edman degradation (*4*) on an Applied Biosystem Model 492 Sequenator, courtesy of Dr. Robert Schackmann of the DNA/Peptide Facility, University of Utah. The 3-phenyl-2-hydantoin derivatives were identified by HPLC.

Peptide Synthesis. Linear αS-RVIIIA was synthesized by standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry using an ABI Model 430A Peptide Synthesizer at the University of Utah Core Facility. The peptide was folded either by air oxidation (0.1 M Tris-HCl at pH 7.0), oxidation in the presence of oxidized glutathione (0.1 mM GSSG and 0.1 M Tris-HCl at pH 7.5), or oxidation in the presence of a combination of oxidized and reduced glutathione (1 mM GSSG, 2 mM GSH, and 0.1 M Tris-HCl at pH 7.5). In each case, the oxidation was allowed to progress at room temperature over a time course from 15 min to 48 h. The oxidation products were sampled at multiple time points and separated by reverse-phase HPLC. In each case, several oxidation products were obtained.

Biological Assays. Toxin was delivered to mice (12−24-day-old) either by intracranial (i.c.) or intraperitoneal (i.p.) injection. Native purified αS-RVIIIA (20 μL) was dissolved in normal saline solution (NSS) and injected using a 29-gauge insulin syringe as described earlier (*5*). The mice were exposed to auditory stimulation by hitting the cage cover continuously immediately after injection. Goldfish were injected with peptide solution intramuscularly (i.m.) or i.p. Control mice and fish were similarly injected with NSS.

Electrophysiology. Capped RNA was prepared and injected into *Xenopus* oocytes as described previously (*6*). Oocytes were injected 1−2 days after harvesting and used for voltage clamp recording 2−6 days after injection. Voltage clamp recording was done as described previously (*6*). Briefly, oocytes were clamped at −70 mV with a two-electrode system and perfused with ND96 (96 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5 mM Hepes at pH 7.1−7.5) containing 1 μM atropine to block endogenous muscarinic acetylcholine receptors and containing 0.1 mg/mL bovine serum albumin (BSA) to reduce nonspecific adsorption of the toxin. Native purified αS-RVIIIA toxin was dissolved in ND96 buffer containing 0.5 mg/mL BSA prior to its application in a static bath. Acetylcholine (ACh)-gated currents were elicited by the following concentrations of ACh: 1 or 2 μM ACh for the human neuromuscular nAChR subtypes ($\alpha1\beta1\epsilon\tilde{\delta}$ or $\alpha1\beta1\gamma\delta$ subunits) and 100 μM ACh for all neuronal nAChR subtypes. ACh was applied at a frequency of once/min in most cases. In some cases, to avoid receptor desensitization, ACh was applied at a frequency of once/2 min. Serotonin was applied to oocytes expressing the 5-HT_3 receptor at a concentration of 10 μM once/min. Toxin was applied to a given oocyte in a static bath (30 μL volume). The peptide was allowed to equilibrate with the receptors expressed on the oocytes for 5−10 min prior to pulsing with ACh. Each toxin at a particular concentration was tested against each receptor at least twice to ensure reproducibility.

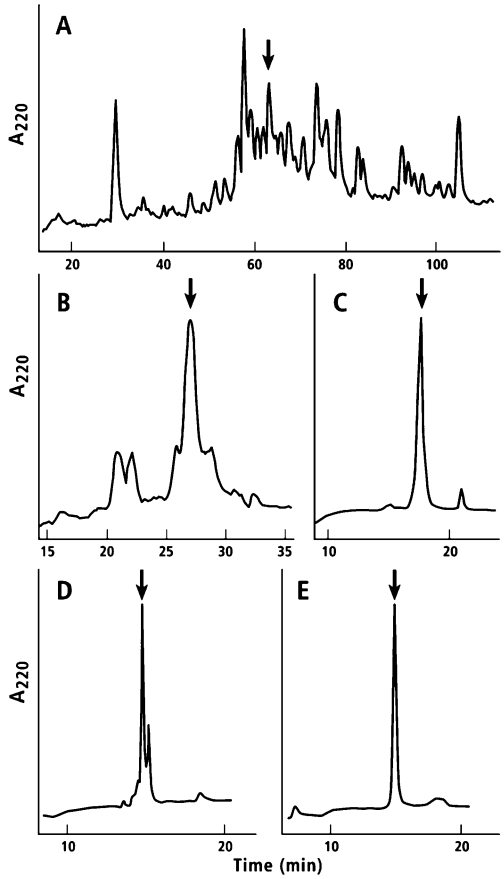

FIGURE 1: Purification of αS-RVIIIA by reverse-phase HPLC. (A) Fractionation of crude venom extract using a C_{18} semipreparative column eluted with a gradient of 0−60% solvent B_{90} (0.085% TFA in 90% acetonitrile) over 120 min at a flow rate of 5 mL/min. The solid arrow indicates the fraction containing αS-RVIIIA. (B) Elution of the fraction indicated by an arrow in A using a C_{18} analytical column at 20−35% solvent B_{90} over 45 min at a flow rate of 1 mL/min. (C) Elution of the bioactive fraction marked by an arrow in B with a gradient of 20−50% B_{90} over 30 min at 1 mL/min. (D) Further purification of the fraction with an arrow in C with a gradient of 20−60% B_{90} over 20 min at 1 mL/min. (E) Rerun of the fraction indicated by an arrow in D using the same gradient and flow rate.

RESULTS

Purification and Biochemical Characterization of αS-RVIIIA. A fraction of crude venom extract from *Conus radiatus* was identified that appeared to cause audiogenic seizures when injected i.c. in mice. This behavioral phenotype was used to follow the biologically active component of the venom during the purification procedure. The purification of the peptide, which involved venom extraction, and five sequential steps by reverse-phase HPLC is shown in Figure 1. The peptide, designated as αS-RVIIIA after its characterization, was purified to apparent homogeneity, and the highly purified peptide was characterized biochemically.

A standard Edman analysis revealed that the peptide had the following primary sequence, comprising of 47 amino acid residues: K**C**NFDK**C**KGTGVYN**C**G?S**C**S**C**?GLHS**C**R**C**-TYNIGSMKSG**C**A**C**I**C**TYY. Positions 17 and 22 showed low levels of glutamate, which is suggestive evidence for the presence of γ-carboxyglutamate (Gla) residues at those loci. Mass spectrometric analysis showed a mass of 5168.99, which is consistent with Gla residues at positions 17 and 22. The mass spectrometric analysis also showed a compound with a mass of 5080.93, indicating decarboxylation of γ-carboxyglutamate residues (calculated mass = 5082.8), a routine occurrence during MALDI mass spectrometry. Thus, the mass spectrometry established and confirmed the amino acid sequence determined directly. Sequence analysis of a cDNA encoding this peptide revealed a stop signal after Tyr[47], as well as the presence of glutamate codons corresponding to positions 17 and 22. Thus, these data are consistent with a 47-residue peptide with two Gla residues and a free carboxyl terminus. All of the data are consistent with the toxin having the following sequence: K**C**NFDK-**C**KGTGVYN**C**GγS**C**S**C**γGLHS**C**R**C**TYNIGSMKSG-**C**A**C**I**C**TYY (where γ = γ-carboxyglutamate).

Peptide Synthesis. The peptide αS-RVIIIA was synthesized and oxidatively folded as described in the Experimental Procedures. Multiple attempts, using various conditions, failed to produce a folded αS-RVIIIA product that coeluted with the native peptide by reverse-phase HPLC. Products of the oxidative folding that eluted near the retention time of native αS-RVIIIA were tested for activity by electrophysiology as described below under electrophysiological characterization. None of these products shared the activity of native αS-RVIIIA. Consequently, all subsequent characterization was performed with limited quantities of native peptide.

Biological Assays. An observation from i.c. injections into mice is that older mice (22−24 days old) were more sensitive to αS-RVIIIA than younger mice (12 and 17−18 days old). In 22−24-day-old mice, a dose of 1.0 nmol of peptide injected i.c. reproducibly elicited seizures followed by death. In 17−18-day-old mice, a dose of 1.0 nmol caused seizures but did not lead to lethality, while in 12-day-old mice, no seizures were observed when mice were injected with the same dose. Auditory stimulation had a profound effect on seizures in mice injected with αS-RVIIIA. When 22−24-day-old mice injected with 1.0 nmol of the peptide were subjected to auditory stimulation, seizures and death occurred within 2 min after injection. Without auditory stimulation, however, >20 min was required prior to the onset of seizures in the injected mice, with lethality occurring about 30 min after injection.

When injected i.p. in 13-day-old mice, αS-RVIIIA caused paralysis at a dose of ~1 nmol/g. When injected i.m. or i.p. in goldfish, 1 nmol/g of the peptide caused paralysis in ~5 min and led to lethality within 30 min after injection; 500 pmol/g of the peptide caused paralysis ~17 min after injection.

Electrophysiological Characterization. We tested αS-RVIIIA against a variety of ligand-gated ion channels by two-electrode voltage clamping *Xenopus* oocytes. A concentration of 1 μM αS-RVIIIA resulted in nearly complete inhibition of both the human adult ($\alpha1\beta1\epsilon\delta$ subunits) and human fetal ($\alpha1\beta1\gamma\delta$ subunits) neuromuscular nAChRs after allowing the toxin to equilibrate with receptors for 10 min. In each case, the inhibition was only very slowly reversible, as shown in Figure 2. Because of limited quantities of native toxin, a full dose−response curve was not generated. However, using native toxin, an approximate IC_{50} of 33 nM was obtained for αS-RVIIIA on the fetal muscle nAChR (Figure 2A), while an approximate IC_{50} of 100 nM was obtained for αS-RVIIIA on the adult muscle nAChR (data not shown) with a 10-min equilibration. These results are consistent with the bioassay data in which paralysis is induced in fish and mice by i.m. and i.p. injections.

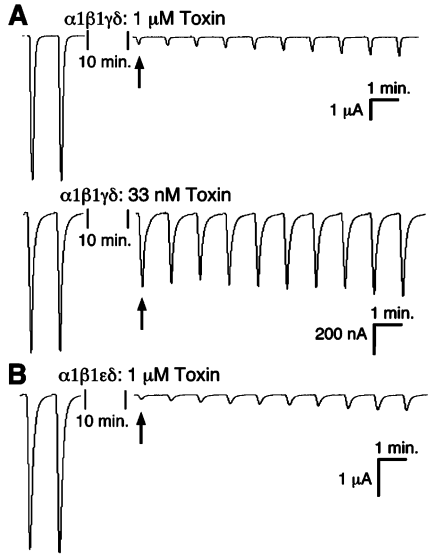

FIGURE 2: αS-RVIIIA was tested for a functional block of both the human adult ($\alpha1\beta1\epsilon\delta$ subunits) and fetal ($\alpha1\beta1\gamma\delta$ subunits) neuromuscular nAChRs by two-electrode voltage clamping *Xenopus* oocytes expressing the respective receptor clones. ND96 buffer was perfused over the oocytes, which were pulsed with ACh once/min. Toxin was applied in a static bath to the oocytes and allowed to equilibrate with receptors for 10 min prior to pulsing with ACh and resuming perfusion of ND96 buffer. In each case, the two traces to the left are control currents that were elicited by pulsing oocytes with ACh prior to application of toxin. Subsequent traces represent currents elicited by pulsing the oocytes with ACh once/min after the 10-min equilibration with toxin. The first trace after the 10-min equilibration is identified by an arrow in each case. (A, top) Concentration of 1 μM αS-RVIIIA blocked currents from the fetal muscle nAChR ($\alpha1\beta1\gamma\delta$ subunits) almost completely, and the toxin dissociated very slowly from the receptor. (A, bottom) Approximate IC_{50} value was obtained with a concentration of 33 nM αS-RVIIIA. (B) Concentration of 1 μM αS-RVIIIA blocked currents from the adult muscle nAChR ($\alpha1\beta1\epsilon\delta$ subunits) almost completely, and the toxin dissociated very slowly from the receptor.

αS-RVIIIA also blocked a number of neuronal nAChRs, with varying degrees of affinity. At a concentration of 1 μM, the toxin reproducibly blocked approximately 70% of elicited currents from oocytes expressing the human α7 nAChR; however, in contrast to the block of the neuromuscular nAChR, this inhibition was rapidly reversible (Figure 3A). αS-RVIIIA is a competitive antagonist of the α7 receptor. Preincubation of αS-RVIIIA with the α7 receptor blocked the slowly dissociating α-bungarotoxin binding to its ligand-binding site (Figure 4). A 10 μM concentration of αS-RVIIIA also blocked >50% of elicited currents from the human $\alpha3\beta2$ (Figure 3B) and rat $\alpha3\beta2$ (data not shown) nAChRs and additionally a chimeric rat $\alpha6/\alpha3\beta2\beta3$ nAChR (extracellular domain is α6) (Figure 5). Despite the relatively low affinity

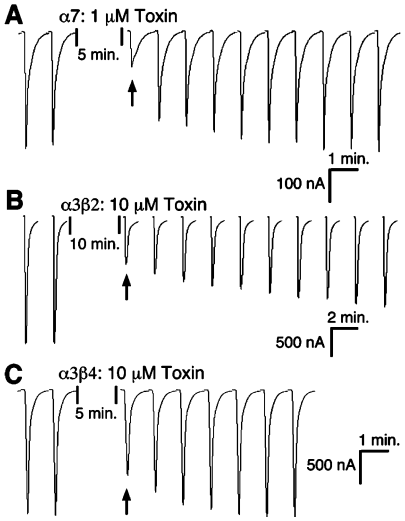

FIGURE 3: αS-RVIIIA was tested for a functional block of various neuronal nAChRs by two-electrode voltage clamping *Xenopus* oocytes expressing the respective receptor clones. ND96 buffer was perfused over the oocytes, which were pulsed with ACh once/min. Toxin was applied in a static bath to the oocytes and allowed to equilibrate with receptors for 5−10 min prior to pulsing with ACh and resuming perfusion of ND96 buffer. In each case, the two current traces to the left are control currents that were elicited by pulsing oocytes with ACh prior to application of the toxin. Subsequent traces represent currents elicited by pulsing the oocytes with ACh after the 5−10-min equilibration with toxin. The first trace after the equilibration is identified by an arrow in each case. (A) Concentration of 1 μM αS-RVIIIA blocked ~70% of ACh-elicited current from the human α7 nAChR after a 5-min equilibration. The block was rapidly reversible, in contrast to the block of the neuromuscular nAChRs (see Figure 2). (B) 10 μM concentration of αS-RVIIIA blocked ~60% of current from the human α3β2 nAChR after a 10-min equilibration. Toxin was allowed to equilibrate for 10 min because of the slow off-rate. The oocyte was pulsed with ACh once/2 min to avoid receptor desensitization. The block was more slowly reversible than the block of the α7 nAChR but more rapidly reversible than the block of the neuromuscular nAChRs. (C) 10 μM concentration of αS-RVIIIA blocked less than half of ACh-elicited currents from the human α3β4 nAChR.

of αS-RVIIIA for these receptors, the toxin blocked with slow reversibility both α3β2 (see Figure 3B) and α6/α3β2β3 (data not shown) nAChRs. The similar block and slow reversibility may be due to the high degree of sequence similarity between α3 and α6 subunits, in combination with the β2 subunits. At a 10 μM concentration, the toxin blocked 30% or less of elicited currents from the human α3β4 nAChR (Figures 3C and 5). Because α7 and α4β2 are the most abundant nAChRs in the CNS and both receptors have been implicated in cholinergic agonist/antagonist-induced seizures in mice (*7*−*10*), we also tested αS-RVIIIA against the human and mouse α4β2 nAChR expressed in oocytes. αS-RVIIIA (10 μM) blocked less than 25% of the elicited currents from the α4β2 nAChRs (Figure 5).

A 10 μM concentration of toxin blocked all nAChRs at least to a small degree (Figure 5). However, the toxin appears to be specific for nAChRs. At a concentration of 10 μM, αS-RVIIIA failed to block the mouse 5-HT_3 receptor or a *Caenorhabditis elegans* glutamate-gated chloride channel (Figure 5). The ability of αS-RVIIIA to elicit seizures (the CNS symptomatology used to purify the peptide) is presumably due to its ability to inhibit one or more of the neuronal nAChR subtypes in the CNS.

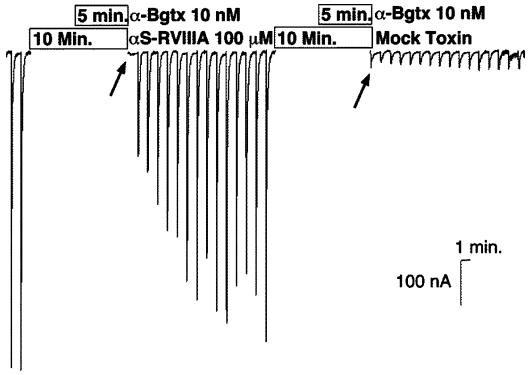

FIGURE 4: Oocytes expressing the human α7 nAChR were voltage-clamped. ND96 buffer was perfused over the oocytes, which were pulsed with ACh once/min. αS-RVIIIA was applied to an oocyte in a static bath at a concentration of 100 μM and allowed to equilibrate with receptors for 10 min. After 5 min, α-bungarotoxin was applied to the same static bath at a concentration of 10 nM. α-Bungarotoxin, which binds with near irreversibility to the α7 nAChR, was blocked from its binding site by αS-RVIIIA, which dissociates rapidly from the α7 nAChR. After the recovery from the block, a control was done using a mock toxin equilibration for 10 min, followed by a 5-min equilibration with 10 nM α-bungarotoxin, which caused a nearly irreversible block, as expected.

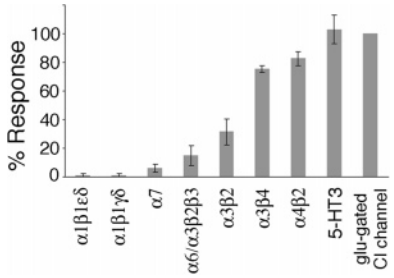

FIGURE 5: Variety of ligand-gated receptors were tested with αS-RVIIIA by expressing the receptor subunit clones in *Xenopus* oocytes and testing each receptor by two-electrode voltage clamping. In each case, toxin was applied to oocytes in a static bath at a concentration of 10 μM for a 5-min equilibration period prior to pulsing oocytes with ACh. The columns represent the mean ACh-elicited current response after toxin application as a percentage of the control response prior to toxin application. Error bars are SEM (n = 2 for each receptor). αS-RVIIIA at a 10 μM concentration inhibited all of the nAChRs to varying degrees. All responses to nAChRs shown are from human clones, with the exception of the α6/α3 chimera, which is from rat clones. The toxin failed to block the mouse 5-HT_3 receptor or a *C. elegans* glutamate-gated chloride channel.

DISCUSSION

The conopeptide characterized in this work, αS-conotoxin RVIIIA (αS-RVIIIA), is novel both with respect to its biochemistry and its targeting specificity. Although the peptide belongs to the same conotoxin superfamily as the previously characterized σ-GVIIIA (see Table 1 for amino acid sequence comparison with αS-RVIIIA) from *Conus geographus*, it differs in its primary amino acid sequence, in lacking the post-translational modification present in σ-GVIIIA, 6-bromotryptophan, and in its targeting specificity. A different, very distinctive post-translational modification was found, the carboxylation of Glu residues to γ-carboxyglutamate (Gla). Although σ-GVIIIA is shorter than αS-RVIIIA, there is a striking alignment of the last eight Cys residues (see Table 1). In particular, the CXC motif for Cys^4/Cys^5, Cys^6/Cys^7, Cys^8/Cys^9, and Cys^9/Cys^{10} is conserved, as well as the size of the large loop, - -$C(X)_{10}C$−

Table 1: Comparison of Amino Acid Sequences[a]

σ-conotoxin GVIIIA	G**C**TRT-**C**GGOK- - -**C**TGT**C**T**C**TNSSK**C**G**C**RYNVHPSG<u>W</u>G**C**G**C**A**C**S#
αS-conotoxin RVIIIA	K**C**NFDK**C**KGTGVYN**C**GγS**C**S**C**γGLHS**C**R**C**TYNIGSM<u>K</u>SG**C**A**C**I**C**TYY∧

[a] ∧, C-terminal free acid; #, C-terminal amidation; γ, γ-carboxyglutamate; <u>W</u>, 6-Br-tryptophan; O, 4-*trans*-hydroxyproline.

Table 2: Comparison of nAChR Antagonists from Fish-Hunting *Conus* Clades

	peptide	reference	superfamily	amino acid sequence[a]	nAChR subtype
Clade I[b]					
Conus magus	α-MI	*18*	A	GR**C**C**C**HPA**C**GKNYS**C**#	NMJ
	α-MII	*6*, *19*	A	G**C**C**C**SNPV**C**HLEHSNL**C**#	α6β3β2, α3β2
Conus striatus	α-SIA	*20*	A	Y**C**C**C**HPA**C**GKNFD**C**#	NMJ
Clade II[b]					
Conus geographus	α-GI	*21*	A	E**C**C**C**NPA**C**GRHYS**C**#	NMJ
Conus obscurus	αA-OIVB	*22*	A	**C**C**C**GVONAA**C**PO**C**V**C**NKT**C**G#	fetal NMJ
Clade III[b]					
Conus purpurascens	α-PIA	*19*	A	RDP**C**C**C**SNPV**C**TVHNPQI**C**#	α6β3β2
	αA-PIVA	*23*	A	G**C**C**C**GSYONAA**C**HO**C**S**C**KDROSY**C**GQ#	NMJ
	ψPIIIE	*24*	M	HOO**C**C**C**LYGK**C**RRYOG**C**SSAS**C**C**C**QR#	NMJ noncompetitive
Conus ermineus	α-EI	*25*	A	RDO**C**C**C**YHPT**C**NMSNPQI**C**#	NMJ
	αA-EIVA	*26*	A	**C**C**C**GPYONAA**C**HO**C**G**C**KVGROOY**C**DROSGG#	NMJ
Clade IV[b]					
Conus radiatus	αS-RVIIIA	this paper	S	K**C**NFDK**C**KGTGVYN**C**GγS**C**S**C**γGLHS**C**R **C**TYNIGSMKSG**C**A**C**I**C**TYY∧	NMJ, α7, α3β2, α6β3β2, etc.

[a] ∧, C-terminal free acid; #, C-terminal amidation; γ, γ-carboxyglutamate; O, 4-*trans*-hydroxyproline; NMJ, neuromuscular junction. [b] Nomenclature used is adopted from Espiritu et al. (*13*).

between Cys[7] and Cys[8]. σ-GVIIIA was the defining peptide for the S-conotoxin superfamily (*2*, *11*, *12*). The similarity in the number and spacing of Cys residues in αS-RVIIIA and σ-GVIIIA is consistent with their belonging to the same conotoxin superfamily. A recent comprehensive analysis of the S-conotoxin superfamily to be presented elsewhere (M. Watkins et al., manuscript in preparation) has provided further compelling evidence that σ-GVIIIA and αS-RVIIIA belong to the same superfamily; the precursors for the two peptides have identical signal sequences. αS-RVIIIA is only the second peptide characterized that belongs to this gene superfamily. It is notable that the two peptides, σ-GVIIIA and αS-RVIIIA, while having divergent targeting specificity (to the 5-HT_3 and nicotinic receptor families, respectively), are both antagonists of ligand-gated ion channels. Thus, the S-superfamily of conopeptides may be predominantly targeted to the ligand-gated ion-channel superfamily.

αS-RVIIIA most potently inhibits neuromuscular nAChRs but additionally inhibits a variety of neuronal nAChRs with varying degrees of affinity. In this respect, it is strikingly different from many other nicotinic receptor-targeted conotoxins, which are often narrowly targeted to a single nAChR subtype (see Table 2). However, αS-RVIIIA did not inhibit the 5-HT_3 receptor (unlike σ-GVIIIA, which also belongs to the S-superfamily of conotoxins). The two S-superfamily conotoxins, σ-GVIIIA and αS-RVIIIA, are from two fish-hunting species, *Conus geographus* and *Conus radiatus*, that belong to different groups of *Conus* fish hunters (*13*). All fish-hunting *Conus* venoms characterized to date have produced at least one toxin that is an antagonist of the nAChR at the neuromuscular junction; indeed, many venomous animals that paralyze their prey have evolved an antagonist to this obvious molecular target. In previously characterized *Conus* venoms, inhibitors of the neuromuscular nAChR have been primarily α- and αA-conotoxins that are members of the A-conotoxin gene superfamily (Table 2). We have cDNA and genomic clones encoding numerous A-superfamily conotoxins from *Conus radiatus* and related species. The most prominent group of α-conotoxin-like peptides were chemically synthesized and tested on muscle nAChRs expressed in oocytes but had no activity (R. Teichert, unpublished results). Additionally, numerous components (~20) have been purified and characterized from *Conus radiatus* venom. αS-RVIIIA is the only component so far that inhibits the neuromuscular nAChR. Our working hypothesis is that S-superfamily conotoxins are utilized by *Conus radiatus* and related fish-hunting species as their primary antagonists of muscle nAChRs.

Thus, αS-RVIIIA is a unique nicotinic antagonist from *Conus*. It defines a new family of conopeptides and appears to be much less subtype selective than other nAChR-targeted conotoxins. Previously characterized peptides from *Conus* such as α-conotoxin MI (which is specific for the α1δ nAChR subunit interface) are among the most selective nicotinic ligands known; in contrast, αS-RVIIIA is arguably the least selective polypeptide antagonist of the nAChR so far characterized. The discovery of αS-RVIIIA underlines the accelerated evolution of the pharmacological agents in *Conus* venoms; a glance at Table 2 clearly shows that this group of snails has evolved an amazing variety of nicotinic ligands, of which αS-RVIIIA is the most divergent.

The seizure activity elicited by i.c. injection of αS-RVIIIA in mice may be rationalized by its antagonism of α7 nAChRs (*9*, *10*, *14*). However, nicotinic receptors may play many complex roles in seizure activity. For example, investigators have demonstrated seizure activity by i.c. injection of both nicotinic agonists and antagonists in mice (*7*, *8*). Furthermore, several recent studies implicate a number of nAChR subtypes in seizure generation in addition to the α7 nAChR. These include the α3 subunit (*15*), α4 subunit (*16*), α5 subunit (*17*), β4 subunit (*15*, *17*), and α4β2 nAChR (*8*). Given this complexity, additional tools such as αS-RVIIIA may play a useful role in the molecular dissection of these various pathways.

To determine whether the seizure activity of αS-RVIIIA is likely to be mediated entirely through its interaction with the α7 nAChR, we performed i.c. injections with a few known inhibitors of α7 nAChRs in parallel on 18-day-old Swiss-Webster mice (10.0−10.4 g). We injected various quantities of either αS-conotoxin RVIIIA, α-conotoxin ImI (α-ImI), or α-bungarotoxin and subjected the mice to repetitive auditory stimulation following the i.c. injection. Interestingly, in our hands, a greater quantity of α-bungarotoxin (>2 nmol) and a much greater quantity of α-conotoxin ImI (>10 nmol) were required to elicit full tonic-clonic seizures in these mice than αS-RVIIIA (<2 nmol). Additionally, the onset of a seizure with α-ImI always required more time (>10 min) than the onset of a seizure with α-bungarotoxin or αS-RVIIIA (<2 min). These results were unexpected because α-bungarotoxin is a more potent, irreversible inhibitor than αS-RVIIIA of the α7 nAChR (see Figures 3 and 4), while αS-RVIIIA and α-ImI have approximately the same affinity for α7 (see Figure 3 and ref *9*). Because both α-bungarotoxin and α-ImI have greater specificity for the α7 nAChR in the brain than αS-RVIIIA, we hypothesize that αS-RVIIIA's greater potency in seizure generation is due to interactions with nAChRs in addition to the α7 subtype. With all toxins, at a sufficiently high dosage, the onset of a seizure appeared to be inducible by auditory stimulation, which may indicate that, when the seizure threshold is reduced sufficiently by the toxin interaction with receptors, any additional excitatory stimuli may evoke a seizure.

Future studies utilizing αS-RVIIIA as a tool may help to elucidate the mechanisms of seizure generation involving the various neuronal nAChRs. Furthermore, the very low dissociation rate from skeletal muscle nAChRs and its intermediate size between the α-conotoxins (10−20 amino acids) and the α-neurotoxins from snakes (60−80 amino acids) give it potential utility for additional classes of biochemical and biomedical applications.

ACKNOWLEDGMENT

We thank Vijay Vishwanath and Mike Ellison for helping us with some of the electrophysiological experiments. We thank Maren Watkins for providing us with the sequence data for the αS-RVIIIA cDNA clone.

REFERENCES

1. Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. E., Woodward, S. R., Hillyard, D. R., and Cruz, L. J. (1990) Diversity of *Conus* neuropeptides, *Science 249*, 257−263.
2. England, L. J., Imperial, J., Jacobsen, R., Craig, A. G., Gulyas, J., Akhtar, M., Rivier, J., Julius, D., and Olivera, B. M. (1998) Inactivation of a serotonin-gated ion channel by a polypeptide toxin from marine snails, *Science 281*, 575−578.
3. Jimenez, E. C., Olivera, B. M., Gray, W. R., and Cruz, L. J. (1996) Contryphan is a D-tryptophan-containing *Conus* peptide, *J. Biol. Chem. 281*, 28002−28005.
4. Edman, P., and Begg, G. (1967) A protein sequenator, *Eur. J. Biochem. 1*, 80−91.
5. Clark, C., Olivera, B. M., and Cruz, L. J. (1981) A toxin from *Conus geographus* venom which acts on the vertebrate central nervous system, *Toxicon 19*, 691−699.
6. Cartier, G. E., Yoshikami, D., Gray, W. R., Luo, S., Olivera, B. M., and McIntosh, J. M. (1996) A new α-conotoxin which targets α3β2 nicotinic acetylcholine receptors, *J. Biol. Chem. 271*, 7522−7528.
7. Stitzel, J. A., Lu, Y., Jimenez, M., Tritto, T., and Collins, A. C. (2000) Genetic and pharmacological strategies identify a behavioral function of neuronal nicotinic receptors, *Behav. Brain Res. 113*, 57−64.
8. Dobelis, P., Hutton, S., Lu, Y., and Collins, A. C. (2003) GABAergic systems modulate nicotinic receptor-mediated seizures in mice, *J. Pharmacol. Exp. Ther. 306*, 1159−1166.
9. Johnson, D. S., Martinez, J., Elgoyhen, A. B., Heinemann, S. F., and McIntosh, J. M. (1995) α-Conotoxin ImI exhibits subtype-specific nicotinic acetylcholine receptor blockade: Preferential inhibition of homomeric α7 and α9 receptors, *Mol. Pharmacol. 48*, 194−199.
10. McIntosh, J. M., Yoshikami, D., Mahe, E., Nielsen, D. B., Rivier, J. E., Gray, W. R., and Olivera, B. M. (1994) A nicotinic acetylcholine receptor ligand of unique specificity, α-conotoxin ImI, *J. Biol. Chem. 269*, 16733−16739.
11. Olivera, B. M., and Cruz, L. J. (2001) Conotoxins, in retrospect, *Toxicon 39*, 7−14.
12. Terlau, H., and Olivera, B. M. (2004) *Conus* venoms: A rich source of novel ion channel-targeted peptides, *Physiol. Rev. 84*, 41−68.
13. Espiritu, D. J. D., Watkins, M., Dia-Monje, V., Cartier, G. E., Cruz, L. J., and Olivera, B. M. (2001) Venomous cone snails: Molecular phylogeny and the generation of toxin diversity, *Toxicon 39*, 1899−1916.
14. Cohen, S. L., Morley, B. J., and Snead, O. C. (1981) An EEG analysis of convulsive activity produced by cholinergic agents, *Prog. Neuropsychopharm. 5*, 383−388.
15. Salas, R., Cook, K. D., Bassetto, L., and De Biasi, M. (2004) The α3 and β4 nicotinic acetylcholine receptor subunits are necessary for nicotine-induced seizures and hypolocomation in mice, *Neuropharmacology 47*, 401−407.
16. Fonck, C., Nashmi, R., Deshpande, P., Damaj, M. I., Marks, M. J., Riedel, A., Schwarz, J., Collins, A. C., Labarca, C., and Lester, H. A. (2003) Increased sensitivity to agonist-induced seizures, straub tail, and hippocampal θ rhythm in knock-in mice carrying hpersensitive α4 nicoitinic receptors, *J. Neurosci. 23*, 2582−2590.
17. Kedmi, M., Beaudet, A. L., and Orr-Urtreger, A. (2004) Mice lacking neuronal nicotinic acetylcholine receptor β4-subunit and mice lacking both α5- and β4-subunits are highly resistant to nicotine-induced seizures, *Physiol. Genomics 17*, 221−229.
18. McIntosh, J. M., Cruz, L. J., Hunkapiller, M. W., Gray, W. R., and Olivera, B. M. (1982) Isolation and structure of a peptide toxin from the marine snail *Conus magus*, *Arch. Biochem. Biophys. 218*, 329−334.
19. 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 α6 subunit-containing nicotinic acetylcholine receptors, *J. Neurosci. 23*, 8445−8452.
20. Myers, R. A., McIntosh, J. M., Imperial, J., Williams, R. W., Oas, T., Haack, J. A., Hernandez, J.-F., Rivier, J., Cruz, L. J., and Olivera, B. M. (1990) Peptides from *Conus* venoms which affect Ca^{2+} entry into neurons, *J. Toxicol., Toxin Rev. 9*, 179−202.
21. Gray, W. R., Luque, A., Olivera, B. M., Barrett, J., and Cruz, L. J. (1981) Peptide toxins from *Conus geographus* venom, *J. Biol. Chem. 256*, 4734−4740.
22. Teichert, R. W., Rivier, J., Torres, J., Dykert, J., Miller, C., and Olivera, B. M. (2005) A uniquely selective inhibitor of the mammalian fetal neuromuscular nicotinic acetylcholine receptor, *J. Neurosci. 25*, 732−736.
23. Hopkins, C., Grilley, M., Miller, C., Shon, K., Cruz, L. J., Gray, W. R., Dykert, J., Rivier, J., Yoshikami, D., and Olivera, B. M. (1995) A new family of *Conus* peptides targeted to the nicotinic acetylcholine receptor, *J. Biol. Chem. 270*, 22361−22367.
24. Shon, K., Grilley, M., Jacobsen, R., Cartier, G. E., Hopkins, C., Gray, W. R., Watkins, M., Hillyard, D. R., Rivier, J., Torres, J., Yoshikami, D., and Olivera, B. M. (1997) A noncompetitive peptide inhibitor of the nicotinic acetylcholine receptor from *Conus purpurascens* venom, *Biochemistry 36*, 9581−9587.
25. Martinez, J. S., Olivera, B. M., Gray, W. R., Craig, A. G., Groebe, D. R., Abramson, S. N., and McIntosh, J. M. (1995) α-Conotoxin EI, a new nicotinic acetylcholine receptor-targeted peptide, *Biochemistry 34*, 14519−14526.
26. Jacobsen, R., Yoshikami, D., Ellison, M., Martinez, J., Gray, W. R., Cartier, G. E., Shon, K. J., Groebe, D. R., Abramson, S. N., Olivera, B. M., and McIntosh, J. M. (1997) Differential targeting of nicotinic acetylcholine receptors by novel αA-conotoxins, *J. Biol. Chem. 272*, 22531−22537.

BI047274+