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Isolation and Characterization of a Novel α-Conotoxin from the Venom of Vermivorous Cone Snail *Conus parvatus*

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In this paper, the isolation, characterization and total synthesis of a small peptide ligand for nicotinic acetylcholine receptors (nAChRs) are reported, which targets the neuromuscular nicotinic acetylcholine receptor with high affinity in frog. The new peptide is α-conotoxin obtained from the snail *Conus parvatus* which preys on polychaete worms. The sequence of the peptide is: Gly-Cys-Cys-His-Pro-Arg-Cys-Gly-lys-Arg-Tyr-Asp-Cys-NH_2. The peptide shows slight homologous sequence difference from all α-conotoxins of fish-hunting *Conus*, but its disulfide-bridging is similar: [2-7; 3-13], It is suggested that cone venoms may provide an array of ligands with selectivity for various nAChR subtypes.

Key Words: Conotoxin, Ion channel, Amino acid sequence, Peptide synthesis.

INTRODUCTION

Acetylcholine acts on nicotinic acetylcholine receptors (nAChRs)[1] to mediate fast excitatory neurotransmission or to modulate neurotransmitter release. Mammalian nicotinic acetylcholine receptors can be divided into two groups: 1) those found in skeletal muscle, which have the subunit composition of $(a)_2\beta\gamma\delta$ in developing muscle and $(\alpha)_2\beta\in\delta$ in mature muscle; and 2) those found in neurons, which are much more diverse and believed to be made up of combinations of $\alpha(\alpha_2\text{-}\alpha_7)$ and β $\beta(\beta_2\text{-}\beta_4)$ subunits[1].

A number of valuable nicotinic antagonists have been described[2,3], few are highly subtype selective, particularly in the case of neuronal nAChRs. d-Tubocurarine, an alkaloid from the *Chondrodendron tomentosum* bush, used for centuries as an arrow poison to kill wild game, blocks both muscle and neuronal nAChRs. In addition it binds to all neuronal nicotinic-receptors with more or less similar affinities. Likewise, dihydro-β-erythroidine, the hydrogenated derivative of erythroidine, isolated from trees and shrubs of the genus *Erythrina* is a competitive antagonist at both muscle and neuronal nAChRs. *Lophotoxin*, a small cyclic diterpene, is used by the soft shell coral *Lophogorgia rigida* to discourage its consumption by fish. This toxin forms a covalent bond with Tyr^{190} of the α-subunit of Torpedo nAChRs,

irreversibly blocking the binding of ACh to the receptor. Neosuragatoxin, α-glycoside from the gastropod *Babylonia japonica*, potently but non-selectively blocks $\alpha_x\beta_2$ nAChRs expressed in oocytes, where x is 2, 3 or 4. The synthetically derived small molecules trimethaphan and mecamylamine discriminate between ganglionic and neuromuscular nAChRs and are used clinically as ganglionic blocking agents.

Conus is a large genus of predatory snails that feed on fish, snails and marine worms. Their venoms are complex, often containing hundreds of different peptidic and non-peptidic components. The peptides are notable for their small size, which allows for straightforward chemical synthesis, potency and receptor subtype selectivity[4-7]. For instance, ω-conotoxins are now the defining ligand for N-type (neuronal) calcium channels[8-11] and have become standard reagents for inhibiting neurotransmitter release. Similarly, μ-conotoxins selectively antagonize muscle sodium channels[12,13] and conantokins specifically target the N-methyl-D-aspartate-subtype of glutamate receptors[14-16] and appear to distinguish between subtypes of N-methyl-D-aspartate receptors[17]. These toxins are available in synthetic form and are routinely used by numerous investigators due to their specificity and ready availability. Nevertheless, only a small fraction of the hundreds of conus venom have been analyzed.

Another series of structurally and functionally related peptides from *Conus* venoms are the α-conotoxins. These are small, disulfide-rich peptides that target to nAChRs of vertebrates. Like the α-neurotoxins from snake venoms, previously isolated α-conotoxins block the nAChR at the mammalian neuromuscular junction, inducing paralysis. Compared to the snake toxins however (*ca.* 80 amino acids in length), the α-conotoxins are much smaller (typically 12-15 amino acids in length)[5,7,18] and readily synthesized.

Several α-conotoxins have been characterized to date from the venoms of the fish-eating cone snails (*C. geographus, C. magus* and *C. striatus*). The great majority of the 500 species of cone snails, however, do not use their venom to paralyze fish, but rather to paralyze polychaete worms (>100 species), other molluscs (50-100 species) and various invertebrates such as echiuroid worms and hemichordates. Each cone species is typically very specialized, sometimes eating only one prey species. Since, the venoms are essential for paralyzing prey and many of the known prey of *Conus* use cholinergic transmission at their neuromuscular junctions, it seems likely that the venoms of each cone snail species will have at least one nAChR antagonist. Correspondingly, because of the wide diversity of different prey and presumably of their respective nAChRs, the cholinergic peptides found in *Conus* venoms are likely to include ligands for the ontogenetically diverse array of receptors within the mammalian system. As part of our broader effort to identify toxins that might serve as ligands for muscle nAChRs in

the mammalian neuromuscular junction, a venom is analyzed from a non-fish-hunting *Conus* species to explore whether it might have significantly different α-conotoxins. In this report, the purification and characterization of α-conotoxin isolated from a vermivorous cone snail, *Conus parvatus* is described and to demonstrate the biochemical and pharmacological properties of the characterized α-conotoxin.

EXPERIMENTAL

Crude venom from dissected ducts of *Conus parvatus* was collected in the Porto nova, Chidambaram, India lyophilized and stored at -20 °C until use. J3-Mer-captoethanol was from SD Fine Chemicals; dithiothreitol was from Ranbaxy laboratories; trifluoroacetic acid (sequencing grade) was from Aldrich; acetonitrile (UV grade) was from Qualigens Iodoacetamide and α-bungarotoxin (α-BTX) were from Sigma. α-Conotoxin GI (tt-CTx-GI) was synthesized as described previously[19]. *tris*-(2-Carboxyethyl)phosphine (TCEP) was synthesized by the method of Burns *et al.*[20].

Peptide isolation and sequencing

Peptide enrichment: Crude *Conus parvatus venom* (100 mg) was extracted with 0.1 % trifluoroacetic acid at 4 °C as previously described[21]. The supernatant from this extraction was transferred to a Centri-con 3 Microconcentrator (Amicon, Beverly, MA) which has a 3,000 Mr cut-off. The Centricon Microconcentrator was centrifuged at 7,500 rpm in a Sorvall SS-34 rotor overnight at 4 °C and the filtrate was used for further purification.

HPLC Purification: For isolation of peptide from venom, buffer A consisted of 0.1 % trifluoroacetic acid and buffer B was 0.1 % trifluoroacetic acid, 90 % acetonitrile. For purification and analysis of synthetic peptide, buffer A consisted of 0.1 % trifluoroacetic acid and buffer B was 0.1 % trifluoroacetic acid, 60 % acetonitrile. Peptides were fractionated on C_{18} Vydac.

Pyridylethylation and purification of modified peptide: Peptide from the final purification was stored in the 0.1 % trifluoroacetic acid/acetonitrile elution buffer. 150 µL of this purified peptide solution was combined with 30 µL of trifluoroacetic acid (0.1 %) and to this was added 60 µL of reducing buffer (0.25 M Na_2HPO_4, 100 min/3-mercaptoethanol, 10 mM EDTA and 10 mM dithiothreitol). The reducing buffer was sufficient to raise the pH to a value between 7 and 8 as measured by pH paper. The reaction vessel was flushed with argon and the reaction incubated at 65 °C for 15 min. The solution was allowed to cool; 2 µL of 4-vinyl pyridine was added and the solution was reacted for an additional 25 min at room temperature in the dark. The solution was then diluted 3-fold with 0.1 % trifluoroacetic acid and the alkylated peptide was purified on the C_{18} Vydac column with gradient program 2.

Sequence analysis: Sequencing was performed with Edman chemistry on an applied biosystems model 477A protein sequencer.

Peptide synthesis and disulfide analysis

Linear peptide: Linear peptide was built by the manual solid phase method following a butoxycarbonyl/benzyl strategy and employing a *p*-methylbenzhydrylarmine resin to obtain the peptide amide. All amino acids were purchased from Nice chemicals Ltd. Side-chain protection used was: S-methylbenzyl-L-cysteine, β-O-benzyl-L-serine, L-aspartic acid β-cyclohexyl ester and N^g-toluenesulfonyl-L-arginine. Coupling reactions were carried out using 2-(1*H*-benzotriole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)[22]. Prior to HF cleavage, the α-amino function of the last amino acid was deprotected by treatment of the resin with trifluoroacetic acid/ethane dithiol/dichloromethane (50/5/45) for 0.5 h. For cleavage, 3 g of peptidyl-resin was treated with liquid HF at 0 °C in the presence of anisole (10 %) and dimethylsulfide (3 %) for 75 min. This procedure simultaneously cleaved peptide from the resin and deprotected all side chains.

Peptide cyclization: After removal of HF under vacuum, the linear peptide and resin were washed with ether and the mixture (peptide + resin) was introduced into 3500 mL of isopropanol/H_2O (50/50) and gently stirred. The pH (checked by pH paper) was maintained at 8-9 by adding diisopropylethylamine during the air oxidation reaction. Progress of the oxidation was monitored by HPLC and the Ellman test[23] and was judged to be complete after 5 d. The solution was filtered to remove the resin, the pH was adjusted to 5 by the addition of acetic acid and isopropanol was evaporated. After dilution with water, the peptide was purified by preparative reversed-phase HPLC on a Vydac C_{18} column, using a gradient of acetonitrile in 0.1 % trifluoro-acetic acid. Its identity with natural peptide was verified by co-elution experi-ments on HPLC and capillary zone electrophoresis (CZE), by mass spectro-metry and by disulfide "fingerprinting" analysis.

Disulfide analysis: The partial reduction strategy of Gray[24] was used. 20 nanomoles of synthetic peptide, as eluted from HPLC, was incubated at room temperature with 10 mM TCEP in 0.1 M citrate, pH 3. After 2-5 min the reaction mixture was diluted with 0.1 % trifluoroacetic acid to decrease acetonitrile concentration and immediately injected onto the HPLC column. Peptide fractions, as identified by UV absorbance were collected manually into 1.5 mL polypropylene centrifuge tubes. Monocyclic intermediates were alkylated with 100 mg of iodoacetamide using the rapid alkylation method[24]. Labelled peptides were submitted to determine sequence analysis to determine the locations of S-carboxamidomethyl-L-cysteine residues and hence, the disulfide connectivity.

Disulfide fingerprinting: Approximately 200 pmol of peptide in 50 µL of HPLC eluent were mixed with 50 µL TCEP (20 mil in 0.2 M citrate, pH

3). After brief incubation at room temperature, the mixtures were analyzed by HPLC to compare product distributions of natural and synthetic materials.

Electro physiology

Intracellular recording from *R. pipiens* muscle fibers: Freshly dissected cutaneus pectoris muscle was pinned on a thinly Sylgard-coated (< 0.5 mm) 25 mm diameter #1 coverslip and placed in an *ca.* 1.2 mL of chamber, which was perfused with Ringer's solution consisting of Na HEPES (10 mM), pH 7.2, NaCl (111 mM), $CaCl_2$ (1.8 mM) and KC1 (2 mM). Intracellular recording and iontophoretic application of carbamylcholine were performed under visual control as described previously[25].

Iontophoretic application of carbamylcholine (CCh) and focal toxin application: The tip of the iontophoretic CCh pipette (containing 1 M CCh) was placed near a neuromuscular junction and CCh was ejected with current pulses (*ca.* 50 nA, 5 ms duration) applied at a frequency of 0.1 Hz. The response to each pulse was recorded and the peaks of the response amplitudes were plotted. A puffer-pipette was used to apply peptide toxin as previously described[26]. Specifically here, a glass micropipette (tip inner diameter, 6 μm) was filled with 50 μm new-toxin in Ringer's. Its tip was placed about 50 μm away from the tip of the CCh pipette at the neuromuscular synapse and peptide was ejected by application of puffs of pressure (2 psi, 50 ms duration) to the pipette. The puffer pipette was placed at a fairly large distance (50 μm) to insure that (1) the toxin diffused to a postsynaptic area which encompassed the area affected by iontophoresed CCh and (2) the puff of toxin did not mechanically disturb the position of the tip of the CCh pipette relative to the postsynaptic membrane. Data were acquired on a computer with an A/D board (Lab NB, National Instruments,) and our own virtual instrument (constructed with the graphical programming language LabVIEW also from National Instruments).

Toxin effect on miniature endplate potentials (MEPPs): Spontaneous MEPPs were recorded intracellularly as above. To facilitate recording of MEPPs, their amplitudes and durations were increased by irreversibly blocking acetylcholinesterase in the preparation by exposing the muscle to 1 mM of melathion (SD Fine Chemicals Ltd.) in Ringer's for 1 h[27]. Spontaneous MEPPs were acquired on a computer as described above with a virtual instrument which captured all transients with amplitudes exceeding 0.4 mV. The recordings of individual transients were visually inspected off-line and all those which did not have profiles characteristic of MEPPs were culled out.

Extracellular recording in *R. pipiens*: Extracellular recording of post-synaptic responses to motor nerve stimulation in a cutaneus pectoris muscle from *R. pipiens* was performed essentially as described previously[28]. Specifically, the muscle was placed in a Sylgard recording chamber (15 mm

long × 1 mm wide × 2.5 mm deep); the motor nerve was passed through a petroleum jelly (Vaseline®) gap into an adjacent well containing embedded Pt stimulating electrodes. The muscle was bathed with frog Ringer's solution supplemented with 0.25 mg/mL lysozyme to minimize possible non-specific absorption of the conotoxin. α-Conotoxin was dissolved in normal frog Ringer's containing 0.25 mg/mL lysozyme. Toxin was applied to the preparation and a plastic coverslip was placed over the recording chamber to minimize buffer evaporation. The motor nerve was electrically stimulated about once every 30-60 s with a supramaximal 0.1 ms pulse. The resulting action potential in the muscle was recorded extracellularly by means of Pt electrodes (embedded in the Sylgard chamber) connected to a Grass amplifier (model P15B). Data was captured by computer as described earlier.

Extracellular recording from electrocytes in eigenmannia: Fish were anesthetized by chilling in ice-cold water and decapitated and the distal *ca.* 4 cm of the tail was removed and further prepared as follows: starting at the anterior end of the piece of tail, the entire skin surrounding the tail was peeled away from the immediately underlying tissue (consisting of muscle/electrocytes). The tissue surrounding the anterior end of the spinal column was removed over a 2-4-mm length *ca.* 14 mm from the distal end of the tail to yield a spinal stump. The posterior tip (14 mm) of the tail was severed away and the preparation was placed in a recording chamber constructed of Sylgard (15 mm long × 2 mm wide × 2 mm deep) filled with Hickman's solution (110 mM NaCl, 2 mM KC1, 3 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM $NaHCO_3$, 1 mM $Na_2H_2PO_4$, pH 7.2) plus 0.25 mg/mL lysozyme. The spinal stump was placed across a petroleum jelly gap and electrically stimulated (*via* Pt electrodes on either side of the gap) every 30-60 s with a supramaximal 0.1 ms pulse. The resulting action potential from the electrocytes or electric organ discharge (EOD) was recorded extracellularly with Pt electrodes embedded in the chamber. Recording electronics were same as described for the *R. pipiens* preparation above. Toxins were dissolved in the Hickman's solution plus lysozyme buffer and applied directly to the bath. Recordings were taken for 20-25 min.

RESULTS AND DISCUSSION

Isolation of α-conotoxin: The main aim is to identify α-conotoxin type peptides from non-fish-hunting *Conus* venoms, with an emphasis on those which might act on subtypes of mammalian muscular nicotinic acetylcholine receptors. The initial venom examined and the subject of this report, was that of the *Conus parvatus* obtained by dissecting venom ducts from specimens mostly collected off Porto nova, Chidambaram, India (Fig. 1). This species hunts the polychaete worm, *Eurythoe complanata*.

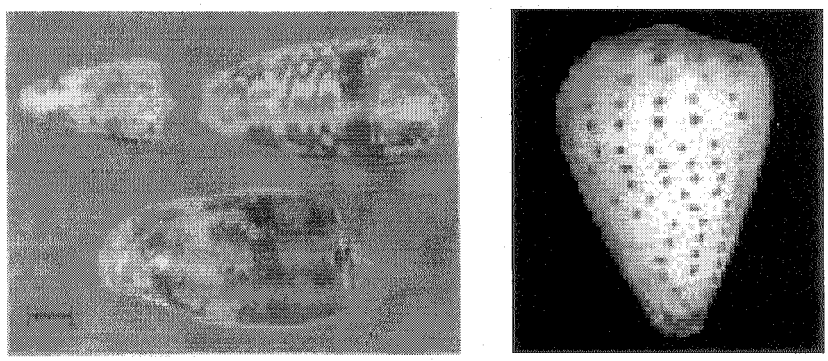

Fig. 1. Cone snails from which α-conotoxins have been isolated. Bottom panel, *C. parvatus*. Top panel, three fish-eating cone snails: *C. magus* (top left), *C. striatus* (top right) and *C. geographus* (bottom)

Several guidelines were used to identify candidate peptide with the desired characteristics. Since, it was likely to be related structurally to the α-conotoxins, which are the smallest of the major toxins in fish-hunting cone venoms. The smaller peptides found in *C. parvatus* venom was examined first. The first step in purification involved filtration through a membrane with a cut-off of 3000 daltons (the Centricon 3 microcon-centrator).

The filtrate was then purified using a C_{18} reverse phase column (Fig. 2A). The UV absorbance pattern is relatively simple, since larger peptides and polypeptides were retained by the filtration procedure.

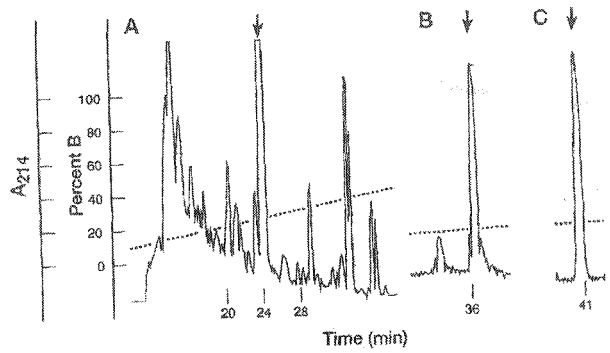

Fig. 2. Purification of new α-conotoxin from *C. parvatus* venom Buffer A = 0.1 % trifluoroacetic acid; buffer B = 0.1 % trifluoroacetic acid, 90 % acetonitrile. Panel A, venom fraction from ultra filtration (see text) was applied to a C_{18} Vydac column. Gradient program 1 was 0-45 % B/40 min, followed by 45-100 % B/15 min, Panel A material eluting at 23.9 min (arrow in panel A) was rerun on the same column with gradient program 2 (2-10 % B/3 min; 10-25 % B/57 min). Panel C, material eluting at 36.4 min (arrow in panel B) was rerun on the Microsorb column with gradient program 2 to obtain the final purified product. The solid line is the absorbance at 214 nm and the dashed line is the gradient of acetonitrile

Synthetic peptide prepared as described in methods co-eluted with natural peptide on HPLC and CZE (Fig. 3) and had the correct molecular mass (monoisotopic MH^+: calculated, 1351.48; found, 1351.4). Partial reduction by TCEP of 200 pmol of natural and synthetic peptides gave essentially identical product profiles on HPLC (Fig. 4). This is a sensitive test of identity of disulfide bridging. The two monocyclic peptides R1A and RIB were isolated from a larger scale reduction of synthetic material and alkylated with iodoacetamide. Sequence analysis showed that R1A

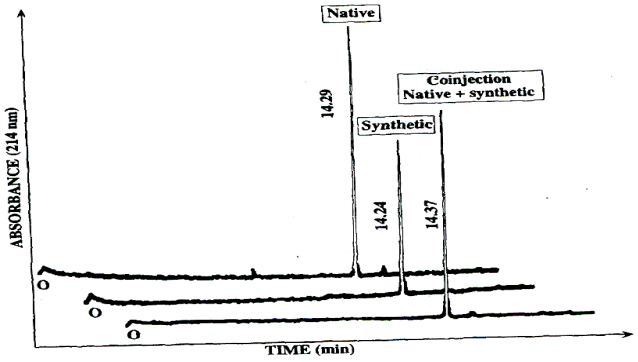

Fig. 3. Comparison of natural and synthetic new α-conotoxin by capillary zone electrophoresis (CZE). Analysis was performed at 30 PC on a Beekman P/ACE System 2060, monitored at 214 nm, with Spectra Physics ChromJet SP4400 integrator. Peptides were applied by pressure injection onto fused silica capillaries from Beckman (50 am × 75 um). Buffer was 100 MM H_3PO_4 adjusted to pH 2.5 by addition of 2 2 M NaOH. Analysis was carried out at 15 kV, 80 mA

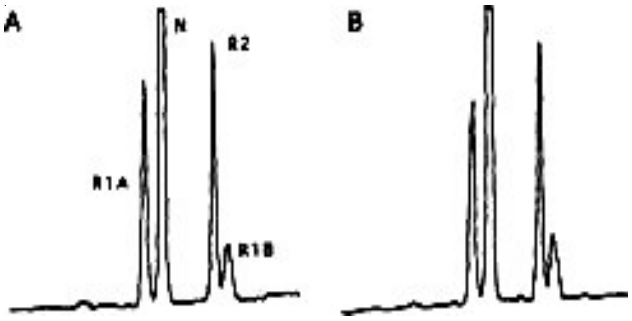

Fig. 4. Disulfide fingerprinting of synthetic (panel A) and natural (panel B) new α-conotoxin partial reduction was carried out by incubating 200 pmol of peptide with 10 μm TCEP in 100 mL of 0.1 M sodium citrate, pH 3, for 4 min at 25 °C. Samples were analyzed by reverse-phase HPLC as described under 'experimental procedures'. Gradient was 15-35 % B in 40 min; segment depicted in each case is from 27-35 % B. Peptides corresponding to the absorbance peaks

was alkylated exclusively at Cys-2 and Cys-7, while RIB was alkylated exclusively at Cys-3 and Cys-13. Thus the disulfide bridging pattern is [2-7; 3-13], similar to that in α-conotoxin GI: [2-7; 3-13], where brackets enclose pairs of numbers denoting the partners in a disulfide (Table-1).

TABLE-1
SEQUENCE ANALYSIS OF NEW α-CONOTOXIN FROM *C. parvatus*
PTH, PHENYLTHIOHYDANTOIN DERIVATIVE

Step	Assigned residue	Yield (pmol of PTH)
1	Gly	118.2
2	Cys	240.4
3	Cys	253.5
4	His	64.0
5	Pro	87.1
6	Arg	92.3
7	Cys	76.2
8	Gly	114.3
9	Lys	88.6
10	Arg	17.8
11	Tyr	50.3
12	Asp	30.0
13	Cys	40.0

Thus, although most of the sequence is slight divergent and the number of amino acids within the disulfide loops is same from those in previously isolated peptides, the disulfide arrangement clearly places the peptide in the α-conotoxin class.

Activity of α-conotoxin at neuromuscular junctions: The cysteine framework and biological activity of new α-conotoxin strongly suggested that it is a ligand for an nAChR. To test this hypothesis electrophysiologically, experiments were performed on the frog neuromuscular junction. Application of toxin from a pipette containing 50 μm toxin blocked depolarizations induced by iontophoretically applied carbamylcholine (Fig. 5). Fig. 6 shows that bath-applied peptide (0.2 μm) blocks MEPPs. At this concentration of toxin, the mean MEPP amplitude was reduced to 29 % of the control value. There was no effect on MEPP frequency indicating that the peptide has no apparent presynaptic effects. When higher concentrations of toxin were used (*e.g.* 50 μm applied through a puffer pipette), complete but reversible block of MEPPs occurred (results not shown). These results indicate that the toxin blocks the postsynaptic nAChR.

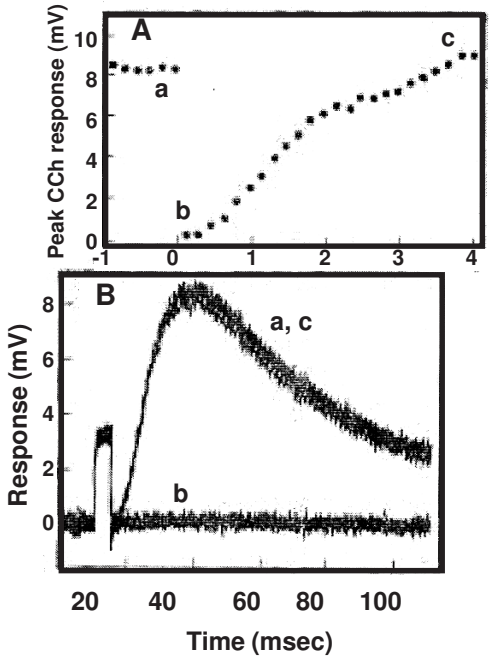

Fig. 5. Panel A, time course of block by new α-conotoxin. CCh was ionto-phoretically applied every 10 g throughout the experiment. At time zero, a puff of new α-conotoxin was delivered from a pipette filled with 50 μm peptide. The response to CCh immediately declined, then gradually recovered, a, just before toxin puff; b, immediately after toxin puff; c, after recovery. Panel B, sample responses to CCh before, during and after toxin application. Two responses at each time point are shown (time correspond to points indicated by a, b and c in Panel A). The fixed, 5 ms iontophoretic pulse of CCh was applied 10 ms into the trace (note rectangular artifact with fixed amplitude and duration from ms 10 to 51). The application of toxin totally blocked the CCh response[6]. The response recovered completely when toxin had diffused away. Note that traces a (before toxin) and c (after recovery) are virtually superimposable. The resting potential remained essentially constant throughout this period, indicating that the toxin had no untoward effects on the muscle membrane *per se*

Since new α-conotoxin was isolated from a worm-hunting cone, it was of interest to compare its phylogenetic activity profile to that of α-CTx-GI (isolated from a fish-hunter). Extracellular recording of synaptically evoked action potentials at the frog neuromuscular junction and electrocytes from the weakly electric fish *Eigenmannia* was used for this purpose.

The IC_{60} of new α-conotoxin was 250-500 nM in frog. Under the same conditions, α-CTx-GI caused inhibition of the action potential with an IC_{50} of 2-4 μm. Effects of both toxins were reversible with 0.5 h of washing (results not shown). Thus, new α-conotoxin is significantly more potent than α-CTx-GI at the frog neuromuscular junction.

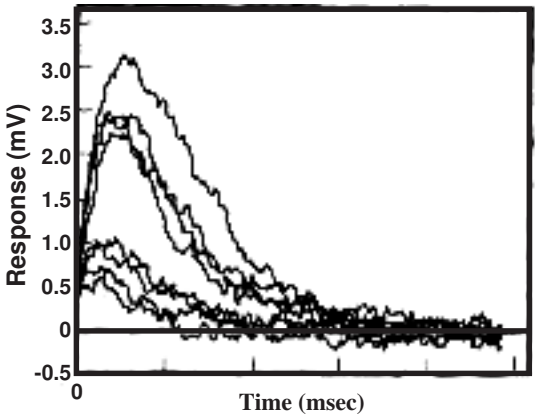

Fig. 6. New α-conotoxin reduces MEPP amplitude but not frequency. Shown are sample traces of MEPPs before (largest four responses) and during (smallest four responses) exposure to 200 nM toxin. Mean amplitude ± SD of MEPPs was 2.37 ± 0.97 mV (control, n = 59) and 0.69 ± 0.26 (toxin present, n = 67). The frequency of MEPPs was essentially unaffected by toxin; MEPP frequency was 6.8/min in control Ringer's and 6.3/min when toxin was in the bath. This slightly lower apparent MEPP frequency in the presence of toxin is thought to result from an increased frequency of unrecorded MEPPs, whose amplitudes were too low to trigger capture by the computer

However, the reverse order of potency was found when these peptides were tested on electrocutes from *Eigenmannia*, new α-conotoxin had an IC_{50} of 25-50 μm, while α-CTx-GI had an IC_{50} of 50-100 nM. Thus, α-CTx-GI is *ca.* 50 times more potent than new α-conotoxin in blocking a teleost neuromuscular preparation. while in the amphibian system, new α-conotoxin is 8-fold more potent than α-CTx-GI.

Relative potencies of new α-conotoxin and α-conotoxin GI: The potency of new α-conotoxin and α-CTx-GI was compared electrophysiologically in fish and frog. The concentration (in μm) of toxin required to inhibit the synaptically mediated action potential by at least 50 % was determined new α-conotoxin is 50-fold more potent in frog than in fish. In contrast, α-CTx-GI is 40-fold less potent in frog thain fish.

The venom of *C. parvatus* is a complex mixture of biologically active agents. This report describes the paralytic peptide characterized from *C. parvatus* the novel nAChR antagonist. Numerous other peptides are present which remain to be fully characterized. Since ACh is the neurotransmitter at annelid neuromuscular junctions, it is reasonable to suppose that a peptide targeted to the nAChR would be a major component of the venom of a vermivorous species such as *C. parvatus*. On the other hand, since the invertebrate prey of *C. parvatus* are quite distinct phylogenetically from

the fish prey of previously characterized cones (*e.g.*, *C. geographus*), it is not surprising that their respective α-conotoxins would differ both structurally and functionally.

Structural relationships: Previously characterized α-conotoxins can be divided into two structural classes. These two classes have distinctive sequence features, particularly in the second disulfide loop where the presence of a proline residue in α-CTx-SI and α-CTx-SII is a diagnostic feature. In other respects however, all previously characterized α-conotoxins have a core sequence CC(N/H)PACGXX(Y/F)XC in common.

Phylogenetic specificity for muscle nAChRs: In view of the slight structural divergence, it is not surprising that new α-conotoxin has a unique functional profile. Based on their activity in phylogenetically distinct organisms, the two previously characterized groups of α-conotoxins have distinctive specificities. The first group (typified by α-CTx-GI and α-CTx-MI) are active in all vertebrates tested including mammals, while the second group (typified by α-CTx-SI and α-CTx-SII), are highly potent in teleosts, but have little activity in mammals.

The cutaneus pectoris preparation from *R. pipiens* have been used to demonstrate that new α-conotoxin is a potent inhibitor of the nAChR in frog neuromuscular junction. In contrast, however, new α-conotoxin two orders of magnitude less potent in blocking the synaptically mediated action potential in electrocytes from weakly electric fish. Thus, in accord with its very distinct amino acid sequence, the phylogenetic activity profile of new α-conotoxin places this peptide into a novel category. α-BTX has at least two different mammalian nAChR targets, the peripheral neuromuscular receptor and a central nervous system nAChR subtype(s), which recent work suggests includes α_7-containing nAChR complexes. α-CTx-GI appears to selectively block the neuromuscular receptor[29,30] and has no activity in the central nervous system. In contrast, new α-conotoxin inhibit the mammalian neuromuscular nAChR, but is centrally inactive.

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