Studies of Conorfamide-Sr3 on Human Voltage-Gated Kv1 Potassium Channel Subtypes

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Abstract: Recently, Conorfamide-Sr3 (CNF-Sr3) was isolated from the venom of Conus spurius and was demonstrated to have an inhibitory concentration-dependent effect on the Shaker K+ channel. The voltage-gated potassium channels play critical functions on cellular signaling, from the regeneration of action potentials in neurons to the regulation of insulin secretion in pancreatic cells, among others. In mammals, there are at least 40 genes encoding voltage-gated K+ channels and the process of expression of some of them may include alternative splicing. Given the enormous variety of these channels and the proven use of conotoxins as tools to distinguish different ligand- and voltage-gated ion channels, in this work, we explored the possible effect of CNF-Sr3 on four human voltage-gated K+ channel subtypes homologous to the Shaker channel. CNF-Sr3 showed a 10 times higher affinity for the Kv1.6 subtype with respect to Kv1.3 (IC50 = 2.7 and 24 µM, respectively) and no significant effect on Kv1.4 and Kv1.5 at 10 µM. Thus, CNF-Sr3 might become a novel molecular probe to study diverse aspects of human Kv1.3 and Kv1.6 channels.

Keywords: Conus spurius; FMRFamide peptides; conorfamides; CNF-Sr3; Shaker K+ channels; Kv1 voltage-gated K+ channels

1. Introduction

CNF-Sr3 is a peptide belonging to the Conorfamides family within the diverse classes of conotoxins [1]. Conotoxins are short peptide toxins, usually 25 amino acids in length, contained in the venom of the predator marine snails of the genus Conus [2]. A common characteristic of these peptides is the presence of cysteine residues in their primary structure that usually form disulfide bonds during their maturation. This feature has been used to name the toxins as conopeptides (disulfide-poor peptides: zero or one) or conotoxins (disulfide-rich peptides: two or more) [3–5]. CNF-Sr3 has no cysteine residues and its C-terminus is similar to the molluskan cardioexcitatory tetrapeptide Phe-Met-Arg-Phe-NH2, initially found in the class Bivalvia of the phylum Mollusca, and afterward in other invertebrate and vertebrate phyla [6]. CNF-Sr3 presents a high similitude in sequence with
respect to CNF-Sr1 and CNF-Sr2; these conorfamides elicit hyperactivity upon injection in mouse [7,8]. CNF-Sr3 was tested on the complete Drosophila family of voltage-dependent K⁺ channels and it was shown to inhibit the Shaker subtype and not to have any significant effect on the other major families of voltage-activated K⁺ channels: Shab, Shaw, Shal, and Eag [1].

The Shaker K⁺ channel was identified by electrophysiological analyses of an unusual leg-shaking Drosophila melanogaster fruit fly strain (under ether anesthesia) [9]. Once a K⁺ channel gene was identified, additional voltage-activated potassium genes have been discovered in the fruit fly and in many other species, including humans [10–13]. In mammals, there are eight members related to the Shaker family (Kv1.1–Kv1.8). Mutations in some of these members lead to several diseases in humans. To mention just a few examples, changes in Kv1.1 channel may cause episodic ataxia type 1 [14], whereas the Kv1.3 channel has been associated with multiple sclerosis, type-1 diabetes, and rheumatoid arthritis, but relatively little is known about Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv1.7, or Kv1.8 channels in human disorders [15]. However, Martel et al., by electrochemical recordings at rat dorsal striatum, confirmed a role for Kv1.2 and Kv1.6 in dopamine release [16] and other authors have shown that Kv1.4 and Kv1.5 are related to cardiac effects [17–19].

The present study arises from our previous finding that the Shaker K⁺ channel is inhibited by CNF-Sr3. Here, we report the effects of CNF-Sr3 on human Kv1.3, Kv1.4, Kv1.5, and Kv1.6 channels.

2. Results

Evaluation of CNF-Sr3 on Kv1 channels Expressed in Oocytes

Previously, it was reported that CNF-Sr3 reversibly inhibits the Shaker K⁺ channel with no activity over other Drosophila families of voltage-dependent K⁺ channels: Shab, Shaw, Shal, and the distant hEag K⁺ channel [1]. Therefore, we decided to examine the effect of CNF-Sr3 on four members of the human Shaker-related subfamily, the Kv1.3, Kv1.4, Kv1.5, and Kv1.6 channels, due to their functional implications in human diseases, particularly for Kv1.3 [20]. Since CNF-Sr3 has a Kd = 2.7 μM for the Shaker channel and there is evidence that some conotoxins, like κ–PVIIA, bind to Shaker but not to Kv1 channels [18,19], we decided to test CNF-Sr3 at higher final concentrations from 10 to 30 μM. At 10 μM, CNF-Sr3 was capable of abolishing up to 20% and 80% of the current for Kv1.3 and Kv1.6, respectively (Figure 1A,D), but decreased the currents for Kv1.4 and Kv1.5 channels by less than 5% (Figure 1B,C). Therefore, we decided to increase the concentration in those which were most sensitive (Kv1.3 and Kv1.6) and we found that almost all the current was inhibited irreversibly at 30 μM. In Figure 1E,F is shown the time course of inhibition of Kv1.3 and Kv1.6 by CNF-Sr3, respectively. Then, concentration–response curves were generated for the Kv1.3 and Kv1.6 channels. CNF-Sr3 blocked Kv1.6 with an ~10-fold greater affinity than Kv1.3 (IC₅₀ 2.7 ± 0.89 μM and 24 ± 4.06 μM, respectively; mean ± SEM; n=3) (Figure 1G) and the Hill equation yielded n=1.08 for Kv1.6 and n=2.1 for Kv1.3.
Figure 1. Inhibition of human Kv1 channels by Conorfamide-Sr3 (CNF-Sr3). (A) \( \text{Kv}_{1.3} \), control current (○); current in presence of CNF-Sr3 30 µM (●). (B) \( \text{Kv}_{1.4} \), control current (○); current in presence of CNF-Sr3 10 µM (●). (C) \( \text{Kv}_{1.5} \), control current (○); current in presence of CNF-Sr3 10 µM (●). (D) \( \text{Kv}_{1.6} \), control current (○); current in presence of CNF-Sr3 30 µM (●). (E,F) Time course of inhibition by CNF-Sr3 on \( \text{Kv}_{1.3} \) and \( \text{Kv}_{1.6} \), respectively. CNF-Sr3 takes twice as much to block the current for \( \text{Kv}_{1.3} \) than \( \text{Kv}_{1.6} \). (G) CNF-Sr3 only inhibited \( \text{Kv}_{1.6} \) and \( \text{Kv}_{1.3} \) channels with low affinity: IC\(_{50}\) of 2.7 ± 0.89 µM and 24 ± 4.06 µM, respectively (n=3 for each concentration; error bars = standard error of the mean (SEM)).
3. Discussion

In this work, we provide more evidence on the effect of CNF-Sr3 on voltage-gated potassium channels; specifically, we studied the effect on the human Kv1.3–Kv1.6 subtypes, closely related to the Shaker channel. We decided to start studying the effect of CNF-Sr3 on these subtypes because of their similarity to the Shaker channel, their impact on several diseases in humans, and because few conotoxins target these subtypes preferentially [20,21]. Until now, 21 conotoxins or conopeptides (including CNF-Sr3) have been reported to have activity on K+ channels [21–24]; 17 of them are disulfide-containing peptides, but four, including CNF-Sr3, do not contain Cys residues; interestingly, all of them are expressed by vermivorous species (Mo1659, by C. monile [25]; CPY-Fe1, by C. ferrugineus [26]; CPY-Pl1, by C. planorbis [26]; and CNF-Sr3, by C. spurius [1]). The first and most studied conotoxin with activity on K+ channels is κ-PVIIA isolated from the venom of C. purpurascens, which belongs to the superfamily O1. κ-PVIIA has an IC\textsubscript{50} of 60 nM for the Shaker K+ channel expressed in oocytes, with no activity for the mammalian Kv1.1 and Kv1.4 subtypes [27]. Additionally, superfamilies A, M, J, and I\textsubscript{2} have toxins that block the Shaker channel in the µM range, with a little effect on Kv1 channels [20,25–30]. In this sense, only κM-RIIIJ and κJ-PIXIVA, isolated from C. radiatus and C. planorbis (fish- and worm-hunting snails, respectively) have inhibitory activity on Kv1.6 channels with IC\textsubscript{50} of 8 µM and 1.59 µM, respectively, whereas κ-ViTx, isolated from C. virgo (worm-hunting snail), has an IC\textsubscript{50} of 2.09 µM for the Kv1.3 channel. ViTx has 31% amino acid percent identity with SrXIA, the very first conotoxin isolated from C. spurius with activity on Kv1 channels. SrXIA blocks 58% of the current elicited by Kv1.6 at 640 nM after 23 min in the recording chamber [31].

Like CNF-Sr3, conopeptides CPY-Fe1 and CPY-Pl1, isolated form C. ferrugineus and C. planorbis, respectively, also block the Kv1.6 subtype, with the latter having a higher affinity (IC\textsubscript{50} = 170 nM), compared to CPY-Fe1 (IC\textsubscript{50} = 8.8 µM) [26].

In this work, we show that CNF-Sr3 is capable of inhibiting the human Kv1.6 and Kv1.3 channels at the same order of magnitude, like conotoxins or conopeptides previously reported to have activity on voltage-dependent K+ channels. Further characterization will be needed to understand the mechanism of action of this toxin, such as determination of I/V curves to assess whether the binding of CNF-Sr3 is voltage-dependent.

A possible explanation for the low affinity of this toxin against Kv1 channels in this work might be the use of mammalian K+ channels. The effect of CNF-Sr3 may be expected to be in the µM range, as it was demonstrated for κM-RIIIK, whose IC\textsubscript{50} for the Shaker channel is 1.21 µM and this value decreases one order of magnitude when determined on TSha1 from the fish O. mykiss (IC\textsubscript{50} = 70 nM) [32].

Even though CNF-Sr3 has a rather low affinity for human Kv1 channels, its short sequence (constituted by 15 amino acid residues, with no cysteine residues), in comparison with the conotoxins or conopeptides described above, which have an average of 25 amino acids in length with four to eight cysteine residues, might allow it to become a useful molecular tool to elucidate the role of Kv1.6 and Kv1.3 channels in human and other mammalian species, and/or as a scaffold for the design and synthesis of more specific probe molecules for these channels. Regarding conotoxins without Cys residues, CNF-Sr3 might have an advantage as a molecular tool over CPY-Pl1 and CPY-Fe1, because it does not induce a leak current in skinned Xenopus oocytes (and, probably, other cell types) [26].

4. Materials and Methods

4.1. Synthetic CNF-Sr3

CNF-Sr3 was synthesized using an Apex 396 automated peptide synthesizer (AAPPTec; Louisville, KY, USA) applying standard solid-phase Fmoc (9-fluorenylmethyloxy-carbonyl) protocols and quantified as was described recently [1].
4.2. Electrophysiology Assay in Oocytes

cDNA of the clones for human Kv1.3 to Kv1.6 channels in vector pSGEM, resistant to ampicillin, were linearized with NotI and SpHI. Then, the products were purified using a QIAquick PCR purification Kit according the protocol by Qiagen (Hilden, Germany). cDNAs were transcribed in vitro with the T7 polymerase (mMessage mMachine Kit; Ambion Inc., Austin, TX, USA). cRNA was purified using a Qiagen RNaseasy kit (Hilden, Germany). Five nanograms of cRNA was injected into each oocyte and used for voltage-clamp recording 1–2 days after injection. Sexually mature *Xenopus laevis* female frogs were kept and handled according to the institutional bioethics committee requirements. Whole-cell currents were recorded under two-electrode voltage clamp control using an OC-725C clamp amplifier (Warner Instruments, CT, USA). The intracellular electrodes were filled with 3 M KCl. Potassium currents were generated by de-polarizations to 10 mV for 1 s every 20 s with a membrane potential held at -80 mV. The bath solution was ND96, consisting of 96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, and 5 mM HEPES (pH 7.5). CNF-Sr3 was diluted in ND96 and directly applied (3 µL) to the (static) bath chamber (30 µL). All electrophysiological measurements were performed at room temperature (~22 °C).

Measurements are reported as the mean ± SEM of three independent experiments. Dose–response curve data were fit to the equation: IC$_{50}$ = fraction of channels blocked (fb) as a function of [CNF-Sr3], at 0 mV. fb = 1 − (IC$_{CNF-Sr3}$/IC), where IC$_{CNF-Sr3}$ toxin is the current in the presence of CNF-Sr3 in log µM. IC is the control IK current (SigmaPlot, Systat Software Inc., San Jose, CA, USA).

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References

1. Campos-Lira, E.; Carrillo, E.; Aguilar, M.B.; Gajewiak, J.; Gómez-Lagunas, F.; López-Vera, E. Conorfamide-Sr3, a Structurally Novel Specific Inhibitor of the Shaker K+ Channel. *Toxicon* **2017**, *138*, 53–58. [CrossRef]

2. Puillandre, N.; Duda, T.F.; Meyer, C.; Olivera, B.M.; Bouchet, P. One, four or 100 genera? A New Classification of the Cone Snails. *J. Molluscan Stud.* **2015**, *81*, 1–23. [CrossRef]

3. Terlau, H.; Olivera, B.M. *Conus* Venoms: A Rich Source of Novel Ion Channel-Targeted Peptides. *Physiol. Rev.* **2004**, *84*, 41–68. [CrossRef]

4. Akondi, K.B.; Muttenthaler, M.; Dutertre, S.; Kaas, Q.; Craik, D.J.; Lewis, R.J.; Alewood, P.F. Discovery, Synthesis, and Structure–Activity Relationships of Conotoxins. *Chem. Rev.* **2014**, *114*, 5815–5847. [CrossRef]

5. Lebbe, E.K.M.; Tytgat, J. In the picture: Disulfide-poor conopeptides, a class of pharmacologically interesting compounds. *J. Mollasscan Stud*. **2015**, *81*, 1–23. [CrossRef]

6. Lópes-Vera, E.; Aguilar, M.B.; Heimer de la Cotera, E.P. FMRFamide and Related Peptides in the Phylum Mollusca. *Peptides* **2008**, *29*, 310–317. [CrossRef]

7. Maillo, M.; Aguilar, M.B.; López-Vera, E.; Craig, A.G.; Bulaj, G.; Olivera, B.M.; Heimer De La Cotera, E.P. Conorfamide, a *Conus* Venom Peptide Belonging to the RFamide Family of Neuropeptides. *Toxicon* **2002**, *40*, 401–407. [CrossRef]

8. Aguilar, M.B.; Luna-Ramirez, K.S.; Echeverría, D.; Falcón, A.; Olivera, B.M.; Heimer de la Cotera, E.P.; Maillo, M. Conorfamide-Sr2, a gamma-carboxyglutamate-containing FMRFamide-related peptide from the Venom of *Conus spurius* with activity in mice and mollusks. *Peptides* **2008**, *29*, 186–195. [CrossRef]
9. Kaplan, W.D.; Trout, W.E. The Behavior of Four Neurological Mutants of Drosophila. Genetics 1969, 61, 399–409.
10. Kamb, A.; Iverson, L.E.; Tanouye, M.A. Molecular characterization of Shaker, a Drosophila gene that encodes a potassium channel. Cell 1987, 50, 405–413. [CrossRef]
11. Swanson, R.; Marshall, J.; Smith, J.S.; Williams, J.B.; Boyle, M.B.; Folander, K.; Luneau, C.J.; Antanavage, J.; Oliva, C.; Buhrow, S.A.; et al. Cloning and expression of cDNA and genomic clones encoding three delayed rectifier potassium channels in rat brain. Neuron 1990, 4, 929–939. [CrossRef]
12. Papazian, D.M.; Schwartz, T.L.; Tempel, B.L.; Jan, Y.N.; Jan, L.Y. Cloning of genomic and complementary DNA from Shaker, a putative potassium channel gene from Drosophila. Science 1987, 237, 749–753. [CrossRef]
13. Roberds, S.L.; Tamkun, M.M. Cloning and tissue-specific expression of five voltage-gated potassium channel cDNAs expressed in rat heart. Proc. Natl. Acad. Sci. USA 2006, 88, 1798–1802. [CrossRef]
14. Wang, H.; Jen, J.C.; Lee, H.; Baloh, R.W.; Nelson, S.F.; Sabatti, C. A novel mutation in KCNA1 causes episodic ataxia without myokymia. Hum. Mutat. 2004, 21, 536.
15. Frollov, R.V.; Bagati, A.; Casino, B.; Singh, S. Potassium Channels in Drosophila: Historical Breakthroughs, Significance, and Perspectives. J. Neurogenet. 2012, 26, 275–290. [CrossRef] [PubMed]
16. Martel, P.; Lee, D.; Fulton, S.; Béard, M.; Trudeau, L.E. Role of Kv1 Potassium Channels in Regulating Dopamine Release and Presynaptic D2 Receptor Function. PLoS ONE 2011, 6, e20402. [CrossRef] [PubMed]
17. Suzuki, S.; Baba, A.; Kaida, K.; Utsugisawa, K.; Kita, Y.; Tsugawa, J.; Ogawa, G.; Nagane, Y.; Kuwana, M.; Suzuki, N. Cardiac involvements in myasthenia gravis associated with anti-Kv1.4 antibodies. Eur. J. Neurol. 2014, 21, 223–230. [CrossRef]
18. Ding, W.G.; Xie, Y.; Toyoda, F.; Matsuura, H. Improved Functional Expression of Human Cardiac Kv1.5 Channels and Trafficking-Defective Mutants by Low Temperature Treatment. PLoS ONE 2014, 9, 1–9. [CrossRef]
19. Nattel, S.; Yue, L.; Wang, Z. Cardiac Ultrarapid Delayed Rectifiers. Cell. Physiol. Biochem. 1999, 9, 217–226. [CrossRef]
20. Comes, N.; Serrano-Albarrás, A.; Capera, J.; Serrano-Novillo, C.; Condom, E.; y Cajal, S.R.; Ferreres, J.C.; Felipe, A. Involvement of potassium channels in the progression of cancer to a more malignant phenotype. Biochim. Biophys. Acta 2015, 1848, 2477–2492. [CrossRef]
21. Shon, K.J.; Stocker, M.; Terlau, H.; Stühmer, W.; Jacobsen, R.; Walker, C.; Grilley, M.; Watkins, M.; Hillyard, D.R.; Gray, W.R.; et al. κ-Conotoxin PVIIA is a peptide inhibiting the shaker K⁺ Channel. J. Biol. Chem. 1998, 273, 33–38. [CrossRef] [PubMed]
22. Mahdavi, S.; Kuyucak, S. Why the Drosophila Shaker K⁺ Channel Is Not a Good Model for Ligand Binding to Voltage-Gated Kv1 Channels. Biochemistry 2013, 52, 1631–1640. [CrossRef]
23. Imperial, J.S.; Bansal, P.S.; Alewood, P.F.; Daly, N.L.; Craik, D.J.; Sporning, A.; Terlau, H.; López-Vera, E.; Bandypadhyay, P.K.; Olivera, B.M. A Novel Conotoxin Inhibitor of Kv1.6 Channel and nAChR Subtypes Defines a New Superfamily of Conotoxins. Biochemistry 2006, 45. [CrossRef] [PubMed]
24. Lewis, R.J.; Dutrê, S.; Vetter, I.; Christie, M.J. Conus Venom Peptide Pharmacology. Pharmacol. Rev. 2012, 64, 259–298. [CrossRef]
25. Sudarsal, S.; Singaravadivelan, G.; Ramasamy, P.; Ananda, K.; Sarma, S.P.; Sikdar, S.K.; Krishnan, K.S.; Balarangam, P. A novel 13 residue acyclic peptide from the marine snail, Conus monile, targets potassium channels. Biochem. Biophys. Res. Commun. 2004, 317, 682–688. [CrossRef] [PubMed]
26. Imperial, J.S.; Chen, P.; Sporning, A.; Terlau, H.; Daly, N.L.; Craik, D.J.; Alewood, P.F.; Olivera, B.M. Tyrosine-rich Conopeptides Affect Voltage-gated K⁺ Channels. J. Biol. Chem. 2008, 283, 23026–23032. [CrossRef]
27. Terlau, H.; Shon, K.J.; Grilley, M.; Stocker, M.; Stühmer, W.; Olivera, B.M. Strategy for rapid immobilization of prey by a fish-hunting marine snail. Nature 1996, 381, 148–151. [CrossRef]
28. Craig, A.G.; Zafaralla, G.; Cruz, L.J.; Santos, A.D.; Hillyard, D.R.; Dykert, J.; Rivier, J.E.; Gray, W.R.; Imperial, J.; DelaCruz, R.G.; et al. An O-Glycosylated Neuroexcitatory Conus Peptide. Biochemistry 1998, 37, 16019–16025. [CrossRef] [PubMed]
29. Ferber, M.; Sporning, A.; Jeserich, G.; DeLaCruz, R.; Watkins, M.; Olivera, B.M.; Terlau, H. A Novel Conus Peptide Ligand for K⁺ Channels. J. Biol. Chem. 2003, 278, 2177–2183. [CrossRef]
30. Kauferstein, S.; Huys, I.; Lamthan, H.; Stöcklin, R.; Sotto, F.; Menez, A.; Tytgat, J.; Mebs, D. A novel conotoxin inhibiting vertebrate voltage-sensitive potassium channels. Toxicon 2003, 42, 43–52. [CrossRef]
31. Aguilar, M.B.; Pérez-Reyes, L.I.; López, Z.; de la Cotera, E.P.H.; Falcón, A.; Ayala, C.; Galván, M.; Salvador, C.; Escobar, L.I. Peptide Sr11a from Conus spurius is a novel peptide blocker for Kv1 potassium channels. *Peptides* 2010, 31, 1287–1291. [CrossRef]

32. Al-Sabi, A.; Lennartz, D.; Ferber, M.; Gulyas, J.; Rivier, J.E.F.; Olivera, B.M.; Carlomagno, T.; Terlau, H. kM-Conotoxin RIIIK, Structural and Functional Novelty in a K⁺ Channel Antagonist. *Biochemistry* 2004, 43, 8625–8635. [CrossRef]

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