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Asteropsin A: An unusual cystine-crosslinked peptide from porifera enhances neuronal Ca²⁺ influx

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Abstract

Background—Herein we report the discovery of a cystine-crosslinked peptide from Porifera along with high-quality spatial details accompanied by the description of its unique effect on neuronal calcium influx.

Methods—Asteropsin A (ASPA) was isolated from the marine sponge *Asteropus* sp., and its structure was independently determined using X-ray crystallography (0.87 Å) and solution NMR spectroscopy.

Results—An *N*-terminal pyroglutamate modification, uncommon *cis* proline conformations, and absence of basic residues helped distinguish ASPA from other cystine-crosslinked knot peptides. ASPA enhanced Ca²⁺ influx in murine cerebrocortical neuron cells following the addition of the Na⁺ channel activator veratridine but did not modify the oscillation frequency or amplitude of neuronal Ca²⁺ currents alone. Allosterism at neurotoxin site 2 was not observed, suggesting an alternative to the known Na⁺ channel interaction.

Conclusions—Together with a distinct biological activity, the origin of ASPA suggests a new subclass of cystine-rich knot peptides associated with Porifera.

General significance—The discovery of ASPA represents a distinctive addition to an emerging subclass of cystine-crosslinked knot peptides from Porifera.

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Keywords

Porifera; Ion channelopathy; Natural product; Bioactive peptide; X-ray crystallography; NMR spectroscopy

1. Introduction

The discovery of a considerable amount of asteropsin A (ASPA) from a marine sponge of the genus *Asteropus* (Class: Demosponiae; Order: Astrophorida; Family: Ancorinidae) validates Porifera as the source of an unusual variety of cystine-crosslinked knot peptides. Cystine knot peptides are a class of peptides that share the rigid molecular “knot” disulfide arrangement [1,2]. A dominant feature of all knot peptides is their cystine arrangement (III–VI through I–IV, II–V) and, although this framework exists in ASPA, sequence analysis using software designed to classify knot peptides suggested that it is markedly dissimilar from any other peptide within the class. Several other characteristics, such as the absence of any basic residues, intra-cystine loop length, the presence of a modified *N*-terminus, and two *cis*-proline moieties contribute to the uniqueness of ASPA and results in a distinct biological activity.

Many natural knot peptides including those of marine origin affect ion channel function. Such peptides can significantly affect the buildup of the action potential in excitatory cells which are considered to be targets for drug discovery. Neuronal ion channels (e.g., Na⁺, K⁺, and Ca²⁺) have become a therapeutic option for a variety of acquired diseases and genetic abnormalities (i.e., ion channelopathies). Ion channel drug discovery has expanded into areas beyond neuropathic pain, such as cardiac arrhythmia, inflammation, tumor proliferation, and neurodegenerative disorders, including Alzheimer’s and Huntington’s diseases. The potential of ion channel modulators is rooted in the fact that ion channels play important roles in numerous and diverse physiological systems [3]. Our understanding of drug-channel interactions has expanded to include allosteric effects that prefer the open state to the closed state [4,5]. Most of the Na⁺ channel-interacting peptides act independently as channel inhibitors, but no Na⁺ channel effects were observed for ASPA alone. However, ASPA notably enhanced Ca²⁺ influx during veratridine-induced Na⁺ channel activation in murine cerebrocortical neurons.

According to the KNOTTIN database, only a handful of marine-derived knot peptides have been discovered outside the cone snail family [6]. Asteropine A (APA) has been the only knot related peptide discovered from Porifera (*Asteropus simplex*) [7] and one of only four sponge-derived peptides containing a disulfide moiety [8–10]. Although ASPA is from the same sponge genus as APA, it differs from APA not only in structure but also in biological activity, which is a major criterion for classifying knot peptides. However, APA and ASPA together are a potentially valuable pair of ribosome synthesized peptides. They can be useful for the identification of a biosynthetic source through cell sorting and metagenomic approaches [11,12], especially at a time when scientists are likely to presume symbiotic microorganisms to be the “true” producers of natural products in marine sponges [13].

The structure of ASPA was unambiguously assigned by X-ray crystallography and solution NMR spectroscopy providing its absolute configuration and tertiary structure which, considering the prior lack of such high-quality structural details, is a valuable contribution to the cystine knot peptide-based drug development as a model scaffold. Only one other independent crystal structure has been reported for a related cystine-crosslinked peptide [14], and none have been reported from a marine source until now.

2. Materials and methods

2.1. Animal material and peptide purification

The marine sponge *Asteropus* sp. (2.4 kg wet weight) was collected by hand using SCUBA (20 m depth) in 2006 off the coast of Geoje Island, Korea, and stored at -20°C until used. The frozen sponge (2.4 kg wet weight) was extracted with MeOH at room temperature. The extract (166 g) was partitioned between water and CH_2Cl_2 (1:1, v/v) and the aqueous layer was further partitioned with *n*-BuOH and water (1:1, v/v). The organic layer was then subjected to step-gradient MPLC (ODS-A, 120 Å, S-30/50 mesh) eluting with 20–100% MeOH. ASPA (44 mg) was purified by RP-HPLC equipped with an RI detector (YMC ODS-H80 column 250 mm×10 mm, i.d. 4 µm, 80 Å) eluting with 60% MeOH+0.2% HCOOH at a flow rate of 1 mL/min. The MALDI-TOF MS of APSA exhibited a monoisotopic ion peak at m/z 3918.5 $[\text{M}+\text{Na}]^+$.

2.2. Amino acid composition analysis

A 0.3 mg portion of the peptide was dissolved in 1 mL of 6 N HCl and hydrolyzed at 110°C for 24 h, and the HCl was then removed by evaporation by streaming N_2 gas. The residue was redissolved in 0.8 mL of H_2O followed by filtration through a 0.22 µm filter. The filtrate (20 µL) was injected into a Hitachi L-8800 amino acid analyzer at the wavelength of 570 and 440 nm.

2.3. Molecular mass determination

To determine the exact molecular mass of the peptides as well as related derivatives, each sample was applied to a MALDI-TOF instrument (Applied Biosystems 4700 proteomics analyzer, Framingham, MA). The samples were dissolved in DMSO solution and further diluted by an α -cyano-4-hydroxycinnamic acid matrix solution (7 mg/mL in 50% CH_3CN , 0.1% TFA) to a $\times 10$ volume. Each sample/matrix solution (1 µL) was spotted onto the MALDI plate to acquire the MS data.

2.4. Reduction and alkylation of the peptide

A portion of ASPA (100 µg) was dissolved in 100 µL of the denaturation buffer (7 M guanidine hydrochloride in 0.4 M Tris-acetate-EDTA buffer, pH 8.3) and 10 µL of 45 mM dithiothreitol (DTT) was added to the solution. The mixture was incubated at 60°C for 90 min. Then, 20 µL of 100 mM iodoacetamide was added and the mixture was further incubated at room temperature for 45 min. The reaction mixture was purified by RP-HPLC using a UV detector (Waters ODS-2 column 250 mm×4.6 mm, i.d. 5 µm; wavelength: 220 nm) with a linear gradient elution (30–80% solvent B; solvent A: H_2O +0.1% TFA, solvent

B: 90% CH₃CN+0.1% TFA) to produce a hexacarboxamidomethyl derivative (m/z 4266.7 [M+Na]⁺), indicating the presence of three intramolecular disulfide bonds.

2.5. N-Terminal deblocking and sequence analysis

The hexacarboxamidomethyl derivative (<30 µg) of ASPA was digested with 2 mU pyroglutamate aminopeptidase (Takara Bio Inc., Shiga, Japan) dissolved in 100 µL of the supplied buffer (a 50 mM sodium phosphate buffer containing 10 mM DTT and 1 mM EDTA, pH 7.0) containing 5% glycerol at 50 °C for 10 h. The digest was desalted with a sample preparation cartridge (Prosorb, Applied Biosystems, Foster City, CA, USA), and subjected to automatic Edman degradation in a protein sequencing system (Procise 491, Applied Biosystems). Sequence analysis was performed at KBSI (Korea Basic Science Institute, Seoul, Korea). Sequence alignments were performed and images rendered using ClustalX (ver. 2.0, <http://www.clustal.org>).

2.6. NMR spectroscopy

The NMR sample used for acquisition was dissolved in CD₃OH (98%, Sigma Aldrich Chemical Co., St. Louis, MO, USA), degassed, and topped with argon to minimize the absorption of water. Residual water signal suppression was achieved using standard presaturation methods. Standard experimental parameters were used for the acquisition of HSQC (adiabatic de-coupling), z-TOCSY (DIPSI, mixing time=80 ms), DQF-COSY, NOESY (mixing time=100 and 300 ms using a Varian 600 MHz) (¹³C=150 MHz) INOVA at 298 K with reference to the internal lock signal. Processing was completed using NMRPipe (ver. 5.5, NIH) and chemical shift analysis using SPARKY (ver. 3.114, UCSF), with spectra image rendering using MestReNova (ver. 6.2.0, Mestrelab Research, Escondido, CA, USA).

2.7. X-ray analysis and refinement

In the course of gathering NMR data, an aggregate was observed at the bottom of the 3 mm NMR tube containing ASPA in CD₃OH. The tube was allowed to sit undisturbed under ambient conditions for approximately seven days, at which time a mass of blade-shaped crystals was observed by microscopy and indicated diffractive properties. The selected crystal used for the X-ray crystallography studies belongs to the triclinic space group P1 with unit cell dimensions as follows: $a=14.8028(6)$ Å, $b=18.5573(8)$ Å, $c=24.1784(10)$ Å, $\alpha=83.186(4)^\circ$, $\beta=84.097(4)^\circ$, $\gamma=68.038(3)^\circ$. A total of 46544 reflections were measured at 90.0 K to 0.87 Å resolution with CuK α radiation ($\lambda=1.54178$ Å) on a Bruker Kappa Apex-II diffractometer, 27756 reflections were independent ($R_{\text{int}}=0.048$). The structure was solved using the Patterson function to locate the six sulfur atoms, followed by partial structure expansion by direct methods and structure completion by difference Fourier methods. Anisotropic refinement was carried out for all non-H atoms, and H atoms were included except on solvent molecules (MeOH and water). The standard absolute configuration for all residues was confirmed based on 10916 Friedel pairs, giving an acceptable Flack parameter $x=0.049(17)$. Final $R=0.079$, $R_w=0.215$ for 2725 variables.

2.8. Solution NMR structure calculations

Interproton distance constraints were obtained from the 100 ms and 300 ms mixing time NOESY spectra recorded in CD₃OH. The NOESY spectra were analyzed with the program SPARKY. The cross peaks were categorized into four classes according to the peak intensities: 2.5, 3.0, 4.0, and 5.0 Å corresponding to strong, medium, weak, and very weak correlations, respectively. Pseudo-atoms were applied for methyl, ambiguously assigned methylene and aromatic protons according to a standard conventions [15]. Hydrogen bond restraints were collected by long-range NOE correlations together with hydrogen-deuterium exchange experiments using CD₃OH and CD₃OD as solvents. Solution structure calculations were initially performed by CYANA 2.1 [16] using distance, dihedral angle, and hydrogen bond constraints, and further refined by simulated annealing within CNS 1.3 [17]. A final set of 200 structures was calculated within the program CNS, and the 20 structures with lowest energies and no residual restraint violations were used to represent the solution structure of the peptides. The calculated 3D structures were analyzed with the program PYMOL. The structure quality was validated by PSVS (protein structure validation software suite) server (http://psvs-1_4-dev.nesg.org/).

2.9. Sequence and tertiary structure alignments

The KNOTTIN database (<http://knottin.cbs.cnrs.fr/>) FASTA search revealed a handful of reported sequences which aligned with ASPA. Final sequence alignments were performed and images rendered using CLUSTAL X (ver. 2.0, <http://www.clustal.org>). Accessing the database of sequences of the RCSB (www.pdb.org), a FASTA search was initiated with the sequence of ASPA using filters of an E Value of 100, 18–74 chain length, 3–4 disulfide bonds and no helices. Twenty two records were found and coordinates downloaded for tertiary structure comparison using TMalign online (last updated January 30, 2011, <http://zhanglab.ccmb.med.umich.edu/TM-align/>) with scores normalized to the ASPA sequence length (37 residues).

2.10. Electrostatic potential modeling and rendering

Calculation of electrostatic potential was accomplished using the APBS algorithm (Linearized Poisson-Boltzmann Equation) through the PDB ID: 2PQR web portal service (ver. 1.7 <http://kryptonite.nbc.net/pdb2pqr/>). PDB ID: 2PQR converted using AMBER force field and APBS configuration was default with a solvent radius of 1.4 Å, system temperature of 310 K, protein and solvent dielectric constants of 2.0 and 78.0. Rendering of all surfaces and models was completed in Pymol Molecular Graphics System (Ver. 1.3, Schrodinger LLC).

2.11. Accession numbers

The crystal structure and solution structure coordinates of ASPA have been deposited along with their associated structure factors and NMR chemical shifts within the Protein Data Bank (PDB) and Biological Magnetic Resonance Bank (BMRB): (PDB ID: 3Q8J), (PDB ID: 2LQA), (BMRB ID: 18300).

2.12. Intracellular Ca^{2+} monitoring

Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss-Webster mice as previously described [18]. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) measurements were made in DIV-10 cerebrocortical neurons grown in 96-well plates as described previously [19]. In brief, the growth medium was removed and replaced with dye-loading medium (100 μL /well) containing 4 μM fluo-3 AM (Life Technologies, Grand Island, NY, USA) and 0.04% Pluronic acid in Locke's buffer. After 1 h incubation in dye-loading medium, the neurons were washed 4 times in fresh Locke's buffer (200 μL /well) using an automated microplate washer (BioTek Instruments, Winooski, VT, USA) and transferred to a FlexStation II benchtop scanning fluorometer chamber. The final volume of Locke's buffer in each well was 100 μL . Fluorescence measurements were performed at 37 $^{\circ}\text{C}$. The neurons were excited at 488 nm and Ca^{2+} -bound fluo-3 emission was recorded at 538 nm at 1.2 s intervals. The fluorescence was monitored and expressed as $(F_{\text{max}} - F_0)$, where F_{max} is the maximum and F_0 is the baseline fluorescence measured in each well.

3. Results and discussion

3.1. Sequence analysis of ASPA

ASPA was isolated from the MeOH extract of the sponge *Asteropus* sp. via several steps of solvent partitions followed by RP-HPLC. The MALDI-TOF MS of APSA exhibited a monoisotopic ion peak at m/z 3918.5 $[\text{M}+\text{Na}]^+$. The structural elucidation of ASPA in solution was initiated using NMR spectroscopy. The ^1H (600 MHz) NMR spectrum recorded in CD_3OH exhibited well-dispersed amide and aliphatic signals indicative of a peptide. Our previous isolation of pyroglutamyl dipeptides from the same sponge aided confirmation of the NH signal of a pyroglutamyl ring by comparison of the signal shape and chemical shift, as well as the HMBC correlations analogous to those of dipeptides (see Fig. S1) [20]. The NMR evidence combined with the difficulty in performing traditional sequence analysis indicated that the *N*-terminus of the peptide was blocked by pyroglutamic acid (pGlu). Amino acid analysis indicated that the peptide was composed of common amino acids with several cystine residues, indicating a likelihood of disulfide bonds. Before traditional sequence analysis, the peptide required pretreatment for *N*-terminal deblocking and disulfide bond reduction. ASPA was reduced with dithiothreitol and alkylated with iodoacetamide to produce a hexacarboxamidomethyl derivative (m/z 4266.7 $[\text{M}+\text{Na}]^+$), indicating the presence of three disulfide bonds. The derivative was digested with pyroglutamate aminopeptidase to remove the *N*-terminal pGlu before the analysis. The complete amino acid sequence presented in Fig. 1A was corroborated by sequence specific chemical shift assignments obtained through analyses of DQF-COSY, TOCSY, and NOESY spectra. Our attempts to confirm the disulfide arrangement of ASPA by partial reduction, proteolytic enzyme hydrolysis, and high-energy CID- MS^n methods ultimately failed. Only the fully oxidized and fully reduced species were observed after partial reduction procedure in the RP-HPLC traces, and ASPA exhibited extraordinary proteolytic stability against proteinase K (from *Tritirachium album*; 50 $^{\circ}\text{C}$, up to 48 h) and thermolysin (from *Bacillus thermoproteolyticus rokko*; 65 $^{\circ}\text{C}$, up to 12 h).

3.2. Crystal structure of ASPA

In the course of gathering our NMR data, we observed an aggregate at the bottom of the 3 mm NMR tube containing ASPA in CD₃OH. The tube was allowed to sit undisturbed under ambient conditions for approximately seven days, at which time a mass of blade-shaped crystals was observed by microscopy and indicated diffractive properties. X-ray crystallographic studies of ASPA were conducted, which provided an anisotropically refined structure to a resolution of 0.87 Å (Fig. 1B). The surface projection of the electrostatic potential (kT/e) was calculated using APBS software via the PDB ID: 2PQR web portal [21,22]. The resulting map showed an isolated region of positive potential that could potentially facilitate organization into a crystal lattice (Fig. 1C and Table 1).

A crucial benefit of the crystal structure was the unambiguous characterization of the *N*-terminal pGlu, *cis-trans* proline conformations, and proper disulfide pairings (Fig. 1B and D). The *N*-terminal pGlu has been reported to be important for the correct conformation and function of the peptides [23], and is an uncommon moiety among knot peptides. Two of the four prolines (Pro27 and Pro30) in ASPA had *cis* geometry (Fig. 1D), which has been observed seldomly in knot peptide structures [14,24]. The anti-parallel β -sheets were found to be supported by two (Cys17–Cys34 and Cys10–Cys25) of the three disulfide bridges that lie in the middle of the structure. The third disulfide bridge lies closer to the surface near the *N*-terminus.

Details of the H-bonding network of ASPA were elaborated in its crystal structure (see Table S1, supplemental data). After removing disordered solvent using the SQUEEZE procedure [25], 48 H-bonds were assigned to peptide-atom donors. Thirteen intramolecular H-bonds were associated with the solvent and 12 with various symmetry mates. Of the remaining 23 intermolecular H-bonds, 17 were associated with the β -sheet regions described above. Five hydrogen bonds were shown to involve residues of the *N*-terminus, including one from the modified pGlu1 to Phe14 in the first β -turn region.

3.3. Solution structure of ASPA

To compare ASPA in its crystal and solution states, the structure assignment was independently completed using NMR techniques [15]. NOE distance constraints and hydrogen-deuterium exchange experiments using CD₃OH and CD₃OD indicated 3 antiparallel β -strands, consisting of the residues Ser9–Asn11, Leu23–Pro27, and Asp31–Tyr36, and 2 type I β -turns, consisting of the residues Val12–Tyr15 and Gly28–Pro30 (Fig. 2A and B). The three-dimensional solution structure of ASPA was initially calculated by CYANA 2.1 [16] using distance, dihedral angle, and hydrogen bond constraints, and further refined by simulated annealing within CNS 1.3 [17]. Twenty structures with the lowest energies and no residual restraint violations were selected to represent the solution structure of ASPA (Fig. 2C). The average root-mean-square deviations (RMSD) calculated for residues 2–37 was 0.18 Å for the backbone atoms and 0.63 Å for all heavy atoms (N, C ^{α} , and C). The Ramachandran plot summary from PROCHECK showed that 85.8% of the residues were in the most favored and 14.2% in the additionally allowed regions [26]. The structural statistics for the 20 lowest energy structures are summarized in Table 2.

The crystal and solution structures were successfully superimposed with a backbone RMSD value of 0.66 Å (Fig. 2D). However, the solution structure deviates from the crystal structure at its *N*-terminus (ca. pGlu1), Gln13 in loop 2, and Pro19–Leu21 in loop 4 (Fig. 2D). The terminal hydrogen bonds identified in the crystal are assumed to be less ordered in solution and account for the calculated variation. The relative solution flexibility of described residues indicates potential regions possibly involved in its biological mechanism of action. Future engineering of novel bioactivity through the exposed loop 4 in ASPA will be greatly facilitated by the elucidation of its structural features.

3.4. Structural uniqueness of ASPA

The sequence of ASPA was submitted for Knotter1D analysis (KNOTTIN database tool) [6]. Although its cystine arrangement ($-C^I-C^{II}-C^{III}C^{IV}-C^V-C^{VI}-$) was previously observed, ASPA was not recognized as a member of the knottin class, most likely because of the inherent sequence variability between cystine knots. Regardless of the cystine arrangement, the sequence bears little resemblance to any previously isolated peptide. ASPA only shares the Pro–Cys–Cys–Pro homology with a handful of conotoxin sequences identified only at the genomic level and without details of their structures or biological activity (Fig. 3A) [27].

Using a pre-screened group of 22 peptides most closely related to ASPA, the TM-align algorithm was used to assign normalized TM-scores with ASPA which would qualify their backbone alignment (see Table S2, supplemental data) [28]. Any aligned peptides which have scores higher than 0.5 would be considered to have similar backbone folds as ASPA [29]. Hainantoxin IV (HNTX_IV; PDB ID: 1NIY) (Fig. 3A and E) [30] from spider venom scored the highest with a 0.59 TM-score. The conotoxin structures represented in the group were assigned scores between 0.46 and 0.22. The unique fold of ASPA as indicated by the modest TM-scores could be influenced by the over 400 million year evolutionary gap between sponges and higher order sources of knot peptides, which may also indicate an alternative benefit for the sponge relative to other knot peptide sources, and explain any potential difference between their biological effects.

Furthermore, most of the reported knot peptides display a positive charge, while ASPA has no basic residues and was instead acidic, and this phenomenon was also observed in APA. Absence of basic residues together with highly acidic nature is very rare in knot peptide families, thus, it is speculated to be one of the unique characteristics of sponge-derived knot peptides, which could account for differences in their potential therapeutic use. However, several distinctions exist between ASPA and APA in addition to its obvious differences in sequence. ASPA is relatively proline rich and two of these prolines have *cis* geometry (Fig. 1D), while APA contains only one *trans*-Pro. The *cis*-Pro conformer has a less than 10% occurrence in nature compared with the *trans* conformer [31,32]. Among the cyclotide knot peptides, *cis*-Pro is the criterion that distinguishes the two main subfamilies [14]. The presence of multiple *cis*-Pro is uncommon among knot peptides and because of its constrained nature, *cis*-Pro is likely to contribute to the stability of the protein fold. Most importantly, the bioactivity of APA and ASPA are distinctly different. APA was reported to be a potent competitive inhibitor of bacterial neuraminidases [7]. However, ASPA showed no inhibition of neuraminidase from *Arthrobacter ureafaciens*, *Clostridium perfringens*,

Vibrio cholera, *Micromonospora viridifaciens*, and recombinant influenza A virus H1N1 up to a 50 μ M concentration. This difference in bioactivity may be explained by their low sequence identity and physical differences in their surfaces (Fig. 3A–C). The acidic residues, which are considered to be necessary for neuraminidase inhibition, are mostly solvent-exposed in APA, especially Asp12, Glu22, and Glu24 (Fig. 3C). However in ASPA, the negatively charged side chains of Glu6, Glu8, and Asp31 are concealed inside the peptide (Fig. 3B) prohibiting them from interactions with neuraminidases. The distinct biological activity together with several other features such as low sequence identity, *N*-terminal pGlu formation, and *cis*-prolines, distinguish ASPA from the other sponge-derived knot peptide APA.

3.5. Effects on neuronal Ca^{2+} influx

The physicochemical characteristics of the peptide surface are justifiably important for peptide-ion channel interaction. Unlike most cation channel blocking knot peptides which produce net (basic) positive charge at their surfaces (Fig. 3D and E), ASPA has no basic residues and its surface is instead negatively charged. Because the sequence and fold of ASPA did not significantly match any known ion channel interacting peptide, its biological activity could not be predicted. However, the association of its cystine arrangement with conotoxins provided some impetus to evaluate its effects on ion channels in addition to a broader evaluation of its cytotoxicity, and antibiotic and antiviral activities.

Neither the inhibition nor the activation of voltage-gated Na^+ ($\text{Na}_v1.2$) or Ca^{2+} ($\text{Ca}_v1.2$) channels was observed at up to micromolar concentrations of ASPA administered alone. However, ASPA enhanced veratridine-induced Ca^{2+} influx in murine cerebrocortical neuron cells with an EC_{50} value of 14 nM (Fig. 4). Veratridine is a neurotoxin that binds to intramembrane receptor site 2 on voltage-gated Na^+ channels (VGSC). It binds preferentially to the activated state of VGSC and causes persistent activation via an allosteric mechanism that leads to the blocking of channel inactivation and a shift of the voltage dependence of activation to more negative potentials [4]. However, allosteric interaction between ASPA and the veratridine binding site of the VGSC was not observed. Other toxins have been suggested to prefer binding to the open state form of ion channels and the enhancement of veratridine-induced Ca^{2+} influx is a potential indicator this phenomenon exists for the ASPA mechanism [33]. Considering that multiple resources for Ca^{2+} regulation exist in neurons and that Ca^{2+} plays a significant role in other signaling processes, the details of the ASPA binding site remain unclear. Early clinical trials of veratridine (i.v. and i.m.) for hypertension revealed a poor therapeutic window due to toxic side effects [34]. However, combination therapy reduced the dosage requirement whilst achieving its desired effect on VGSC [35]. Considering the low toxicity of ASPA (no toxicity or behavior changes were observed in mice [i.p. 40 μ g/g] and chicks [i.c.v. 60 μ g/g]) and the selective veratridine-ASPA effects on Ca^{2+} influx in neuronal cells, ASPA may have utility for a control of hypertension or topical pain when used in combination with veratridine.

4. Conclusions

An unusual cystine-crosslinked peptide, ASPA, was isolated from the marine sponge *Asteropus* sp., which dose dependently enhanced veratridine-induced neuronal Ca^{2+} influx. The absence of basic residues and the acidic nature of ASPA clearly distinguish ASPA from other knot peptides. Meanwhile, ASPA is also distinguished from another sponge-derived knot peptide, APA, because of its *N*-terminal modification, two *cis*-prolines, and most importantly, its bioactivity. Unlike other knot peptides, which work independently as ion channel inhibitors, ASPA affected neuronal Ca^{2+} influx only when it was administered together with the Na^+ channel activator veratridine and is likely to prolong the activation state of Na^+ channels. This result indicates an alternative mechanism of ASPA interaction, which requires further study. The discovery of ASPA, while seemingly routine in the shadow of its class, represents a distinctive addition to this emerging subclass of cystine-crosslinked knot peptides from Porifera.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.2012.11.015>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ASPA	asteropsin A
APA	asteropine A
BMRB	Biological Magnetic Resonance Bank
DTT	dithiothreitol
HNTX_IV	hainantoxin IV
PDB	Protein Data Bank
PSVS	protein structure validation software suite
RMSD	root-mean-square deviations
VGSC	voltage-gated Na^+ channels

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Fig. 1.

The sequence and crystal structure of ASPA. (A) The sequence of ASPA. X: pyroglutamic acid (pGlu). The disulfide bonds are presented as solid lines. (B) The crystal structure of ASPA. The side chain of the *N*-terminal pGlu is indicated by sticks. The three disulfide bonds and β -sheets are colored yellow and blue, respectively. Six Cys residues are labeled. (C) Representation of the ASPA crystal lattice in relation to electrostatic potential. (D) *Cis*-proline moieties of ASPA indicated by sticks. The C ^{α} of the residues Ile26, Pro27, Asp29, and Pro30 are colored yellow. This figure was drawn with the program PYMOL.

Fig. 2.

The secondary and three-dimensional solution structures of ASPA. (A) A summary of the sequential and medium range NOE correlations ($|i-j| < 5$) and hydrogen-deuterium exchange experiments. The NOE intensities are grouped into four classes (2.5, 3.0, 4.0, and 5.0 Å), which are represented by the thickness of solid lines. Filled circles indicate the amide protons observed in the ¹H NMR spectrum recorded in CD₃OD (500 MHz). The arrows at the top of the figure indicate the position of the β-sheets as analyzed by a combination of strong sequential $d_{\alpha N}$, weak d_{NN} , and non-exchanged amide protons. (B) The triple-stranded

antiparallel β -sheet region with hydrogen bonds (dashed line) and relevant NOE (solid arrows). (C) The overlapped backbone atoms of 20 selected models with lowest energy representative of the solution structure of ASPA. The side chains of the *N*-terminal pGlu and Pro residues are indicated by sticks. Four Pro residues are labeled. The RMSD calculated for residues 2–37 were 0.18 Å for the backbone atoms and 0.63 Å for all heavy atoms (N, C $^{\alpha}$, C). (D) Structural alignment of the lowest energy solution (blue) and crystal (red) structures with a backbone RMSD value of 0.66. The side chains of *N*-terminal pGlu and Pro residues are indicated by sticks. pGlu1, Gln13, Leu21, and four Pro residues are labeled. This figure was drawn with the program PYMOL.

Fig. 3.

Sequence alignment and surfaces of selected knottins. (A) Sequence alignment of ASPA with APA [7], CNTX_1 (conotoxin_1) [27], CNTX_MVIIA (ω -conotoxin MVIIA) [36–38], and HNTX_IV (hainantoxin_IV) [30]. Disulfide bonds are presented as solid lines. Residues: red=acidic residues, blue=basic residues, green=hydrophobic uncharged residues, yellow=Cys, purple=Pro. Surface morphology of (B) ASPA, (C) APA, (D) CNTX_MVIIA, and (E) HNTX_IV. Red=acidic residues and C-terminal –COOH; blue=basic residues and N-terminal –NH₂; green=hydrophobic uncharged residues; white=others. The side chains of the acidic residues of ASPA (B) and APA (C) are shown with sticks. The negatively charged side chains of Asp12, Glu22, and Glu24 in APA were solvent-exposed, while the side chains of Glu6, Glu8, and Asp31 in ASPA were almost buried in the peptide. The proposed ion-channel interacting basic residues of CNTX_MVIIA (D) and HNTX_IV (E) are labeled. The sequence alignment was performed with the program ClustalX 2.0 and the surface morphology was drawn with PYMOL.

Fig. 4.

The effects of ASPA on the veratridine (VRT, 3 μM)-induced Ca^{2+} influx in murine cerebrocortical neurons. (A) Time–response relationships for the enhancement of the veratridine-induced Ca^{2+} influx by ASPA. (B) Concentration–response relationships for the enhancement of the veratridine-induced Ca^{2+} influx by ASPA. Each data point represents the mean of three emission determinations.

Table 1

Crystal data and structure refinement for ASPA.

Empirical formula	C181 H308N40 O75 S6	
Formula weight	4437.03	
Temperature	90.0(5) K	
Wavelength	1.54178 Å	
Crystal system	Triclinic	
Space group	P 1	
Unit cell dimensions	$a=14.8028(6)$ Å	$\alpha=83.186(4)^\circ$.
	$b=18.5573(8)$ Å	$\beta=84.097(4)^\circ$.
	$c=24.1784(10)$ Å	$\gamma=68.038(3)^\circ$.
Volume	$6104.0(4)$ Å ³	
Z	1	
Density (calculated)	1.207 Mg/m ³	
Absorption coefficient	1.245 mm ⁻¹	
F(000)	2370	
Crystal size	0.50×0.26×0.13 mm ³	
Theta range for data collection	2.5 to 62.2°	
Index ranges	-16≤ <i>h</i> ≤15, -20≤ <i>k</i> ≤20, -20≤ <i>l</i> ≤26	
Reflections collected	46544	
Independent reflections	27756 [<i>R</i> (int)=0.048]	
Completeness to theta=50.40°	97.70%	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.857 and 0.575	
Refinement method	Full-matrix least-squares on F ²	
Data/restraints/parameters	27756/6/2725	
Goodness-of-fit on F ²	1.06	
Final R indices [<i>I</i> >2σ(<i>I</i>)]	R1=0.0795, wR2=0.2147	
R indices (all data)	R1=0.0927, wR2=0.2300	
Absolute structure parameter	0.049(17)	
Largest diff. peak and hole	0.769 and -0.372 e.Å ⁻³	

Table 2Structural statistics for the 20 lowest energy solution structures of ASPA defined by PSVS^a.

<i>NOE distance constraints</i>	
Total	544
Intra-residue [$i=j$]	112
Sequential [$ i-j =1$]	153
Medium range [$1< i-j <5$]	81
Long range [$ i-j \geq 5$]	198
Dihedral angle constraints	19
Hydrogen bond constraints	28
Violations	
Distance ($>0.1 \text{ \AA}$)	0
Dihedral angle ($>1^\circ$)	0
Van der waals ($<1.6 \text{ \AA}$)	0
<i>RMS deviations from idealized geometry^b</i>	
Bond lengths ($\text{\AA}$)	0.009
Bond angles ($^\circ$)	0.7
RMS of distance violation	0.01
RMS of dihedral angle violation	0.02
RMSD values for residues 2–37 ($\text{\AA}$) ^b	
All backbone atoms	0.18
All heavy atoms	0.63
Ramachandran plot (%)	
Most favored regions	85.8
Additionally allowed regions	14.2

^a Structural statistics were evaluated for the residues 2–37.^b RMSD is given as the mean value.