Structural and functional analysis of natrin, a venom protein that targets various ion channels

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Abstract

Cysteine-rich secretory proteins (CRISPs) are secreted single-chain proteins found in different sources. Natrin is a member of the CRISP family purified from the snake venom of Naja naja atra, which has been reported as a BKca channel blocker. In our study, crystals of natrin were obtained in two different crystal forms and the structure of one of them was solved at a resolution of 1.68 Å. Our electrophysiological experiments indicated that natrin can block the ion channel currents of the voltage-gated potassium channel Kv1.3. Docking analyses of the interaction between natrin and Kv1.3 revealed a novel interaction pattern different from the two previously reported K+ channel inhibition models termed “functional dyad” and “basic ring”. These findings offered new insights into the function of natrin and how the specific interactions between CRISPs and different ion channels can be achieved.

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A family of cysteine-rich secretory proteins (CRISPs), which are characterized by 10 out of 16 strictly conserved cysteine residues residing in their C-terminus, have a wide distribution in many species from Caenorhabditis elegans [1] to snake [2], equine, human [3,4], etc. These CRISPs are divided into many subclasses according to their sequence homology and tissue specificity, which are CRISP-1 in epididymis, CRISP-2 in spermatocytes, CRISP-3 major in salivary glands, and the genes of CRISP-4 members have also been found recently in mouse and rat epididymis [5,6].

In the snake venom of many species, a number of CRISP-3 family members including trifin, abomin,
latisemin, tigrin, pseudecin, piscivorin, orphamin, catrin, stecrisp, and natrin have been reported. The sequence alignment indicates that CRISP family members compose a new group of snake proteins [2,7,8]. Recently the structures and functions of several CRISP-3 proteins have been explored. Trifin can function as a Ca\(^{2+}\)-channel blocker [9], natrin acts as a blocker to the BK\(\text{Ca}\) channel [10], and stecrisp has a K\(^+\)-channel inhibitor-like fold [11]. These studies have greatly expanded our knowledge of the structure and function of CRISP-3 family members. However the detailed interaction between these proteins and ion channels and the mechanism of blocking are less known.

As a well-known member of the CRISP-3 family, the function of helotermine, which is purified from the Mexican beaded lizard, has been well explored [12]. It can block a variety of ion channels including voltage-gated Ca\(^{2+}\) channels, voltage-gated K\(^+\) channels, and ryanodine receptor (RyR)/Ca\(^{2+}\) releasing channels [13–15]. Natrin is another CRISP-3 member that has been isolated from the snake venom gland of *Naja naja atra* and crystallized by our research group. Although the crystal structure of natrin in a different space group was reported [10] when we were solving the structure, some crucial questions about natrin remained unanswered: (1) Can natrin interact with other channels besides the reported BK\(\text{Ca}\) channel? (2) What is the interaction mode between natrin and its target ion channels? (3) Is there any structural difference of natrin in different crystal forms?

Here we reported the structure of natrin in new crystal form and investigated the interaction of natrin with Kv1 type channel by electrophysiology methods. Furthermore, molecular simulations were carried out to obtain the detailed interaction between natrin and two kinds of K\(^+\) channel molecules.

**Materials and methods**

**Purification, crystallization, and sequence analysis.** Dried crude venom from *Naja naja atra* was obtained from Guangxi province, China, and the protein was purified using a three-step chromatography procedure by using Sephadex G50, DEAE Sepharose Fast Flow, and Resource S (all these from Amersham–Pharmacia Biotech) columns on an FPLC system (Amersham–Pharmacia Biotech). The molecular weights and the N-terminus sequence were determined to identify the purified protein.

The protein was concentrated to about 10 mg/ml in 20 mM MES buffer (pH 6.5) and crystallized using the hanging-drop vapor-diffusion method by using 289 K. We first used 24–28% PEG MME 5000, 0.2 M K/Na\(\text{H}_2\text{PO}_4\), 50 mM NaCl, and 50 mM (NH\(_4\))\(\text{SO}_4\) as precipitant. Single crystals were obtained from dendritic ones through micro-operation and one set of data was collected (Form II). Later we further purified the protein by using Superdex 75 and collected the peak fractions only, then screened again and further optimizing was performed with 30–35% PEG MME 2000 and 0.10–0.15 M KSCN as precipitant. Single crystals were obtained and another set of data was collected from one of these crystals (Form I).

The total mRNA of a single fresh snake (*Naja naja atra*) was isolated using an RNA Trizol agent (Gibco), following the manufacturer’s instruction. Basing on the sequences of N-terminus determined and C-terminus of NIKK, oligonucleotide primers were designed for PCR. The PCR products were purified and ligated with pGEM-T vector (Promega) and then sequenced.

**Data collection and structure determination.** All the crystals of two forms were flash-cooled using paratone as cryo-protection at 79 K and datasets for both crystal forms were collected from frozen crystals at FR-E system (Institute of Biophysics, CAS, Beijing). The datasets were scaled and merged using the CrystalClear program [16]. Crystallographic analysis revealed that both of the two crystal forms belong to space group P2\(_1\) but had different cell parameters, which were \(a = 43.55\) Å, \(b = 58.91\) Å, \(c = 86.87\) Å, \(\beta = 97.18^\circ\) for Form I crystals at a resolution of 1.68 Å and \(a = 44.62\) Å, \(b = 48.11\) Å, \(c = 97.71\) Å, \(\beta = 97.07^\circ\) for Form II crystals at a resolution of 2.30 Å (detailed statistics are not shown).

The structure for crystal Form I was solved by molecular replacement method using the program Molrep from the CCP4i package suite [17] with the structure of stecrisp as an initial model. Further refinement was performed using refmac5 and CNS programs [18]. Manual rebuilding of the model was done using the program COOT [19]. The final \(R\) factor and \(R_{	ext{free}}\) factor are 22.5% and 23.4%, respectively. The detailed statistics of data-collection and the structure are listed in Table 1.

**Cell culture and gene transfection.** CHO-K1 cells were grown in Ham’s F-12 nutrient mixture (Gibco) supplemented with 10% fetal bovine serum. Cells were grown in a 37 °C incubator with a 5% CO\(_2\) humidified environment and passed twice weekly through exposure to 0.05% trypsin and 0.5 mM EDTA in PBS(–) solution. For gene transfection, CHO-K1 cells were transfected to poly-c-lysine (Sigma)-coated coverslips. After the cell density reached 50–70% confluence, pEGFP (Clontech) was transiently coexpressed with the Kv1.3 channel gene (Proteintech Group) which is in mammalian expression vectors pcDNA3 (wt/wt) at a ratio of 5:1 (wt/wt) using LipofectAMINE Plus\(^{TM}\) reagent (Invitrogen). Cells were used for electrophysiological studies 1–3 days after transfection.

**Electrophysiology.** Membrane currents were measured using a conventional tight seal whole cell recording technique at room temperature by using an EPC-9 patch-clamp amplifier (HEKA). Only cells exhibiting bright green fluorescence were used for recordings. Virtually no detectable endogenous K\(^+\) channel activity was observed in non-transfected Chinese hamster ovary (CHO) cells. The pipette solution contained (in mM): 155 KCl, 2 MgCl\(_2\), 0.1 CaCl\(_2\), 1 EGTA, 2 Na\(_2\)ATP, and 10 Hepes (pH 7.3 with KOH), while the extracellular solution was a Hanks’ balanced salt solution (HBSS, Gibco): 1.3 CaCl\(_2\), 0.8 MgSO\(_4\), 5.4 KCl, 0.4 KH\(_2\)PO\(_4\), 136.9 NaCl, 0.3 Na\(_2\)PO\(_4\), 10 n-glucose, and 4.2 NaHCO\(_3\). Compensation for cell capacitance and series resistance was made automatically by the EPC9 amplifier, and only recordings with stable series resistances \(<25\) megohms were included in the study. Currents were amplified using an EPC9 patch-clamp amplifier (HEKA), sampled at 2–5 kHz, and filtered at 1.5–2.9 kHz via a 4-pole low-pass Bessel filter. The steady-state outward IK current

### Table 1

<table>
<thead>
<tr>
<th>Data-collection, phasing and refinement statistics of crystal form I</th>
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<tr>
<td>Space group</td>
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<td>Unit cell dimensions</td>
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<td>Resolution range (Å)</td>
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<td>Total number of reflections</td>
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<td>(R_{\text{merge}})(^a)</td>
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<td>Output ((I/\sigma I))</td>
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<td>(R) factor(^b)</td>
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<td>(R_{\text{free}}) factor(^c)</td>
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<tr>
<td>rmsd bonds</td>
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<td>rmsd angles</td>
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</table>

Note: Values in () are for the last resolution shell.

\(^a\) \(R_{\text{merge}} = \sum_i |I_i - \langle I\rangle| / \sum_i |I_i|\), where \(\langle I\rangle\) is the mean intensity of N reflections with intensities \(I_i\) and common indices \(k\), \(l\).

\(^b\) \(R\) factor = \(\sum_i |I_i - F_{\text{calc}}| / \sum_i |I_i|\), where \(F_{\text{obs}}\) and \(F_{\text{calc}}\) are the observed and calculated structure factors, respectively.

\(^c\) For \(R_{\text{free}}\), the sum is extended over a subset of reflections (10%) excluded from all stages of refinement.

**References:**

1. Can natrin interact with other channels besides the reported BK\(\text{Ca}\) channel?
2. What is the interaction mode between natrin and its target ion channels?
3. Is there any structural difference of natrin in different crystal forms?
(+70 mV) was measured as mean value in a range from 85% to 100% of the current trace.

**Molecular modeling and docking simulation.** The modeling of the Kv1.3 channel was achieved on the basis of the structure of the mammalian Kv1.2 potassium channel (PDB code 2A79, chain B) [20], using the SWISS-MODEL server [21]. The sequence alignment between Kv1.3 and chain B of Kv1.2 showed that residues 358–491 of Kv1.3 and residues 288–421 of Kv1.2, which consisted of the potassium channel pore, shared an identity of 94%. The initial model of the Kv1.3 channel was further refined by rigid-body minimization using the CNS software, until reasonable rmsd bonds and angles were achieved.

The docking simulation was performed using the FTDock program [22]. In the simulation, region 415–459 of each chain of the Kv1.3 model and the whole natrin molecule of Form I was used. The calculated complexes were minimized by CNS software and by LIGPLOT [23].

We also performed a docking simulation between BK_{Ca} and natrin. The modeling of the BK_{Ca} channel [24] was achieved on the basis of the structure of the bacterial KcsA channel (PDB Code 1BL8) [25]. The sequence alignment between BK_{Ca} and KcsA was achieved according to the criteria described by Gao and Garcia [26]. In the simulation, region 324–368 of each chain of the BK_{Ca} model was used.

### Results and discussion

**The overall structure of natrin**

A 25 kDa protein was purified from the crude venom of *Naja naja atra* with an N-terminal sequence of NVDFNSESTRRKKKQKEIVD. According to the sequence blast, we considered that this was the protein of natrin-1 (NA–CRVP1), which was truncated at the 18th residue with molecular weight in range of acceptable errors. The whole sequence was determined from the cDNA cloning sequence and was exactly the same as the translated natrin sequence [gi: 32492059].

The two molecules of natrin in one asymmetrical unit have high structural similarity, with rmsd = 0.081 Å. The natrin molecule can be divided into three regions (Fig. 1A): a pathogenesis-related protein of the group I (PR-1) like domain (residues 6–160), a cysteine-rich domain (CRD) (residues 183–221), and a hinge between them (residues 161–182). The PR-1 domain has an α-β-α sandwich fold as PR-1 like members and the CRD domain contains three short α helixes with several linkers and tails. Structural comparison between different crystal forms revealed that their differences are mainly located at the CRD domain, especially from residue 188 to 209 (shown in Fig. 1B). The structural differences reflect the high flexibility of CRD domain which may play important role in ion channel recognition.

**The blocking of natrin to the Kv1.3 channel**

As the CRD domain of natrin has a tertiary structure similar to those of BgK and ShK, two other confirmed blockers of the voltage-gated potassium channel, we tested its effect on the whole-cell K⁺ current of CHO cells that had been transfected with voltage-gated potassium channel (Kv1.3) genes using the patch-clamp technique. Externally applying natrin in the concentration range of 1–200 nM significantly reduced Kv1.3 channel currents evoked by a depolarizing pulse from −90 to 70 mV with a −60 mV holding potential (Fig. 2A). Application of 1, 10, 50, 100, and 200 nM natrin reduced the steady-state current to 0.69 ± 0.05 (n = 4), 0.67 ± 0.08 (n = 4), 0.68 ± 0.10 (n = 6), 0.62 ± 0.11 (n = 6), and 0.54 ± 0.16 (n = 4) of the control value, respectively.
The docking results revealed a novel interaction model of blocking pattern

In order to get insights into the interactions between natrin and potassium channels, docking simulations were performed and three-dimensional natrin-Kv1.3 complex models were generated. In the simulation, region 415–459 of Kv1.3 instead of the whole model was used [27,28]. At the global scan stage, the angle step was set to nine degrees and 21440 rotations were performed by FTDock. To reduce the number of possibilities, a filter was established: the CRD of natrin must interact with the pore region of Kv1.3 channel. Among the results, only the top four had positive RPscores and thus were further refined by CNS software. The LIGPLOT analysis of the four natrin–Kv1.3 complexes showed that they were almost identical except for some atom-level interactions. The interactions between natrin and the Kv1.3 channel in the top one complex are summarized in Table 1.

Intriguingly, our docking result revealed a novel interaction pattern between K⁺ channel toxin and its target. In the docking simulation, natrin was anchored to Kv1.3 channel mainly by Gln198/Ser200/Asp204/Arg217 through hydrogen bonds. Also Gln202 of natrin seemed to play vital role in blocking the K⁺ channel. It plugged its side-chain into the channel pore and formed hydrophobic contacts with all of the four chains of Kv1.3. Fig. 3A shows the Kv1.3 channel model and the important residues in the natrin–Kv1.3 complex.

Although it has been reported that the “functional dyad” [29] and/or the “basic ring” [30,31] motifs of K⁺ channel toxins played important roles when blocking the channels, we did not find these two kinds of motifs functioning in our docking result. None of the five basic residues (K188/K197/K207/R217/K219) could form “functional dyad” with other hydrophobic residues and plugged into the pore of Kv1.3 channel at the same time (Fig. 3A). K188/K207/R217/K219 did form a ring with an aromatic residue (F215) located in their center, but lying at the side of CRD (Fig. 3B) and could not function as reported [29,32]. K207/F215/K197 were only involved in the hydrophobic contact (data not shown in Table 2), rather than hydrogen bonds. Among these five basic residues, only R217 participated in the hydrogen bonding interactions with chain C of Kv1.3 channel (Fig. 3B). From the sequence alignment of CRD domain of six CRISPs, the first five ones (Fig. 2B) share a higher homology than helothermine especially in the residue Q202 position. This implies that helothermine may have a different interaction pattern. Additionally, Tyr447 of Kv1.3 might play an important role since it is involved in both polar and hydrophobic interactions, and is conservative in Kv1 type channels.

The BKCa–natrin docking did not display results as consistent as Kv1.3–natrin complex. We selected the top three complexes according to the scores, and these
The results suggest that the CRD of natrin may have different interaction behavior from the BKCa channel under the docking conditions and only the results for the third complex showed a similar interaction pattern to that of the Kv1.3–natrin complex.

It has been reported that helothermine, a member of the CRISP-3 family, can block ryanodine receptor (RyR)/Ca\(^{2+}\) release channels. More recently, Gibbs et al., solved the NMR structure of the Crisp domain of TPX-1 (viz. the CRD domain) and found that this exclusive domain could regulate the RyR/Ca\(^{2+}\) release channel [33]. Moreover, our immunocoprecipitation experiment indicated that natrin and RyR receptor could be co-precipitated (data not shown). Therefore, we speculated that natrin could interact with the RyR/Ca\(^{2+}\) release channel and most likely attributed to this CRD domain (or both the linker and the CRD domain).

Collectively, it is demonstrated that natrin could interact with and/or block a variety of ion channels. These functions may be attributed to the C-terminal domain of CRD or both CRD and linker domains independently. The molecular simulation results reveal the crucial residues and suggest a new interaction pattern and these new findings will facilitate further experiments in inspecting the blocking interaction of natrin to various ion channels.

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**References**


