# Sodium channel modulating activity in a δ-conotoxin from an Indian marine snail

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**Abstract A 26 residue peptide (Am 2766) with the sequence CKQAGESCDIFSQNCCVG-TCAFICIE-$NH_2$ has been isolated and purified from the venom of the molluscivorous snail, *Conus amadis*, collected off the southeastern coast of India. Chemical modification and mass spectrometric studies establish that Am 2766 has three disulfide bridges. C-terminal amidation has been demonstrated by mass measurements on the C-terminal fragments obtained by proteolysis. Sequence alignments establish that Am 2766 belongs to the δ-conotoxin family. Am 2766 inhibits the decay of the sodium current in brain rNav1.2a voltage-gated $Na^+$ channel, stably expressed in Chinese hamster ovary cells. Unlike δ-conotoxins have previously been isolated from molluscivorous snails, Am 2766 inhibits inactivation of mammalian sodium channels.**


*Key words:* δ-Conotoxin; Sodium channel; Conus peptide; Mass spectrometry; *Conus amadis*

## 1. Introduction

Conotoxins, a group of pharmacologically active peptides produced by diverse species of conus snails, act with a high degree of specificity on different classes of channels and receptors in excitable cells [1,2]. The evolution of conotoxins in the venom of predator snails may be influenced by selective pressures imposed by the nature of the prey, with peptide mixtures from molluscivorous, piscivorous and vermivorous snails exhibiting differences [3]. Systematic elucidation of structure–activity relationships for all components in a conotoxin mixture is impeded by the difficulties in isolating and identifying every individual peptide. Conotoxins are characterized by multiple disulfide bridges, which provide a relatively rigid peptide backbone framework, upon which amino acid side chains, important for interaction with the pharmacological receptors, are arrayed [4]. The classification of conotoxins has relied on the distribution of Cys residues in the primary sequence, the nature of the disulfide pairing topology and the functional attributes of the peptides [5,6]. As many as 14 classes of conotoxins have thus far been identified (α, αA, δ, ε, γ, κ, λ, λ/χ, μ, μO, ρ, σ, ω and ψ). The δ-conotoxins have been shown to inhibit voltage-gated $Na^+$ channel inactivation. The specific role of the peptide δ P VIA in combination with a $K^+$ channel antagonist κ P VIIA has been shown to be critical for prey capture in the fish-hunting snail, *Conus purpurascens*. Peptide combinations (cabals), which act in concert at distinct target sites, have been suggested to be important in rapid immobilization of prey [7]. The δ-conotoxins identified thus far have polypeptide chain lengths of 27–32 amino acids and have three disulfide bridges with a pattern (1–4; 2–5; 3–6), where 1–6 indicates the six Cys residues starting from the N-terminus. The only other class of conotoxins characterized thus far that target $Na^+$ channels are the μ-conotoxins, which share a similar disulfide-bonding pattern, but have a relatively shorter polypeptide chain length of 17–22 amino acids. The isolation of δ-conotoxins from complex mixtures is rendered difficult due to their hydrophobicity.

As a part of the program to explore diversity of conotoxins produced by conus snail species found off the Indian coast, we report the isolation and characterization of a δ-conotoxin from *Conus amadis*, a hitherto uninvestigated species of snail collected in the Bay of Bengal. (For a description of the geographical distribution of *Conus* species, see www.seashell-collector.com.) The conus peptide Am 2766 is shown to inhibit the delayed inactivation of a mammalian $Na^+$ channel.

## 2. Materials and methods

### 2.1. Isolation of peptides

The conus species *C. amadis* were collected from the southeastern coast of India. The glands after dissection were stored in 100% ethanol and the hydrophobic peptides extracted were subjected to high-performance liquid chromatography (HPLC) purification. The alcohol-extracted venom was preliminarily purified on a HP 1100 series HPLC system, using a $C_{18}$ reverse phase column (Zorbax, 4.6×250 mm, 5 μM particle size, 300 Å pore size). Further purification was effected on a $C_{18}$ reverse phase column affording higher resolution separations (Jupiter, Phenomenex, 10×250 mm, 4 μM particle size, 90 Å pore size). Water and acetonitrile containing 0.1% trifluoroacetic acid (TFA) were used as the mobile phase and a flow rate of 1.5 ml/min was maintained. Linear gradients were run from 20 to 98% acetonitrile. The absorbance was monitored at 226 nm.

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*2.2. Chemical modification*

*2.2.1. Reduction and alkylation.* The purified peptide was dissolved in 30 μl, 0.1 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 1.5 h. To the solution, appropriate iodoacetamide stock solution was added to get a final concentration of 40 mM and the mixture was incubated at room temperature in the dark, for 45 min. The reaction mixture was analyzed by LC-electrophoretic secondary ion mass spectroscopy (ESIMS) through a $C_{18}$ column.

*2.2.2. Acetylation.* The stock acetylation reagent was prepared by mixing 20 μl acetic anhydride and 60 μl methanol. The peptide dissolved in 30 μl, 0.1 M $NH_4HCO_3$, pH 8.0, was mixed with 1 μl stock acetylation reagent and incubated at room temperature for 1 h. The resultant mixture was analyzed by LC-ESIMS using a $C_{18}$ reverse phase column.

*2.3. Proteolytic digestion*

The purified sample of reduced and alkylated peptide was digested with TPCK treated trypsin and TLCK treated chymotrypsin (Sigma, USA) with 10 μg of enzyme in 50 μl of $NH_4HCO_3$, pH 8.0 for 3 h at 37°C. The digest was directly analyzed by online LC-ESIMS.

*2.4. Mass spectrometry (MS)*

Electrospray ionization (ESI) mass spectra were recorded using a Hewlett Packard single quadrupole mass spectrometer (HP 1100 MSD series). The samples were infused into the mass spectrometer through a reverse phase $C_{18}$ column (Zorbax, 4.6×150 mm) with solvent A (0.1% acetic acid) and solvent B (acetonitrile with 0.1% acetic acid) at a flow rate of 0.25 ml/min. The data were acquired over the range *m/z* 50–3000 in positive ion mode and were analyzed using HP LC/MSD Chemstation software.

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS analysis was carried out using a Kompact SEQ (Kratos Analytical, Manchester, UK) mass spectrometer, equipped with a nitrogen laser of wavelength 337 nm. The samples were prepared by mixing an equal amount of peptide (0.5 μl) with a matrix solution (α-cyano-4-hydroxy cinnamic acid) saturated in 0.1% TFA and acetonitrile (1:1).

*2.5. Amino acid sequence*

The sample was reduced with tri-*n*-butyl phosphine and alkylated with 4-vinyl pyridine. The pyridylethylated peptide was repurified by reverse phase HPLC and the amino acid sequence was analyzed by automated Edman degradation on a Shimadzu PPSQ-10 sequencer.

*2.6. Electrophysiology*

Isolated sodium currents were measured from the rat brain IIA sodium channel α-subunit (rNav1.2a), stably expressed in Chinese hamster ovary (CHO) cells [8]. The currents were recorded using the patch clamp technique in the whole cell mode using an EPC-8 amplifier (Heka). Pipettes for patch clamp experiments were made from borosilicate glass (Clark Electromedical Instrument, UK). They were polished to give resistance of 1–3 MΩ. Solutions for patch clamp recordings were (in mM): 116 CsCl, 10 HEPES, 10 ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 $CaCl_2$; 135 NaCl, 5 HEPES, 1 $MgCl_2$, and 1.5 $CaCl_2$, for the pipette and bath solutions, respectively, pH adjusted to 7.4 with NaOH. Data acquisition and pulse protocols were controlled with the pClamp8 software, and Digidata 1320 analog/digital converter (Axon Instruments Inc.). Data were low pass filtered at 3 kHz and sampled at 20 kHz. The recordings were done at 15°C. Cells were held at −80 mV. The toxin was dissolved in 50% ethanol and applied to the bath as a bolus to achieve a final concentration of 200 nM. Modification of the sodium currents was seen about 4 min after toxin application. The final alcohol concentration of 0.5% did not affect the sodium current waveform in separate experiments.

## 3. Results

Fig. 1 shows the HPLC profile of the *C. amadis* venom extract. A large number of peaks were observed, of which Am 2766 is a major peak and is quite hydrophobic as evidenced from the retention time on a $C_{18}$ column. The intact

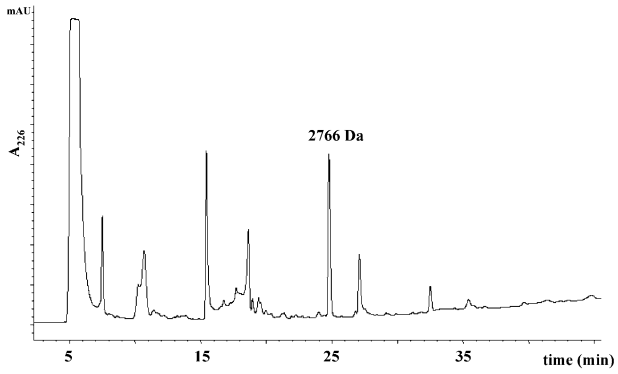


Fig. 1. Purification profile of the *C. amadis* venom extract. The alcohol-extracted peptides were passed through a $C_{18}$ RP column with water and acetonitrile as the mobile phase. A linear gradient of 20–98% acetonitrile was employed for 45 min and the flow rate was maintained at 1.5 ml/min. The Am 2766 peak is marked.

molecular weight of the peptide was determined using ESI and MALDI-MS. ESI-MS reveals the presence of $[M+2]^{2+}$ (1384 Da) and $[M+3]^{3+}$ (923 Da) species, which yield a molecular mass of 2766 Da (Fig. 2A). Simultaneous determination of the mass using MALDI-MS revealed a singly protonated molecule (2767 Da) along with $Na^+$ and $K^+$ adducts. In order to determine the number of Cys residues, the peptide was subjected to reduction with DTT and subsequently alkylated with iodoacetamide. Carboxamidomethylation yields an additional mass of 58 Da for each Cys residue. The ESIMS observed molecular mass for derivatized Am 2766 was 3114 Da (Fig. 2B), showing a mass increment of 348 Da, corresponding to the presence of six Cys residues. Upon acetylation, a mass increment of 84 Da was detected, suggesting the presence of two primary amino groups, which may be tentatively assigned to a free N-terminus and a single Lys residue.

The reduced and pyridylethylated peptide on conventional Edman sequencing yielded the sequence Cys-Lys-Asn-Ala-

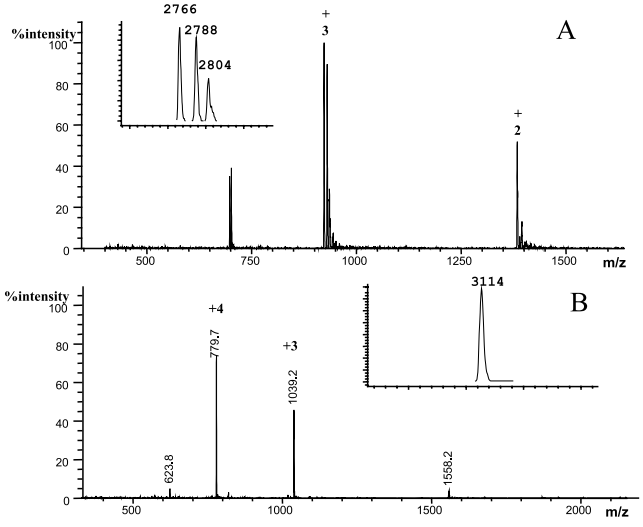


Fig. 2. A: ESI mass spectrum of Am 2766 peak. Inset: Deconvoluted molecular mass is 2766 Da. Adducts $Na^+$ (2788 Da) and $K^+$ (2804 Da) are also observed. B: ESI mass spectrum of the reduced (DTT) and alkylated (iodoacetamide) *C. amadis* toxin, Am 2766. Inset: Deconvolution of charge states yields a mass of chemically modified species, 3114 Da, corresponding to six sites of modification.

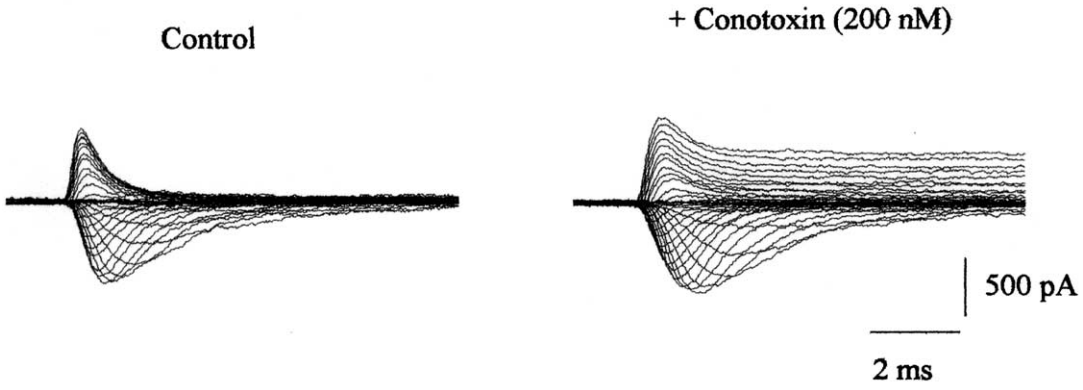


Fig. 3. Families of sodium current traces in the control and in the presence of the toxin following step depolarization potentials between −65 and +80 mV from a holding potential of −80 mV in 5 mV steps. The sodium current traces are corrected for leak and membrane capacitance. Am 2766 slows the decay of sodium current at depolarized potentials.

Gly-Glu-Ser-Cys-Asp-Ile-Phe-Ser-Glu-Asn-Cys-Cys-Val-Gly-Thr-Cys-Ala-Phe-Ile-Cys-Ile-Glu. The precise molecular mass detected by ESIMS was 2766 Da while the Edman sequencing results correspond to a mass of 2767 Da, assuming three disulfide bonds in the molecule. This discrepancy of 1 Da may arise due to C-terminal amidation of the peptide, a common posttranslational modification observed in many conotoxins. In order to confirm the C-terminal amidation, the reduced and alkylated peptide was digested with the sequencing grade trypsin and chymotrypsin. The masses of the observed fragments were compared with those anticipated. It was observed that the mass of the C-terminal peptide (ICIE) was 532 Da whereas the expected value for the tetrapeptide is 533 Da, confirming C-terminal amidation.

### 3.1. Electrophysiology

Fig. 3 shows the effect of Am 2766 on brain rNav1.2 voltage-gated sodium channels stably expressed in CHO cells. Application of the conus peptide (200 nM) resulted in marked slowing of the sodium current decay at depolarization potentials greater than +45 mV, with a slight increase in the peak sodium current. These observations are very similar to that reported for the δ-conotoxins, Gm VIA, Ng VIA and Tx VIA from *Conus gloriamaris*, *Conus nigropunctatus* and *Conus textile* respectively [9–11].

## 4. Discussion

Am 2766, a major peptide from the venom of the molluscivorous snail *C. amadis*, has been shown to exhibit a distribution of Cys residues identical to that observed in previously characterized δ-conotoxins. Our electrophysiological studies demonstrate that Am 2766 targets mammalian voltage-gated sodium channels. The predominant effect on the decaying phase of the sodium current suggests that the toxin inhibits the inactivation phase of sodium channels. Similar effects on the inactivation behavior of $Na^+$ channels have been noted for the δ-conotoxins Tx VIA, isolated from the molluscivorous snail *C. textile*. Interestingly, Tx VIA inhibits $Na^+$ current inactivation only in molluscan neurons and does not appear to have a similar effect on rat brain channels, despite binding strongly to mammalian $Na^+$ channels [11]. In contrast, the δ-conotoxins Ng VIA, isolated from the piscivorous snail *C. nigropunctatus*, inhibits $Na^+$ current inactivation in both molluscan and vertebrate systems [10]. Our observation that Am 2766 inhibits the inactivation phase in cloned rat

| Conotoxin | Sequence | Class | Net charge | No. of residues | Ref. |
|---|---|---|---|---|---|
| Tx VIA | --WCKQSGEMCNL---LDQNCCDGY-CIVLVCT----- | δ | -1 | 27 | [11] |
| Tx VIB | --WCKQSGEMCNV---LDQNCCDGY-CIVFVCT----- | δ | -1 | 27 | [14] |
| Am 2766 | ---CKQAGESCDI---FSQNCCVGT-CAFI-CIE---- | δ | -1 | 26 | |
| Gm VIA | VKPCRKEGQLCDP---IFQNCCRGWNCVLF-CV----- | δ | +3 | 29 | [9] |
| Ng VIA | -SKCFSPGTFCGIKPGL---CCSVR-CFSLFCISFE-- | δ | +3 | 31 | [10] |
| S VIE | -DGCSSGGTFCGIHPGL---CCSEF-CFLW-CITFID- | δ | -1 | 31 | [15] |
| M VIA | -DGCYNAGTFCGIRPGL---CCSEF-CFLW-CITFVDS | δ | -1 | 32 | [15] |
| P VIA | -EACYAPGTFCGIKPGL---CCSEF-CLPGVCFG---- | δ | 0 | 29 | [15] |
| C VIE | -YGCSNAGAFCGIHPGL---CCSEL-CLVW-CT----- | δ | +1 | 27 | [15] |
| Mr VIA | --ACRKKWEYCIVPIIGFIYCCPGLICGPFVCV----- | μO | +3 | 31 | [12] |
| G IIIB | -RDCCTPPRKCK-----DRRCKPMKCCA---------- | μ | +7 | 22 | [16] |
| G IIIC | -RDCCTPPKKCK-----DRRCKPLKCCA---------- | μ | +7 | 22 | [17] |
| G IIIA | -RDCCTPPKKCK-----DRQCKPQRCCA---------- | μ | +6 | 22 | [18] |
| Pn IVB | ---CCKYGWTCWL---GCSPCGC--------------- | μ | +2 | 17 | [13] |

Fig. 4. Sequence alignment of known conotoxins targeting sodium channels. Manual adjustments were effected following initial alignment using Clustal W. Net charges indicated are obtained assuming a positive charge at the N-terminus. The sequences of M VIA, P VIA and C VIE are derived from corresponding cDNA sequences [15].

brain IIA α-subunit channels suggests that conotoxins from some molluscivorous snails may also be active on mammalian $Na^+$ channels.

Fig. 4 presents an alignment of the sequences of δ-conotoxins from both snail-hunting and fish-hunting snails [9–11,14,15]. In addition, some selective sequences of conus peptides exhibiting activity on $Na^+$ channels are also compared [12,13,16–18]. It is clear that, while the Cys framework is completely conserved across the δ-conotoxins, there is a clear grouping of the sequences, with the peptides from molluscivorous and piscivorous snails falling into distinct classes. Particularly noteworthy is the conservation of the stretch of amino acids between the second and third Cys residues in the sequences from piscivorous snails and the invariant Gly residues between the fourth and fifth Cys residues in the sequences from molluscivorous snails. It is conceivable that the nature of the target channels may influence the selection of conotoxin sequences in the predator snail. Overall differences in the distribution of both charged and hydrophobic residues are observed even within the δ-conotoxin subgroups. The μ-conotoxins isolated from *Conus geographus* have a much higher distribution of positive charges, shorter polypeptide chain lengths and a distinctly different pattern of distribution of Cys residues along the sequences. Two recently isolated conotoxins, which appear to affect $Na^+$ channels, are also listed in Fig. 4. The μO-conotoxin Mr VIA, isolated from *Conus marmoreus*, has been shown to be a potent blocker of the $Na^+$ channel in *Aplysia* neurons [12]. Examination of the sequences shows that the Cys frameworks of the μO-conotoxin appear to resemble that of the δ-conotoxins. Further, the μO-conotoxin has a much lower net positive charge density than the μ-conotoxins, resembling the δ-conotoxins in their overall net charge. A significantly shorter conotoxin Pn IVB has been isolated from the species *Conus pennaceus*. Although this peptide possesses a characteristic N-terminus CC doublet, the distribution of the three C-terminal Cys residues does not appear to correspond to the pattern observed for either δ- or μ-conotoxins. This peptide has also been shown to have sodium channel blocking property [13].

The conus peptides, which target diverse $Na^+$ channels, appear to vary significantly in detailed stereochemistry and surface charge distribution. This structural diversity is undoubtedly an advantage to the organism in specifically targeting various subtypes of $Na^+$ channels in their natural prey. Detailed structure–function studies involving specific amino acid replacements together with three-dimensional structure determination are required in order to establish a firm correlation between peptide sequence and physiological function.

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