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A novel 13 residue acyclic peptide from the marine snail, *Conus monile*, targets potassium channels

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Abstract

A novel 13-residue peptide Mo1659 has been isolated from the venom of a vermivorous cone snail, *Conus monile*. HPLC fractions of the venom extract yielded an intense UV absorbing fraction with a mass of 1659 Da. De novo sequencing using both matrix assisted laser desorption and ionization and electrospray MS/MS methods together with analysis of proteolytic fragments successfully yielded the amino acid sequence, FHGGSWYRFPWGY-NH_2. This was further confirmed by comparison with the chemically synthesized peptide and by conventional Edman sequencing. Mo1659 has an unusual sequence with a preponderance of aromatic residues and the absence of apolar, aliphatic residues like Ala, Val, Leu, and Ile. Mo1659 has no disulfide bridges distinguishing it from the conotoxins and bears no sequence similarity with any of the acyclic peptides isolated thus far from the venom of cone snails. Electrophysiological studies on the effect of Mo1659 on measured currents in dorsal root ganglion neurons suggest that the peptide targets non-inactivating voltage-dependent potassium channels.

Keywords: *Conus* peptide; *Conus monile*; Acyclic peptide; Mass spectrometry; Electrophysiology; Non-inactivating voltage-dependent potassium channel

The venoms of cone snails are primarily a complex mixture of biologically active peptides, which target a diverse range of ion channels and receptors, permitting rapid immobilization of prey [1–4]. The polypeptide toxins acting in concert ("a cabal") simultaneously target several critical signaling pathways in the central nervous system of worms, fishes, and molluscs [5,6]. The ability of these peptides to act as selective ligands for receptors and channels in the mammalian nervous system has triggered considerable interest in their chemistry and neuropharmacology. Small, disulfide bonded polypeptides, conotoxins, have been the most extensively studied components of cone snail venom. Some reports of biologically active acyclic peptides have also appeared. These include, the conantokins, a γ-carboxy glutamate containing peptide [7,8], contulakin, a neurotensin receptor antagonist [9], and conorfamide, which appears to act in a manner similar to the FMRF-amide neuropeptide family [10]. The molecular diversity of K^+ channels is larger than any other group of ion channels, with more than 80 different genes and many splice variants. The diversity is strikingly observed in the central nervous system, with numerous subtypes of neurons expressing a unique set of potassium channels. The voltage gated potassium channels are responsible for the repolarization of the action potential in neurons. In this study, we describe the characterization of a novel 13 residue peptide Mo1659 isolated from the venom of *Conus monile*, a vermivorous snail found off the south east coast of India, which targets non-inactivating voltage-dependent K^+ channels. The mass spectrometrically determined sequence FHGGSWYRFPWGY-NH_2

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is remarkable in that it contains a high proportion of aromatic amino acids and is completely devoid of the common hydrophobic, aliphatic amino acids, and disulfide bridges.

Materials and methods

Peptide purification. The specimen, *C. monile*, was collected from the southeast coast of India. The venom ducts after dissection were preserved in ethanol and the venom that oozes out was subjected to HPLC purification after concentration on a rotavapor. Crude venom extract was applied onto a Jupiter 4 μ, Proteo 90 Å, C_{18} column (10 mm × 250 mm) and eluted with a linear gradient of acetonitrile containing 0.1% TFA. The flow rate was maintained at 1 ml min^{-1} and the absorbance was monitored at 226 nm. Fractionation into several peptide components was achieved.

Reduction and alkylation. The purified peptide was dissolved in 30 μL, 0.05 M NH_4HCO_3 buffer, pH 8.0. For the reduction, 200 mM stock dithiothreitol (DTT) was added to a final concentration of 8 mM and incubated at 37 °C for 2 h followed by addition of iodoacetamide stock solution, to get a final concentration of 40 mM. The resulting mixture was incubated at room temperature in the dark, for 1 h and subsequently analyzed by MALDI MS.

Acetylation. The peptide solution was dried and resuspended in a 1:1 ratio of acetic acid and acetic anhydride. After 5 min of incubation at room temperature, the resultant solution was again dried and resuspended in 0.1% TFA and acetonitrile in the ratio 1:1 (v/v) and analyzed using MALDI MS.

Trypsin digestion. The purified sample was digested with TPCK-treated trypsin (Sigma, USA) with 10 μg enzyme in 50 μl of 50 mM NH_4HCO_3, pH 8.0, for 3 h at 37 °C. The digest was analyzed using MALDI and ESI mass spectrometers.

Mass spectrometry. Electrospray ionization (ESI) mass spectra were obtained on a Hewlett Packard, HP 1100 MSD series spectrometer equipped with a single quadrupole analyzer. The data were acquired over a range of 300–1500 m/z in positive ion mode and analyzed using HP LC/MSD Chemstation software. MALDI spectra were collected using a Bruker Daltonics, *Ultraflex* TOF/TOF system, in the reflectron positive ion mode, equipped with a nitrogen laser of 337 nm. The samples were prepared by mixing equal volumes of peptide solution and saturated matrix (α-cyano-4-hydroxycinnamic acid). A standard peptide mixture was used for external calibration. ESI MS/MS data were obtained on *Esquire 3000 plus* LC ion trap mass spectrometer (Bruker Daltonics, Germany). The nitrogen gas pressure and flow rate to the nebulizer were 10 bar and 5 L min^{-1}, respectively, with a drying gas temperature of 300 °C. The scan range was set at 50–1000 m/z. The sample was dissolved in 1:1 (v/v) ratio of water and acetonitrile containing 0.1% HCOOH and was infused directly into the system delivered by a syringe pump (Cole-Parmer, Vernon Hills, IL, USA) at a flow rate of 120 μl h^{-1}. Helium was used as the collision gas for CID experiments. The data were analyzed using Esquire data analysis software, version 3.1.

Edman sequencing. The primary sequence of the peptide was determined by using a Shimadzu PPSQ-10 protein sequencer equipped with an LC-10A HPLC system.

DRG neuron preparation. Dorsal root ganglion (DRG) neurons for electrophysiological studies were prepared following the method reported by Hu and Li [11], with modifications. Postnatal male Wistar rats (P5) were anesthetized with diethyl ether. The whole vertebral column was removed and transferred to a dish containing pre-oxygenated phosphate-buffered saline. While holding the vertebral column a strip of bone from the dorsal root of the vertebral column was cut. The dorsal root ganglia together with dorsal and ventral roots were individually taken out with fine dissecting forceps and transferred into phosphate buffered saline containing 1.5 mg ml^{-1} trypsin (from porcine pancreas, Sigma, USA). The DRGs were minced with dissecting spring scissors and incubated at 37 °C for 30 min. After trypsin treatment, the cells were pelleted by centrifugation at 1000 rpm for 5 min. The supernatant was removed and washed with 1 ml DMEM containing 10% FBS. Following resuspension in fresh DMEM containing 10% FBS, single cell suspension was obtained by trituration using a fire polished Pasteur pipette. To increase the cell density, a locally fabricated 8 mm diameter optically polished glass ring was placed on the bottom of a sterile 35 mm tissue culture dish. The suspended cells were plated into the well formed by the glass ring. The cells were incubated for 1 h at 37 °C. Isolated DRG neurons were used for the electrophysiology experiments.

Electrophysiology. Isolated K^+ currents were recorded from DRG neurons using the patch-clamp technique in the whole-cell mode using an EPC-8 amplifier (Heka). Patch-clamp electrodes with resistance of 1–3 mega-ohm were made from borosilicate glass (Clark Electromedical Instruments, UK). The external bath solution contained (in mM): 130 choline chloride, 3 KCl, 2.5 $CoCl_2$, 0.6 $MgCl_2$, 10 Hepes, 1.2 $NaHCO_3$, and 10 glucose, pH 7.4 with Tris base; osmolarity, 325 mosmol, adjusted with sucrose. The internal solution contained (in mM): 140 KCl, 1 $CaCl_2 \cdot 2H_2O$, 2 $MgCl_2 \cdot 6H_2O$, 11 EGTA, and 10 Hepes, pH 7.2, with Tris base; osmolarity 310 mosmol. The neurons were voltage-clamped at −80 mV in all the experiments. Capacity and leak subtraction was done using a P/4 subtraction protocol. Using *R*s compensation of 50% in all the experiments minimized voltage errors. Data acquisition and pulse protocols were controlled with the pClamp8 software, and Digidata 1320 analog/digital converter (Axon Instruments Inc.). The currents were filtered at 3 kHz, sampled at 50 kHz for the data shown in Fig. 6 and 20 kHz for the data shown in Fig. 7. The bath temperature was maintained at 20 °C. The toxin was dissolved in water. Bolus application of the toxin was employed to achieve a final bath concentration of 200 nM. The effects of Mo1659 on the K^+ currents reported here were recorded 15 min after peptide application.

Peptide synthesis. The peptide was synthesized by standard solid phase peptide synthetic methods using Fmoc chemistry [12]. All amino acids are protected at the N-terminus with the Fmoc group (Nova Biochem). The side chains of Tyr and Ser were protected with the *t*-Bu group, Arg with Mtr group, and His with trityl group. The coupling reactions proceeded using the OPfp esters of the protected amino acids on Fmoc-Rink amide AM resin (200–400 mesh, Nova Biochem). The synthesis was performed with 300 mg resin with a bead capacity of 0.63 mMg^{-1}. The C-terminal amino acid (Tyr) was linked to the resin by the formation of an amide linkage with the amino functional group emanating from the solid support. Ser and His were coupled by using HBTU (*N*-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluoro phosphate *N*-oxide). The Fmoc deprotections were performed with 20% piperidine in dimethylformamide. Peptide was cleaved from 100 mg of resin after synthesis, using 94% TFA (7.52 ml), containing 5% anisole (400 μl) and 1% ethanedithiol (80 μl) as cation scavengers. After 5–6 h, the resin was filtered off, the TFA was removed by evaporation in vacuo, and the peptide was precipitated with ether. The precipitate was repeatedly washed with ether and purified by RP HPLC.

Results

Mo1659 was isolated from the vermivorous snail species, *C. monile*, a common species found off the Cuddalore coast of Tamilnadu, India. The RP HPLC profile of the crude venom extract is shown in Fig. 1A. Several peptide components with masses in the range 1300–2700 Da were identified by MALDI mass spectral

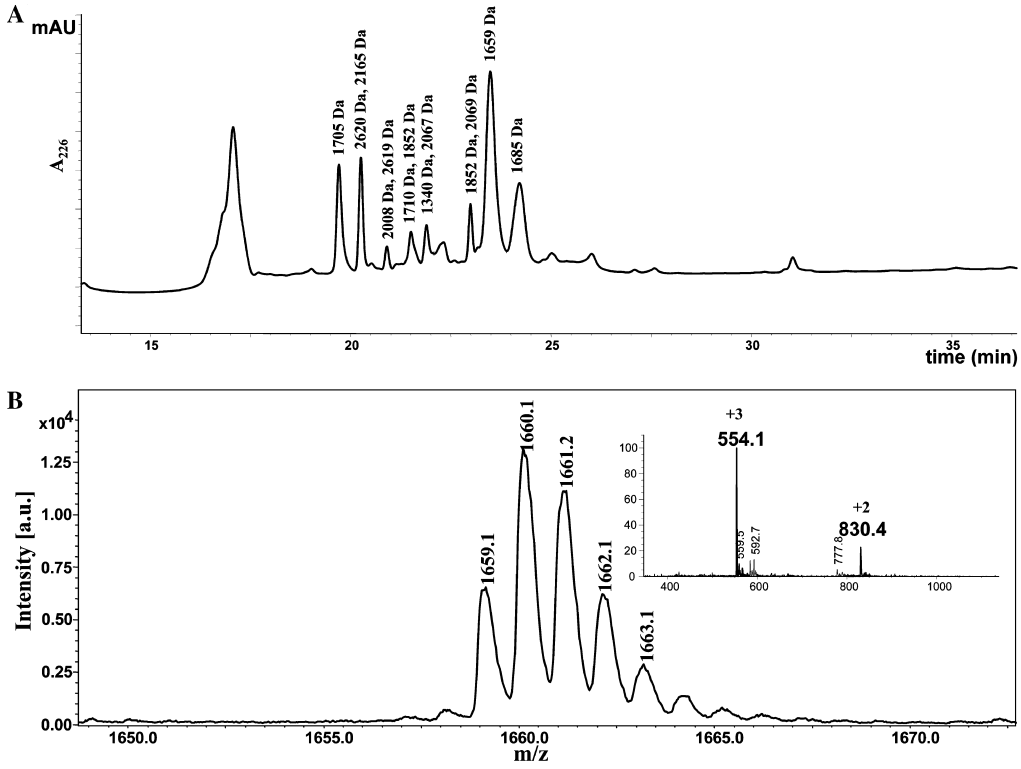

Fig. 1. (A) HPLC purification profile of a *Conus monile* venom extract. Sample was injected onto a Phenomenex C_{18} RP column (10×250 mm, 4 μ particle size and 90 Å pore size) and eluted at 1 ml min^{-1} with a linear gradient of acetonitrile, containing 0.1% TFA. The molecular masses detected by MALDI MS are indicated above each fraction. (B) High-resolution MALDI mass spectrum showing the isotopic profile of the molecular ion of Mo1659 recorded in reflectron, positive ionization mode. Inset: electrospray spectrum of Mo1659, showing the observed charge states and corresponding m/z values.

analysis of individual HPLC fractions. The intense component at the retention time of 23.4 min corresponding to a molecular mass of 1659 Da was chosen for mass spectrometric de novo sequencing. Fig. 1B shows a high resolution MALDI mass spectrum, which establishes $[M + H]^+ = 1659.1$ Da (monoisotopic mass). The inset shows the charge states observed in an electrospray mass spectrum, where the +2 and +3 states are detectable suggesting the presence of at least three protonatable groups in the molecule. Attempted reduction with DTT followed by alkylation with iodoacetamide left the molecular mass unchanged, establishing the *absence* of disulfide bonds. Acetylation with acetic anhydride and acetic acid yielded a product with a mass $[M + H]^+ = 1701.3$ Da ($\Delta m = +42$ Da) indicating the presence of a single primary amino group. UV and fluorescence spectra established the presence of both Trp and Tyr residues.

Peptide sequencing was undertaken using MALDI MS/MS techniques selecting the 1659.1 Da as the precursor ion. Fig. 2A shows the observed fragment ions along with assignments of the b and y ion series [13]. The presence of an intense b_2 ion at 285 Da permitted sequential tracing of the 8-residue segment –GGSWYR FP-. The immonium ions at 70, 110, 136, and 159 suggested the presence of the residues Pro, His, Tyr, and Trp, respectively. The b_2 ion at 285 Da could correspond to the dipeptide –FH- or –HF- at the amino terminus. The observation of mass peaks at 194.9 Da suggested the presence of the dipeptide ion –GH- or –HG-. This supports the assignment of the sequence –FHG- at the N-terminus. The paucity of intense fragments in the mass range 1200–1500 Da limited extension of the sequence at the C-terminus.

Digestion of Mo1659 with trypsin yielded two fragments with masses (MALDI) 1010 and 668 Da, corresponding to the N-terminus and C-terminus fragments, respectively. The 668 Da fragment is assigned to the C-terminus fragment –FPXX-, which has previously been detected as a fragment ion in Fig. 2A [Note that 17 Da (OH) is added to the N-terminus fragment and 1 Da (H) to the C-terminus fragment, upon hydrolysis]. The 668 Da fragment was subjected to high-energy collision induced dissociation (CID) in an ion trap mass spectrometer, using an electrospray ionization source. The ESI MS/MS fragmentation pattern observed is shown in Fig. 2B. The intense peak at 651 Da corresponds to a facile loss of NH_3 from the C-terminus suggestive of the presence of C-terminal amidation. *Conus* peptides are often posttranslationally modified, with amidation being commonly observed [14,15]. The identification of the b_2 ion at 245 Da corresponds to the

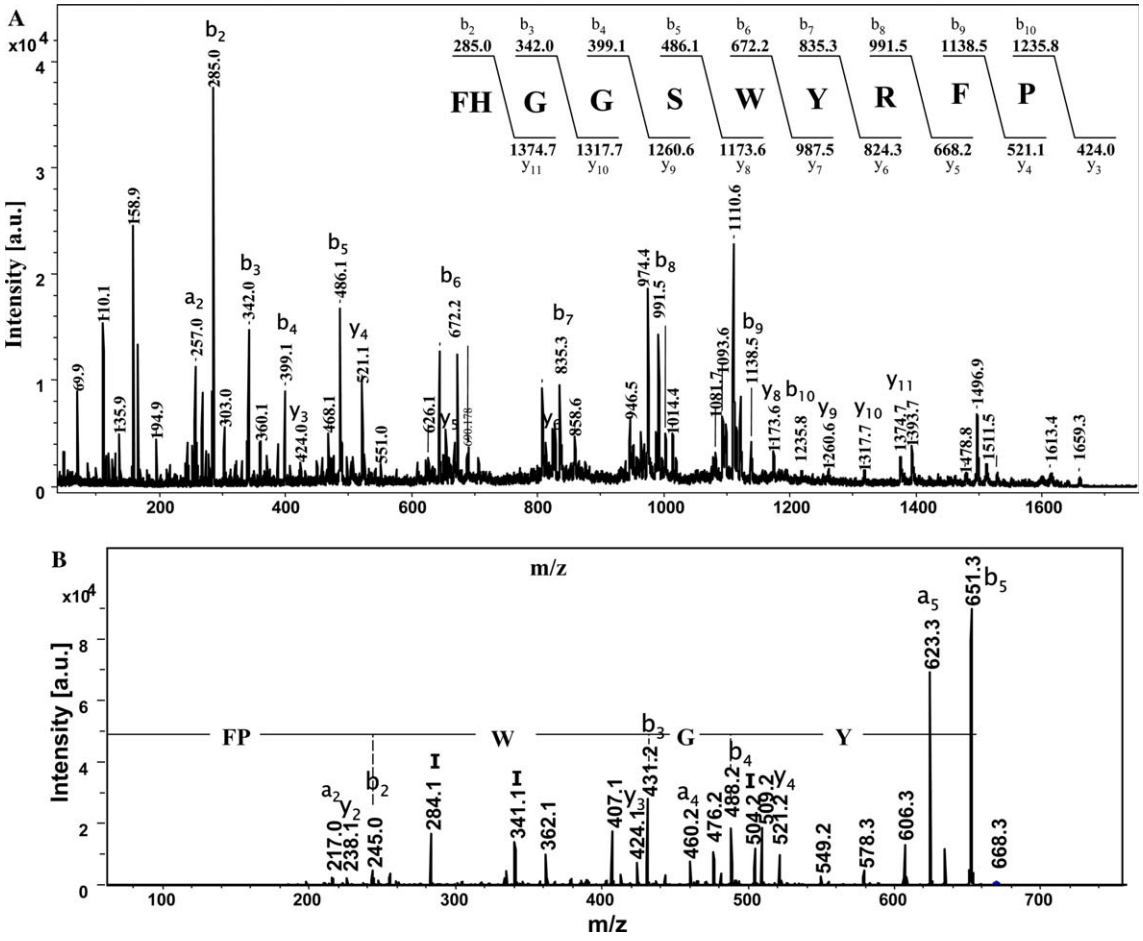

Fig. 2. (A) MALDI MS/MS spectrum of Mo1659. Monoisotopic m/z values of both *b*-series and *y*-series ions (nomenclature as proposed by Biemann [13]) are marked. Inset: the sequence assignment of the N-terminal part of Mo1659. (B) ESI MS/MS spectrum of the tryptic digest of Mo1659 showing fragmentation of the C-terminal peptide. The singly charged peptide at $m/z = 668$ was selected as the precursor ion, on an ion trap system and subjected to high-energy collision induced dissociation (CID) with helium as the collision gas. Peaks marked I, correspond to the internal fragments, –PW- (284.1 Da), –PWG- (341.1 Da), and –PWGY (504.2 Da).

–FP- fragment, already established by MALDI MS/MS, permitting the ready identification of the C-terminus tripeptide as –WGY-amide. The final determined sequence is FHGGSWYRFPWGY-NH_2, corresponding to a calculated average mass of 1659.8 Da (Average mass observed in ESI MS = 1659.3 Da).

While de novo mass spectral sequencing of peptides is an extremely powerful analytical tool, reports of sequence determination of natural peptides are relatively uncommon. Applications of these methods in the area of protein identification are either by coupling database searches or bioinformatic approaches to mass spectral sequencing [16,17]. A search of the existing protein sequence databases reveals no matches for the determined sequence of Mo1659. Confirmation of the determined sequence was achieved by two independent methods. First, a synthetic peptide corresponding to the determined sequence of Mo1659 was prepared and its MS/MS fragmentation pattern was shown to be identical to that of the natural product (Fig. 3). The identity of the synthetic and natural peptides was also established by HPLC analysis (Fig. 3, inset). Second, conventional Edman sequencing using an automated sequenator confirmed the sequence. A notable feature of Mo1659 is the presence of as many as seven aromatic amino acids (F-2, Y-2, W-2, and H-1) in a short stretch of 13 residues. The positively charged peptide is notably deficient in the common aliphatic, hydrophobic amino acids like Ala, Val, Leu, and Ile.

Mo1659 shows K^+ channel modulating activity in DRG neurons. Fig. 4 shows the effect of Mo1659 on the mixed whole-cell outward K^+ currents from a DRG neuron. A marked reduction in the current amplitudes at all the potentials was observed with 200 nM of the Mo1659 in the external bath solution. The mixed K^+ currents have a fast transient current and a sustained current component. The fast transient current component was dissected from the sustained current component using two different pre-pulse voltages followed by identical voltage protocols that are shown schematically in Fig. 5. Although the K^+ current components cannot be fully isolated using the conditioning prepulse voltages

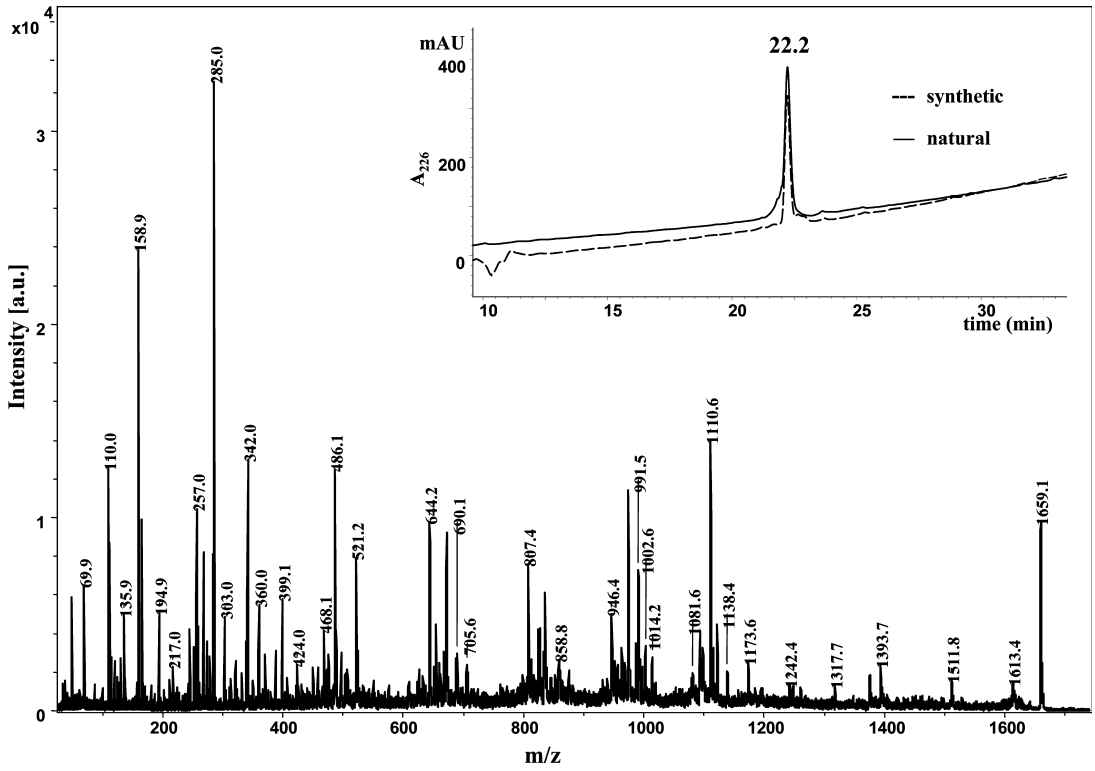

Fig. 3. MALDI MS/MS fragmentation pattern of the synthetic peptide sequence. Note identity to sequence in Fig. 2A. Inset: the superimposed HPLC profiles of natural Mo1659 and the synthetic peptide.

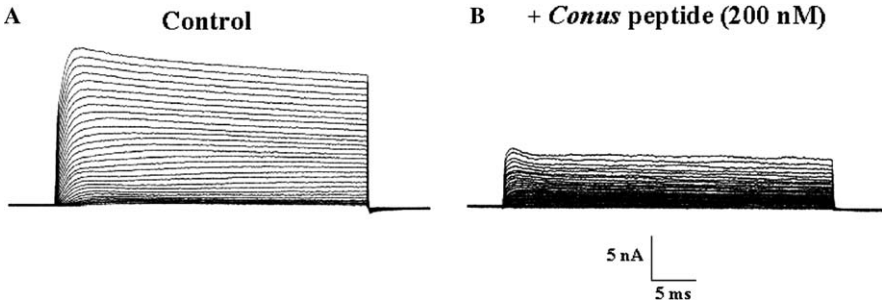

Fig. 4. Reduction of whole cell K^+ currents by Mo1659 in DRG neurons. (A) Family of outward K^+ currents elicited by the voltage steps from −50 to 100 mV in 5 mV steps. (B) Reduction of K^+ currents by Mo1659 in the bath.

alone [18], the results suggest that Mo1659 addition to the external bath solution predominantly affects the sustained K^+ current component. It may be noted that the transient current component, that was obtained following subtraction of current traces, is not significantly affected by Mo1659. Similar results were obtained in 5 different experiments. Mo1659 thus appears to affect non-inactivating voltage-dependent potassium channels.

Discussion

During the course of investigations to systematically characterize peptides from the venom of cone snails found off Indian coast, a novel 13 residue peptide, Mo1659, with the sequence FHGGSWYRFPWGY-NH_2 has been isolated from the venom of a vermivorous snail, *C. monile*. Mo1659 appears to affect non-inactivating voltage-dependent K^+ channels.

Thus far, the number of *Conus* peptides isolated, which inhibit potassium channels, is limited with only four published reports. The kappa conotoxins, which have been shown to inhibit voltage sensitive potassium channels, are 27–34 amino acid residues long and contain 3 or 4 disulfide bonds [19–22]. Mo1659 isolated in this study is unique in that it appears to be the first non-disulfide bonded peptide, which influences K^+ currents in neurons. Notably, the sequence lacks a Lys residue, but has an Arg residue placed in the center of a cluster of aromatic residues –WYRFPWGY. Until

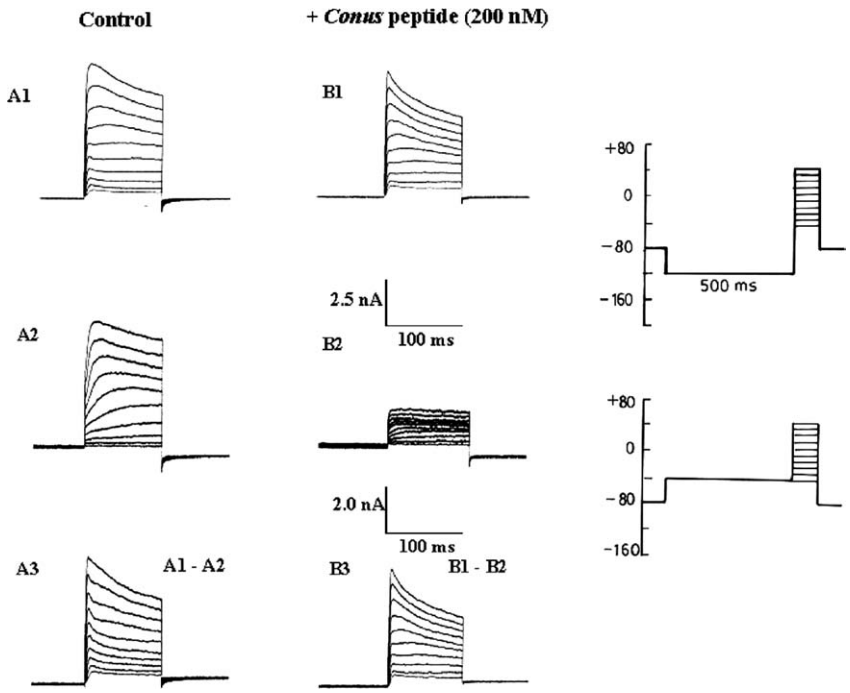

Fig. 5. Mo1659 selectively affects the non-inactivating K^+ currents. A voltage-dependent current isolation protocol was used to isolate the relative contribution of transient and sustained K^+ currents. Following a prepulse voltage step from the holding potential (−80 mV), 100 ms pulses were applied from −40 to +50 mV in 10 mV steps. A1 and B1 show family of current traces with the −120 mV prepulse protocol in control and following the addition of Mo1659 that maximizes the contribution of the transient current component. A2 and B2 show family of current traces using the −40 mV prepulse protocol that isolates the sustained current, by inactivating the transient current component. A3 and B3 show subtracted currents that reveal the transient current component. The voltage stimulation pulse protocols are schematically shown on the right. Note the decrease in non-inactivating K^+ currents in B2.

recently, structure–activity relationship studies on scorpion toxins, that target voltage gated K^+ channels, suggested the importance of a functional dyad consisting of a Lys residue and an aromatic (Tyr or Phe), separated by a distance of about 7 Å [23,24]. A recent study on Pi1, a 35 residue scorpion toxin, that acts on voltage gated K^+ channels however, suggests that the integrity of the functional dyad is not a pre-requisite for the recognition and binding of the toxin to the voltage gated Kv1.2 channels. Computed simulation of peptide docking, however, points to the important role of basic amino acid residues in the toxin binding property [25]. The positive charge of Arg may replace the key Lys of other K^+ channel toxin peptides and may be important in causing a block of the channel pore.

Earlier electrophysiological studies on dorsal root ganglion neurons have indicated the expression of at least six voltage-gated K^+ currents, three transient, and three non-inactivating currents [26]. Our preliminary electrophysiological studies suggest that Mo1659 specifically acts on the non-inactivating K^+ currents. This observation can be potentially used to further differentiate the different types of voltage-gated K^+ channels. Further studies with cloned K^+ channels and investigations of synthetic analogues are necessary to identify the target channel subtype and to establish the molecular mechanism of channel blocking activity. The small size of Mo1659, its unique sequence characteristics, and the absence of disulfide bonds make it a promising lead in the search for small molecules that specifically inhibit K^+ channels.

Acknowledgments

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