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α S-Conotoxin GVIIIB Potently and Selectively Blocks α 9 α 10 Nicotinic Acetylcholine Receptors

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Abstract

Although acetylcholine is widely utilized in vertebrate nervous systems, nicotinic acetylcholine receptors (nAChRs), including the α 9 α 10 subtype, also are expressed in a wide variety of non-neuronal cells. These cell types include cochlear hair cells, adrenal chromaffin cells and immune cells. α 9 α 10 nAChRs present in these cells may respectively play roles in protection from noise-induced hearing loss, response to stress and neuroprotection. Despite these critical functions, there are few available selective ligands to confirm mechanistic hypothesis regarding the role of α 9 α 10 nAChRs. *Conus*, has been a rich source of ligands for receptors and ion channels. Here, we identified *Conus geographus* venom as a lead source for a novel α 9 α 10 antagonist. The active component was isolated and the encoding gene cloned. The peptide signal sequence and cysteine arrangement had the signature of the σ -conotoxin superfamily. Previously isolated σ -conotoxin GVIIIA, also from *Conus geographus*, targets the 5-HT₃ receptor. In contrast, α S-GVIIIB blocked the α 9 α 10 nAChR with an IC₅₀ of 9.8 nM, yet was inactive at the 5-HT₃ receptor. Pharmacological characterization of α S-GVIIIB shows that it is over 100-fold selective for the α 9 α 10 nAChR compared to other nAChR subtypes. Thus, the S-superfamily represents a novel conotoxin scaffold for flexibly targeting a variety of receptor subtypes. Functional competition studies utilized distinct off-rate kinetics of conotoxins to identify the α 10/ α 9 nAChR interface as the site of α S-GVIIIB binding; this adds to the importance of the (+) face of the α 10 rather than the (+) face of the α 9 nAChR subunit as critical to binding of α 9 α 10-targeted conotoxins.

Abstract

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Keywords

protein purification; oocyte; nicotinic receptor; alpha9 alpha10; conotoxin

1. Introduction

Conus are predatory marine mollusks that envenomate prey to facilitate capture. There are an estimated 500-700 species of cone snails. Each cone snail venom is comprised of a unique cocktail of hundreds of components. The *Conus* species therefore represent a natural, evolutionarily refined library of compounds that act on the nervous system (1) (2) (3) (4) (5).

Nicotinic acetylcholine receptors (nAChRs) are a subset of ligand gated ion channels that use acetylcholine (ACh) as its primary natural agonist (6). To date, there are seventeen known nAChR subunits in vertebrates, those found primarily in muscle that include $\alpha 1$, $\beta 1$, δ , γ and ϵ and those found in neuronal as well as non-neuronal tissues, $\alpha 2$ - $\alpha 10$, and $\beta 2$ - $\beta 4$. These subunits combine to form pentamers with varying pharmacology and function that depends on the composition of the individual subunits. Additionally, $\alpha 7$, $\alpha 9$ and $\alpha 10$ can form functional receptors in the absence of β subunits; $\alpha 7$ and $\alpha 9$ form homomers, while $\alpha 9\alpha 10$ can form a functional heteromer. Phylogenetic data of nAChRs has shown that $\alpha 7$, $\alpha 9$ and $\alpha 10$ are closely related compared to the other neuronal subtypes and the muscle subtypes (7).

The $\alpha 9\alpha 10$ nAChR was originally identified as the receptor that mediates synaptic transmission from the olivocochlear efferents to auditory hair cells of the cochlea (8). The $\alpha 9\alpha 10$ nAChR was subsequently identified in adrenal chromaffin cells and is upregulated in response to cold-induced stress (9). Other studies suggest the presence of $\alpha 9\alpha 10$ nAChRs in tissues including immune cells and breast tumors (10) (11). Block of $\alpha 9\alpha 10$ nAChRs has been associated with analgesia (12) (13) (14) (15). Despite the potential importance of this receptor subtype, there are few available ligands with which to characterize the function and pharmacology of $\alpha 9\alpha 10$ nAChRs.

nAChRs are utilized by various prey types hunted by *Conus*. As such, nAChRs provide a rational target for prey immobilization. The α -conotoxins are a well-studied family of *Conus* peptides that have been shown to act on a variety of nAChR subtypes (16) (17) (18) (19)

(20). Recently, however, there have been reports of other families of conotoxins that have activity on nAChRs (21) (22) (23) (24) (25).

The aim of this study was to examine *Conus* venoms for the presence of uncharacterized antagonists of the $\alpha 9\alpha 10$ nAChR. To achieve this goal we screened several venom samples against the $\alpha 9\alpha 10$ nAChR. We then purified and characterized the responsible component from the most potent venom. The novel peptide αS -GVIIIB was identified and characterized.

2. Materials and Methods

2.1. Crude venom extraction

Various species of *Conus* were selected from several clades (26). To 40 mg of each venom was added 800 μ l of B35 (65:35:0.1 H₂O/acetonitrile/trifluoroacetic acid) (Thermo Fisher Scientific). The mixtures were homogenized by hand using a disposable pestle a minimum of thirty rotations or until the tissue appeared to be thoroughly dissociated. The samples were then centrifuged at 13,000 RPM and the supernatant was removed. The venom was then re-extracted a second time and the supernatants from both extractions were pooled for each individual species.

For large scale extraction of *Conus geographus*, 200 mg of lyophilized venom was combined with 2 ml of B35. The sample was homogenized using a Teflon pestle spinning at 1000 RPM. The sample was then centrifuged at 13,000 RPM and the supernatant was removed. An additional 2 ml of B35 was added to the pellet and homogenization and centrifugation was repeated two additional times. The combined supernatants were then diluted to a volume of 50 ml using Buffer A (99.9:0.1 H₂O/trifluoroacetic acid) and loaded onto a semi-preparative C18 HPLC column (Vydac 218TP510, Grace Discovery Sci.). The gradient was 2% to 100% B60 (40:60:0.092 H₂O/Acetonitrile/trifluoroacetic acid) for 98 minutes (1% per min). The flow rate was 5 ml/min; fractions were collected for the length of the run at 2 min intervals.

2.2. Venom purification and isolation

The resulting fractions from the large scale venom extraction described above were assessed for activity on $\alpha 9\alpha 10$ nAChRs. Fractions exhibiting activity were selected for further purification to isolate the active component. The active fraction was run on an analytical HPLC (Alliance, Waters Corp.) using a Vydac C18 column (218TP54, Grace Discovery Sci.) and a gradient of 10% to 50% B60 for 40 minutes (1% per min). The flow rate was 1 ml/min. All peaks were collected and assessed for activity on oocytes expressing $\alpha 9\alpha 10$ nAChRs. Active peaks were then further purified using size exclusion chromatography (SEC) as follows: two columns (Superdex Peptide HR 10/30, GE Healthcare Bio-Sciences Inc.) were attached in series and eluted with B30 (70:30:0.1 H₂O/acetonitrile/trifluoroacetic acid) at a flow rate of 0.5 ml per minute.

2.3. Peptide sequencing

The isolated active peptide was reduced using dithiothreitol (DTT) (Promega, Madison, WI) at a concentration of 20 μ M. To 5 nmols of lyophilized peptide was added 25 μ l of 1 M Tris (pH 7.5) (Sigma-Aldrich), 2.5 μ l of 0.1 M EDTA (pH 7.5) (Sigma-Aldrich), and 1.6 mg DTT dissolved in 223 μ l H₂O. The reaction proceeded for 2 hours at room temperature, and was quenched with 8% formic acid (Sigma-Aldrich) (20 μ l for the 250 μ l reaction volume). Following DTT reduction, the Cys residues were alkylated with 4-vinylpyridine (Sigma-Aldrich) (27). 250 μ l of Buffer A was added to the reaction mix and 3 μ l of 4-vinylpyridine was added. Immediately after addition of the 4-vinylpyridine the reaction mixture was bubbled with Argon and then was incubated at room temperature for 30 min in the dark. The peptide was purified using an analytical HPLC. MALDI-TOF mass spectrometry was performed to confirm the reduction of disulfide bonds and pyridylethylation of the Cys residues. Pyridylethylated peptide was sequenced using Edman chemistry on an Applied Biosystems 477A Protein Sequencer at the Protein/DNA Core Facility at the University of Utah.

2.4. Peptide quantification

The concentration of purified α S-GVIIIIB was quantified using an absorbance measurement with a spectrophotometer at a wavelength of 280 nm and calculated using the Lambert-Beer equation, $A = \epsilon lc$; where A is absorbance, ϵ is the extinction coefficient, l is the cuvette path length and c is the concentration. The cuvette path length is 1 cm and the extinction coefficient for α S-GVIIIIB at a wavelength of 280 nm was calculated as 2480 $\text{cm}^{-1}\text{M}^{-1}$ using a peptide properties calculator found at www.basic.northwestern.edu/biotools/proteincalc.html.

2.5. cDNA cloning

We used the sequence from a previously published *Conus geographus* transcriptome (28) to design two forward primers for carrying out nested polymerase chain reactions, designated Oplus: 5'GCAAGACGTGACGTGCAAG 3' and Iplus: 5'CATGATGTGCGAAAATGGGAGC 3'. First strand cDNA was synthesized from total RNA isolated from venom duct using 3'-RACE CDS primer A (SMARTer™ RACE cDNA Amplification Kit, Clontech Laboratories, Inc.) according to the vendor's instructions. cDNA encoding the conotoxin was isolated by amplification using polymerase chain reaction (PCR). The initial PCR was carried out using Advantage 2 polymerase (Clontech) and oligonucleotides, Oplus and UPM (Clontech, kit mentioned above) as primers. The amplified product was diluted 50 fold and used as template for a subsequent PCR using Go Taq® DNA polymerase (Promega Corporation, WI) and Iplus and NUP (kit mentioned above) as primers (PCR was carried out using buffers and instructions provided by the vendors). The amplified product was purified from an agarose gel using QIAquick Gel Extraction Kit (Qiagen Inc, CA). The isolated DNA was ligated to pGEM®-T Easy vector DNA (Promega, WI) and used to transform E.coli DH10B (New England Biolabs Inc.). Vector DNA carrying the insert was isolated and their sequences determined by Sanger's dideoxy sequencing method at the University of Utah DNA Peptide Core facility.

2.6. Oocyte electrophysiology

Xenopus laevis (*Xenopus* express, FL) oocytes were micro-injected with cRNA of the various rat nAChR subunits as previously described (29). Clones for $\alpha 9$ and $\alpha 10$ were generously provided by AB Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina), high expressing $\beta 2$ and $\beta 3$ were generously provided by C Luetje (University of Miami, Miami, FL) and all other nAChR subunits used were generously provided by S Heinemann (Salk Institute, LaJolla, CA). The 5-HT₃ serotonin receptor was generously provided by AV Maricq (University of Utah, Salt Lake City, UT) (30). Injected oocytes were incubated at 17 °C for 1-3 days. Following that, oocytes expressing the various receptors were voltage clamped at -70 mV using a two-electrode voltage clamp system (Warner Instruments, Hamden, CT) as previously described (29). Briefly, oocytes were placed in a 30 μ l bath and perfused with ND96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, pH 7.5) (Thermo Fisher Scientific) containing 0.1 mg/ml protease-free bovine serum albumin (BSA) (Sigma-Aldrich). ACh (Sigma-Aldrich) was applied for 1 s at 1 min intervals and the peak amplitude was recorded. ACh concentration was 10 μ M for $\alpha 9\alpha 10$ nAChR and human adult muscle; 300 μ M for $\alpha 7$ nAChR; and for all other nicotinic receptors ACh concentration was 100 μ M. For the oocytes expressing the 5-HT₃ receptor a 1 s pulse of 5 μ M serotonin (Sigma-Aldrich) was applied at 1 min intervals (31). After a stable baseline of agonist-responses was achieved, the solution flow was stopped and whole venom or venom fraction was applied and allowed to incubate for five minutes. Following incubation, agonist was pulsed and response was measured. For purified peptides, αS -GVIIIB at concentrations of 30 nM or lower, or αS -RVIIIA at concentrations of 100 nM or lower, a solution of ND96 containing the toxin was perfused onto the oocyte at a flow rate of ~1 ml/min until steady state block was achieved. Response was monitored by applying a 1 s agonist pulse once per minute. For higher toxin concentrations, the flow was stopped and toxin was applied and allowed to incubate for 5 min before flow of buffer was resumed.

3. Results

3.1. Activity of *Conus* crude venoms

Conus venoms are composed of hundreds of individual compounds. Each *Conus* species has its own unique set of peptides, and thus there are tens of thousands of unique peptides present in these venoms. The purification and testing of each of these individual components is impractical. We therefore prepared whole venom extracts from representative species from eight various clades of *Conus* (26) as described in *Experimental Procedures* and tested these for activity against the $\alpha 9\alpha 10$ nAChR heterologously expressed in *Xenopus* oocytes. Venom samples were initially screened using 0.33 μ g/ml of venom extract per test application. Any sample that elicited greater than 80% block of the $\alpha 9\alpha 10$ nAChR was subsequently diluted ten-fold and tested again at a concentration of 0.033 mg/ml. Venom from a single species, *Conus geographus*, blocked greater than fifty percent of the ACh induced current when tested at 0.033 mg/ml (~84 % block), Table I.

3.2. Purification of *Conus geographus* venom

From the initial screening on the $\alpha 9\alpha 10$ nAChR, *Conus geographus* was selected based on its potent block of the receptor and also because the block was slowly reversible following venom washout, suggesting slow off-rate kinetics and possibly high potency of the responsible compound(s). A larger scale extraction of venom (200 mg) was performed and separation of the venom components was done based on hydrophobicity using reversed-phase HPLC (Fig. 1A). Ten ml fractions were collected and lyophilized. In the original assay we had tested 0.033 mg/ml of crude venom extract, a control sample was taken from the 200 mg extraction before fractionation and it exhibited 96.3% block for 0.033 mg/ml. We defined the activity present in 1 μ g of venom as one 'unit'. From a 200 mg crude venom preparation we assumed that we would have 200,000 units of material. Therefore, 100 μ l of each fraction was lyophilized and re-suspended in 60 μ l of ND96 buffer and then serially diluted such that one part in 200,000 of each original fraction was tested for activity on oocytes expressing $\alpha 9\alpha 10$ nAChRs. The fraction eluting at 32-34 min blocked 92.4 % of the ACh response (Fig 1A).

A second round of purification of the identified active fraction was performed based on hydrophobicity using a reversed-phase C18 column (Fig. 1B). Forty nmol of toxin was obtained from the original starting material of 200 mg of crude venom. $\alpha 9\alpha 10$ nAChR expressing oocytes were again used to screen activity and the responsible component was identified (Fig. 1B). The active material was further purified based on molecular mass using a size-exclusion column (Fig. 1C). The resulting fractions were tested on $\alpha 9\alpha 10$ nAChRs. Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry showed a monoisotopic mass of 4435.6 Da (C) for the active component (Fig. 1D), and the two inactive side components shown in figure 1C had respective masses of 1417.2 Da (A) and 3768.8 Da (B). The active component purity was > 98 % based on the UV absorption peaks obtained at 220 nm during HPLC. (Fig. 1D). Mass spectrometry of the active fraction showed a predominant main mass of 4435.6 Da; In addition, there were minor masses of 4419.6 Da and 4451.6 Da, which are -16 and +16 Da. These likely correspond to variable hydroxylation of Pro residues as has been reported for other conotoxins (2).

3.3 Sequencing of the active component

Previously characterized conotoxins generally have multiple Cys residues connected by disulfide bonds. We assumed that this was likely the case with the present compound, and we therefore reduced the compound with DTT and alkylated the active component with 4-vinylpyridine. The resulting product was purified by HPLC and analyzed by mass spectrometry; pyridylethylation resulted in a compound with an increased mass of 1054.2 Da, indicating the presence of 10 cysteine residues. The alkylated peptide was then subjected to Edman degradation sequencing that resulted in only a partial amino acid sequence (Fig. 2A). Using the partial amino acid sequence obtained, search results of previously published *Conus geographus* transcriptomes revealed two precursor proteins that contained a mature toxin sequence identical to that of the partial peptide sequence (28) (32). The two predicted mature toxins from the transcriptomes differed by only a single amino acid in the mature toxin region, a Pro/Arg substitution at the 35th amino acid. One of these sequences (the Pro variant) was identical to that of the sequence of the peptide isolated in

the present work. To confirm the presence of this sequence in our collection of *Conus geographus*, a cDNA library was prepared from venom duct, and cloning was performed using primers designed from the transcriptome sequence as described in the *Experimental Procedures*. The cDNA sequencing results yielded clones with a Pro in the 35th position of the toxin sequence (Fig. 2B). The calculated mass of the two transcriptome sequences are 4462.7 Da for the Arg variant and 4403.7 Da for the Pro variant. The measured mass of the venom isolated peptide was 4435.6 Da, which is +32 Da compared to that of the Pro variant. The increase of 32 Da is most likely attributable to hydroxylation of 2 of the 4 Pro residues in the peptide sequence. Hydroxylation of Pro is a common post-translational modification of *Conus* peptides (2). The sequence of the pre-pro-region of the peptide places this peptide in the S-superfamily (33). The Cys framework is that of conotoxins designated with Roman numeral VIII (34). The peptide was therefore named α S-GVIIIIB according to previously established criteria (35).

3.4. nAChR Subtype selectivity testing

Several concentrations of α S-GVIIIIB were applied to oocytes expressing α 9 α 10 nAChRs. A concentration-response curve was generated yielding an IC_{50} of 9.8 nM (95% confidence interval of 6.6-14.4 nM) (Fig. 3A). Additionally, α S-GVIIIIB was tested on several other neuronal nAChR subtypes, the adult muscle subtype α 1 β 1 $\delta\epsilon$, and also the 5-HT₃ serotonin receptor (Figs. 3B, 3C, and 4). Application of 100 nM α S-GVIIIIB resulted in little or no block: % response was 96.3(2.9)%, α 3 β 2; 89.22(7.7)%, α 6/ α 3 β 2 β 3; 101.34(6.5)%, α 3 β 4; 95.40(5.3)%, α 4 β 2; and 97.22(4.1)% for α 7; (= S.E.M.). At ten-fold higher concentration partial block was observed, with the α 3 β 2 and α 6/ α 3 β 2 β 3 exhibiting 48.3% and 54.0% response to ACh, respectively (Fig. 4, Table II). The small amount of available material prevented more detailed testing at higher concentrations.

3.5. Binding site analysis

We next examined the site of action of α S-GVIIIIB. We specifically tested whether the binding of α S-GVIIIIB is prevented by pre-incubation with the competitive α 9 α 10 antagonist α -conotoxin RgIA (36) (37). Although α -RgIA potently blocks the α 9 α 10 nAChR, it has relatively rapid off-rate kinetics. Ten μ M α -RgIA completely blocked α 9 α 10 nAChRs with full recovery 1-2 min after toxin washout (Fig. 5A) (36). In contrast block by α S-GVIIIIB is much more slowly reversed. One hundred nM α S-GVIIIIB blocked ~90% of the ACh response with a slow recovery after toxin washout, half time to recovery is 7.1 min ($n=3$, 95% confidence interval, 6.2-8.4 min). We took advantage of this difference in off-rate kinetics to examine whether α S-GVIIIIB and α -RgIA bind to the same or overlapping sites on the receptor. Using oocytes expressing α 9 α 10 nAChRs, a baseline ACh response was established; then 10 μ M α -RgIA was applied and allowed to incubate for 1 min. After pre-incubation with α -RgIA, 100 nM α S-GVIIIIB was also added to the oocyte chamber and incubation in the presence of both toxins continued for another 4 min. Following the incubation, ND96 perfusion resumed and ACh pulses were taken at 1 min intervals (Fig. 5B). After application of both toxins, the ACh response was completely blocked. However, after 1 min of toxin washout, the ACh response returned to pre-toxin baseline levels. Thus, washout kinetics of toxin block were the same as that of α -RgIA but not that of α S-GVIIIIB.

Pre-application of α -RgIA prevented the slowly reversible block of α S-GVIIIIB consistent with pre-binding of α -RgIA preventing subsequent binding of α S-GVIIIIB.

We then conducted the converse experiment; that is pre-binding of α S-GVIIIIB followed by subsequent exposure to α -RgIA. 100 nM α S-GVIIIIB was first applied to the oocyte and allowed to incubate for 4 min; then, 10 μ M α -RgIA was applied and the incubation in the presence of both toxins continued for 1 min (Fig. 5C). Under these conditions, the α 9 α 10 nAChR was fully blocked. Toxins were then washed out and further ACh pulses showed slow recovery from toxin block back to baseline levels (Fig. 5C). Thus, the order of toxin exposure determined the recovery from toxin block. When α -RgIA was applied first, the off-rate was similar to that with block by α -RgIA alone. In contrast, when α S-GVIIIIB was applied first, the off-rate was similar to that with block by α S-GVIIIIB alone. These results indicate that pre-binding of α -RgIA prevents subsequent binding of α S-GVIIIIB. These findings are consistent with the toxins binding to the same, or overlapping binding sites. Block by 30 nM α S-GVIIIIB (% response = 18.11 ± 4.0 S.E.M.) could be fully overcome by co-application of 300 μ M ACh and 30 nM α S-GVIIIIB (% response 99.47 ± 0.9 S.E.M.), also consistent with competitive binding.

4. Discussion

In this study we describe the purification and characterization of a novel conotoxin from *Conus geographus*, designated α S-GVIIIIB. *Conus geographus* venom was chosen from the tested venoms based on the high degree of block and slow washout kinetics from application of a small amount of venom. The active peptide was isolated using activity at the α 9 α 10 nAChR as an assay to purify the responsible component. α S-GVIIIIB is a 4 kDa, 10 Cys peptide that blocked the α 9 α 10 nAChR with low nanomolar potency. By comparison, a well-known peptide, α -bungarotoxin from the snake venom of *Bungarus multicinctus*, also potently blocks the α 9 α 10 nAChR. However, the larger (~ 8 kDa) α -bungarotoxin also potently blocks the α 7 nAChR as well as the muscle nAChR subtype. In contrast, α S-GVIIIIB is over 100-fold selective for α 9 α 10 nAChRs compared with other nAChR subtypes (Fig. 4). α 9, α 10 and/or α 7 subunits may represent primordial subunits of the nAChR subunit family (38). As such, α 9, α 10 and/or α 7 are more closely related and may explain why snake venom toxins and plant alkaloids have difficulty pharmacologically distinguishing among nAChRs containing these subunits.

Other previously characterized conotoxins also block the α 9 α 10 nAChR. These peptides come from the worm-hunting *Conus regius* and *Conus vexillum*, and the mollusk-hunting *Conus victorea*. In contrast, α S-GVIIIIB comes from a fish-hunting cone snail. The sequence and Cys arrangement of α S-GVIIIIB are entirely unrelated to the α 9 α 10 nAChR blocking peptides from the aforementioned species (Table III). This demonstrates an example of convergent evolution, as the snail venom components diversify to different gene superfamilies with activity for the same receptor.

Recent work on conotoxins has indentified whether venom components are used for prey capture or for defensive strategies. The study performed by Dutertre et. al., (32) focused on the classification of *Conus geographus* venom. The previously characterized σ -GVIIIA was

identified among the defensive venom components, as were several α -conotoxins that target nAChRs. Due to these reported findings, α S-GVIIIIB may also be one of the toxins utilized by *C. geographus* to defend against predators.

The precursor sequence and arrangement of Cys residues place α S-GVIIIIB in the sigma conotoxin family (Table IV). The selectivities of the three characterized σ -conotoxins are completely different. Whereas α S-GVIIIIB selectively targets the $\alpha 9\alpha 10$ nAChR, σ -GVIIIA targets the 5-HT₃ receptor and α S-RVIIIA blocks multiple nAChR subtypes. It is of note that α S-GVIIIIB comes from the same species as σ -GVIIIA and is from the same gene superfamily, yet the two toxins target two different classes of ligand-gated ion channels. Typically, the venom of cone snails will contain multiple toxins from the same gene family, but these toxins target the same receptor or ion channel class. The previous study on σ -GVIIIA tested activity on several nAChR subtype combinations, but did not test the $\alpha 9\alpha 10$ nAChR (39). We therefore tested σ -GVIIIA on $\alpha 9\alpha 10$ nAChRs, and observed no block at a concentration of 1 μ M (Fig. 3A). When 1 μ M of α S-GVIIIIB was applied to oocytes expressing the 5-HT₃ receptor no block was observed (Fig. 3B). These results illustrate the stark preference for one type of ligand-gated ion channel between the two toxins from the same gene family, produced by the same *Conus* species. α S-RVIIIA, is a sigma family conotoxin from the fish-hunting *Conus radiatus*. Concentration response analysis indicated that α S-RVIIIA most potently blocked the muscle nAChR with an IC₅₀ of 43 nM (Fig. 3C). α S-RVIIIA also blocked the $\alpha 9\alpha 10$ nAChR with an IC₅₀ of 187 nM (Fig. 3A).

Neuronal nAChRs typically consist of both an α and a β subunit. A ligand binding interface occurs between these two subunits, with agonist binding that occurs between the (+) face of the α subunit and the (–) face of the β subunit. Binding of agonist causes closure of a loop of amino acids present in the α subunit (the C- loop) of the nAChR; movement of this loop is believed to facilitate subsequent opening of the ion channel. For these and other reasons, the α subunit is often referred to as the ‘principal’ binding subunit, whereas the β subunit is referred to as the ‘complementary’ binding subunit. The $\alpha 9\alpha 10$ nAChR is unique in that it is formed by two different α subunits and thus an asymmetrical binding interface is formed between two α subunits. However, if injected alone, the $\alpha 9$ subunit will form a functional homopentamer (40). In contrast, the $\alpha 10$ subunit does not form a functional receptor when injected in the absence of other nAChR subunits (41). For this reason, it was previously assumed that the $\alpha 9$ subunit was analogous to other nAChR α subunits in providing the principal binding site, whereas the $\alpha 10$ subunit functions similar to neuronal nAChR β subunits in providing a complementary binding site. However, α -conotoxins that bind to the $\alpha 9\alpha 10$ nAChR appear to bind to an interface formed by the $\alpha 10/\alpha 9$ subunit interface rather than the $\alpha 9/\alpha 10$ interface (37) (42) (43). We therefore sought to examine whether block of the $\alpha 9\alpha 10$ nAChR by α S-GVIIIIB occurs at the same or different binding site than the α -conotoxins. Block by α -RgIA reverses quickly after toxin washout. In contrast, block by α S-GVIIIIB is more slowly reversed. We took advantage of the difference in off-rate kinetics to assess whether α S-GVIIIIB and α -RgIA have similar binding sites. Pre-incubation of α -RgIA prevented subsequent block by α S-GVIIIIB suggesting that the two peptides have the same or overlapping binding sites. Thus, an entirely different antagonist, α S-GVIIIIB, also appears to bind to the $\alpha 10/\alpha 9$ rather than the $\alpha 9/\alpha 10$ subunit interface. Thus, at least for the

tested conotoxins, the ‘principal’ face of the $\alpha 9\alpha 10$ nAChR appears to be provided by the $\alpha 10$ rather than the $\alpha 9$ subunit (44) (45) (46).

The 5-HT₃ receptor and nAChRs are members of the Cys-loop family of ligand-gated ion channels (47) (48). Both members of this family are composed of five symmetrically arranged subunits that surround an ion-conducting pore. In both cases, the extracellular domain is the site of action of agonists and competitive antagonists. The binding site for agonist and antagonists lies between the faces of two adjacent subunits and is formed by the convergence of three ‘loops’ (A-C) from the ‘principal’ subunit and three ‘loops’ (D-F) from the adjacent or ‘complementary’ subunit. These similarities likely allow the σ -conotoxins to be able to target one or another of these receptor types. However, significant differences are present with respect to key binding determinants of the 5-HT₃ receptor compared to the $\alpha 10$ and $\alpha 9$ subunits (Fig. 6) (49) (44). Although the binding determinants of αS -GVIIIB have yet to be determined, similar differences may account for specificity differences among sigma conotoxins.

In conclusion, we have isolated and characterized a novel peptide from a fish-hunting cone snail. Although this peptide belongs to the σ -conotoxin superfamily, the selective block of the $\alpha 9\alpha 10$ nAChR makes it unique among previously characterized σ -conotoxins. Other cloning studies have identified several other σ -conotoxin sequences (50) (33); the pharmacological activity of these other toxins is unknown. The principal challenge to the synthesis of σ -conotoxins is the presence of 10 Cys residues. For ten cysteine residues, there are 945 unique possible ways to arrange the disulfide bonds. To-date, the disulfide arrangement has not been solved for any conotoxin superfamily containing more than eight cysteine residues. σ -conotoxins have now been discovered in cone snails that hunt a wide diversity of prey type (fish, mollusk and worm). The diversity of *Conus* species that use σ -conotoxins and the array of activity at various receptors, suggest that the σ -conotoxin superfamily has substantial potential to provide many more novel ligands for probing the structure and function of a variety of ligand-gated ion channel subtypes.

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Abbreviations

nAChR	nicotinic acetylcholine receptor
ACh	acetylcholine
HPLC	high performance liquid chromatography
SEC	size-exclusion chromatography
TFA	trifluoroacetic acid

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Figure 1.

HPLC purification of α S-GVIII B. A. Purification of crude venom extract of 200 mg of lyophilized *Conus geographus* venom. Arrow indicates the fraction that blocked α 9 α 10 nAChRs. B. Analytical scale purification of 100 μ l of the active fraction from panel A; Peaks A and B were active. C. Size-exclusion chromatography of the active components shown in panel B. Peak C was the active component. D. RP-HPLC of purified α S-GVIII B (peak C).

Figure 2.

Sequence of α S-GVIII B. A. Partial toxin sequence obtained from Edman degradation sequencing. B. Gene sequence for α S-GVIII B obtained from cDNA cloning. The first arrow represents the end of the signal sequence and start of the pro-peptide region. The second arrow represents the proteolytic processing site, and the mature toxin sequence is underlined. The C-terminus is predicted to be amidated, represented by #. The * represents the stop codon.

Figure 3.

Concentration-response of native sigma-conotoxins. α S-GVIIIIB as well as two previously isolated sigma conotoxins were each tested on A. $\alpha 9\alpha 10$ nAChRs, B. the 5-HT₃ serotonin receptor, and C. the muscle subtype ($\alpha 1\beta 1\delta\epsilon$) nAChR, expressed in *Xenopus laevis* oocytes. α S-GVIIIIB blocked the $\alpha 9\alpha 10$ nAChR with an IC₅₀ of 9.8 (6.6-14.3) nM and a Hill slope of 1.32 (0.7-1.9); for 5-HT₃ and $\alpha 1\beta 1\delta\epsilon$ nAChR 1 μ M toxin blocked less than 50% of the response. σ -GVIII A blocked the 5-HT₃ receptor with an IC₅₀ of 86.8 (56-135) nM and a Hill slope of 1.00 (0.6-1.4); for $\alpha 9\alpha 10$ and $\alpha 1\beta 1\delta\epsilon$ nAChRs, 1 μ M toxin blocked less than half of the response. α S-RVIII A blocked the $\alpha 9\alpha 10$ nAChR with an IC₅₀ of 187 (138-255) nM and a Hill slope of 1.9 (1.0-2.9), $\alpha 1\beta 1\delta\epsilon$ with an IC₅₀ of 43.1 (17-108) nM and a Hill slope of 0.71 (0.3-1.1); 1 μ M toxin blocked less than 50% of the serotonin-evoked response of the 5-HT₃ receptor.

Figure 4.

Activity of α S-GVIIIIB on other neuronal nAChRs. Oocytes expressing different nAChR subtypes were exposed to 100 nM and 1 μ M applications of α S-GVIIIIB for 5 min; after toxin incubation, a 1 s pulse of ACh was applied. A. Each bar represents the average percentage of ACh response for the corresponding receptor subtype and α S-GVIIIIB concentration ($n = 3-5$ oocytes per toxin concentration). B. A representative trace for each receptor subtype exposed to 100 nM α S-GVIIIIB. For each subtype, the first response, labeled C, is the control ACh response. The oocyte was then incubated for 5 min with 100 nM α S-GVIIIIB, followed by another 1 s pulse of ACh, labeled T.

Figure 5.

Competition of α -RgIA and α S-GVIIIB for binding the α 9 α 10 nAChR. A. Washout kinetics of toxin block by 10 μ M α -RgIA. A baseline control response was established for α 9 α 10 nAChRs; the ND96 flow was then stopped for 5 min, and α -RgIA was added for the last minute. One s ACh pulses were then given at 1 min intervals. Note the rapid recovery from toxin block. B. Washout kinetics of toxin block following pre-incubation with RgIA. Baseline control responses to ACh were established and then ND96 flow was stopped. Ten μ M α -RgIA was then applied to the bath, with a 1 min incubation. Subsequently, 100 nM α S-GVIIIB was added for an additional 4 min. ACh was pulsed at 1 min intervals. Note the rapid recovery from toxin block. C. Washout kinetics of toxin block following pre-incubation with α S-GVIIIB. Oocytes were incubated with 100 nM α S-GVIIIB for 4 min. Ten μ M α -RgIA was added to the bath for the final minute; then ACh pulses were resumed and given at 1 min intervals. Note the slow recovery from toxin block; note that washout kinetics are dependent on order of addition of toxins. Traces shown are representative, all experiments were repeated for a n = 3.

Figure 6.

Extracellular binding domain comparison of the 5-HT₃ receptor and α9α10 nAChR. The binding loops of the 5-HT₃ receptor were aligned with the corresponding loops of the α9 and α10 nAChR subunits. For the α9 nAChR subunit, loops D, E and F from the 'complimentary face' are indicated; for the α10 nAChR subunit the A, B and C loops from the 'principal face' are shown. * = residues shown to be important for agonists and competitive antagonists of the 5-HT₃ receptor. # = residues shown to be important for the α9α10 selective antagonist α-conotoxin RgIA. Conserved residues are shaded.

Table ICrude *Conus* venoms activity on $\alpha 9\alpha 10$ nAChRs

species	10 μ g % block	SEM	1 μ g % block	SEM
<i>C. aulicus</i>	46.7	1.4	ND	
<i>C. quercinus</i>	53.7	7.4	ND	
<i>C. virgo</i>	76.5	1.5	ND	
<i>C. litteratus</i>	15.7	4.6	ND	
<i>C. geographus</i>	98.8	0.4	84.7	3.4
<i>C. brettehami</i>	91.0	1.2	13.8	2.4
<i>C. radiatus</i>	82.3	5.4	21.6	8.1
<i>C. consors</i>	96.1	0.8	38.1	6.6

SEM, standard error of the mean; ND, not determined.

Table II α S-GVIIIB selectivity

nAChR subtype	IC ₅₀
α 3 β 2	~1 μ M
α 6/ α 3 β 2 β 3	~1 μ M
α 3 β 4	> 1 μ M
α 4 β 2	> 1 μ M
α 7	> 1 μ M
α 9 α 10	9.79 (6.6-14.4) nM
α 1 β 1 δ ϵ	> 1 μ M
5-HT ₃	> 1 μ M

(), 95% confidence interval

Table III $\alpha 9\alpha 10$ nAChR-targeting conotoxins

Conotoxin	Superfamily	Sequence	IC ₅₀	Ref
α -RgIA	A	GCCSDPRCRYRCR	4.6 nM	(26)
α -Vc1.1	A	GCCSDPRCNYDHPEIC-amide	109 nM	(32)
α B-VxXXIVA	B3	VRCLEKSGAQPNKLFPPCCQKGPSFARH SRCVYYTQSRE	1.2 μ M	(35)
α S-GVIIIIB	S	SGSTCTCFTSTNCQGSCECLSPPGCYCSN NGIRQRGCCTCPGT-amide	9.8 nM	This study

Table IVSequence alignment of venom purified σ -conotoxins

Toxin	Signal Sequence	Propeptide
α S-GVIII B	MMSKMGAMFVLLLLFTLASS	QEGDVQARKTRPKSDFYRALPR
σ -GVIII A	MMSKMGAMFVLLLLFTLASS	LQEGDVQARKTRLKSDFYRALARDDR
α S-RVIII A	MMSKMGAMFVLLLLFTLASS	QEGDVQARKTHPKREFQRILLRSGR
Mature Toxin		
α S-GVIII B	SGSTCT---CFTSTN--CQGSCECLSPPGCYCSNNGIRQR-GCSCTCPGT	
σ -GVIII A	---GCTRT-CGGPK---CTGTCTCTNSSKCGCRYNVHPSGWGCGCAC	
α S-RVIII A	---KCNFDKCKGTGVYNCGESCSCEGLHSCRCTYNIGSMKSGCACICTYY	