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Nematostella vectensis resistance to their own neurotoxin: Exploring the coevolutionary resistance of ion channels to neurotoxins in venomous animals

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Abstract
Nematostella vectensis (Starlet Sea Anemone) provides valuable insights into the evolution of nervous system development and excitability. For example, *Nematostella* has all types of voltage-gated K+ channels (Kvs) found in higher animals, demonstrating that these critical regulators of neuronal excitability evolved prior to the divergence of cnidarians and bilaterians roughly 600 million years ago. It is also well known that anthozoans such as *Nematostella* contain diverse voltage gated potassium channel (Kv) toxins that they use to paralyze prey or defend themselves in their natural habitats. This raises the question of whether anthozoans have developed resistance to their own toxins at the level of the channel targets themselves. To test this, we are examining whether *Nematostella* Shaker family Kv channels are resistant to a Shaker-like 2 toxin (Shkl2) present in their own venom. We expressed the *Nematostella* Shaker channels in *Xenopus* oocytes to expose them to Shkl2 to assess functional block using two electrode voltage clamp. Results obtained from this experiment suggest resistance in two different *Nematostella* shaker channels (NvKv1 and NvKv5) to Shkl2 which provides insight into the co-evolution of toxins and their target channels in venomous anthozoans.

Introduction

The phylum Cnidaria contains animals that are characterized by their radial symmetry and two celular germ layers (Frazao et al. 2012). Within the phylum, there are four well known taxonomic classes of cnidarians which are the Anthozoan, (sea anemones or corals) Hydrozoan, (hydras) Scyphozoa, (true jellyfish) and Cubozoa (box jellyfish). They differ with the shape of their mitochondrial DNA and life cycles with Anthozoans being the basal evolutionary group (Moran et al. 2011, Frazao et al. 2012, Jouiaei et al. 2015). Cnidarians are carnivorous, and the predatory success of these organisms is determined by their toxin delivering cells known as cnidocytes which contain tiny harpoon like organelles called nematocysts that rapidly project proteinaceous toxins into their prey (Moran et al. 2011). Given Cnidarians place in the taxonomic hierarchy within metazoa they provide some of the earliest insights into the evolution, development, and excitability of the nervous system. *Nematostella vectensis* (Starlet Sea Anemone) in particular has been found to have all the voltage gated potassium channels found across metazoa. (Jegla et al. 2012)

Voltage gated potassium channels are an important constituent of the nervous system because they help hyperpolarize bioelectrical activity. In bilaterians, there are three major gene families which include Shaker, KCNQ, and Ether-a-go-go (EAG) that encode all voltage gated potassium channels (Martinson et al. 2013 and Li et al. 2015). Shaker channels are found across the metazoa. They were found in *Drosophila* and contain four subfamilies (Shak, Shab, Shal, and Shaw) (Li et al. 2015). Kv channels assemble as heterotetramers which are promoted by the T1 domain in the Shaker family. Heterotetramers can only form between the same gene subfamilies, but not across different gene subfamilies. *Nematostella vectensis* was used as a model cnidarian that provided evolutionary genetic views into the first nervous systems (Jegla et al. 2012). 43 of 46 ion channel families have been conserved across bilateria and found to also be present in *Nematostella.* This implies that various voltage gated potassium channel proteins have been highly conserved throughout the evolution of the metazoans (Jegla et al. 2012). *Nematostella* Shaker Channels also showed a variety of fast inactivating or non-inactivating functional properties that resemble those of *Drosophila* or mammalian Shaker channels (Jegla et al. 2012).

Anthozoans like *Nematostella* are also well known to contain a cocktail of proteinaceous venom that they use for predatory and defensive interactions. Anthozoan toxins have been observed to induce a number of diverse toxicological effects that include cardiotoxicity, paralysis, and necrosis (Frazao et al. 2012). The best characterized toxins found within their venom are neurotoxins. They are low molecular weight peptides that allow sessile Anthozoans to immobilize their prey. They modify sodium channel gating or block potassium channel gating by prolonging the action potentials of myocytes, osteocytes, or the excitable or non-excitable membranes of sensory neurons (Jouiaei et al. 2015). Hyperactivity is followed by high release of neurotransmitters in synapses and neuromuscular junctions that eventually induce paralysis. The two types of Anthozoan neurotoxins are voltage-gated sodium channel toxins (Nav channel toxins) and voltage-gated potassium channel toxins (Kv channel toxins) (Gasparini et al. 2004, Moran et al. 2011, Frazao et al. 2015 Orts et al. 2013, Jouiaei et al. 2015). Kv channel toxins work by blocking or modulating the activity of various types of voltage gated potassium channels (Orts et al. 2013).

Since Anthozoans like *Nematostella* have developed lethal toxins throughout evolutionary history, they may have developed resistance to them at the ion channel target level. In this study we looked at this idea by examining if *Nematostella* Shaker family Kv channels are resistant to

Shaker Like 2 toxin (Shkl2) found within their venom. The results obtained in this study should provide insight into the co-evolution of toxins and target channels in venomous Anthozoans.

In this study we utilize extensive knowledge of *Nematostella vectensis* Kv channel evolution with an attempt to see if their Kv channels are resistant to their recently extracted Shk toxin (Sachkova et al. 2020). Here, we expressed NvKv1 and NvKv5 in *Xenopus* oocytes to undergo voltage clamp experiments with exposure to Shk toxin and they were compared to controlled conditions with not exposure to the toxin. Overall, we expected the Kv channel kinetics to not be affected by exposure to the toxin because anemones that contain toxins should be resistant to toxins at an ion channel level. We show here that this was the case.

Materials and Methods

1 Shkl2 Toxin Preparation

Shkl2 toxin was supplied by Maria Sachkova of Yehu Moran´s Laboratory in Tel Aviv University. 10 ug stock solution was diluted to 100 nM for use in subsequent two electrode voltage clamp experiments.

2 Electrophysiology

Xenopus laevis ovaries were used and supplied by Xenopus 1. To enzymatically defolliculate mature oocytes from the ovaries, type II collagenase was administered to the ovaries at 1 mg/mL in Ca2+ free ND98 solution (98mM NaCl, 2mM KCl, 1 mM MgCl2, 5mM Hepes, pH 7.2). After defolliculation the mature oocytes were stored in ND98 culture solution. (98mM NaCl, 2mM KCl, 1 mM MgCl2, 2.5mM Na-pyruvate, 100U/mL penicillin, 100ug/mL streptomycin, 5mM Hepes, pH 7.2) cDNA used in this study have been cloned for previous studies (Jegla et al. 2012, Li et al. 2015). Mature oocytes were injected with 50nL of transcripts and incubated at 18C in ND98 culture solution for 2 days before recording.

Two electrode voltage clamps were conducted at room temperature (22-24C) with a Dagan CA-1B amplifier. A pClamp 10 acquisition suite was used to analyze and collect data. Recordings were carried out under constant perfusion using pH 7 solution that served as the control. Experimentation followed by switching to perfusion of 100nM Shkl2 with pH7 solution. The circuitry of the bath clamp was isolated using a 1M NaCl agarose bridge. 3 M KCl was used to fill the electrodes and they had resistances of 0.4-1 MΩ.

Data obtained from individual cells from Boltzmann fits of voltage activation used the equation $g(V) = (A1-A2)/(1+ e((V- V_{50})/s)) + A2$, where g(V) is the conductance at voltage V, V_{50} is the half-maximal conductance value, s is the slope factor and A1 is the minimum and A2 is the maximum. Mean +/- S.E.M. with a simulated Boltzmann fit ($G/Gmax = 1/(1+ e-((V- V_{50})/s))$ was used to display normalized data. Fits of data from individual cells were used to obtain fixed mean values of V_{50} and s. Two tailed t-test data analysis was used to compare sets of data.

Results

Functional expression of truncated *Nematostella vectensis* Shaker 1 and 5 genes (NvShak1 ΔN and NvShak5 ΔN) were tested to determine if there was Shkl2 resistance found within these channels. Voltage activation curves for NvShak1 ΔN and NvShak5 ΔN are shown in Figures 1 and 2 respectively with parameters of Single Boltzmann Fits shown on Table 1. Overall, the curves showed no pattern of decreased activation (decreased slope) of either NvShak channel when exposed to the Shkl2 toxin (Figure 1 and 2). In fact, there was a slight increase in activation in NvShak 1 when exposed to Shkl2 toxin (Figure 1). Isochronal tail currents were used to obtain data and they followed 100ms depolarization voltage steps from a resting potential of -60mV. The

truncated versions of NvShak1 ΔN and Nv Shak5 ΔN were used to eliminate N type inactivation that otherwise would create uncertainty in determining activation of these channels.

To determine if there is any statistical difference between the voltage activation of NvShak1 ΔN with pH7 and pH7 with Shkl2 exposure and NvShak5 ΔN with pH7 and pH7 with Shkl2 exposure, individual two tailed t-tests were made on individual Shaker Channel gene V_{50} datasets. The results of the t-tests are displayed in Table 2 and showed no statistical significance between the activation of each channel when compared to their control and toxin exposed conditions.

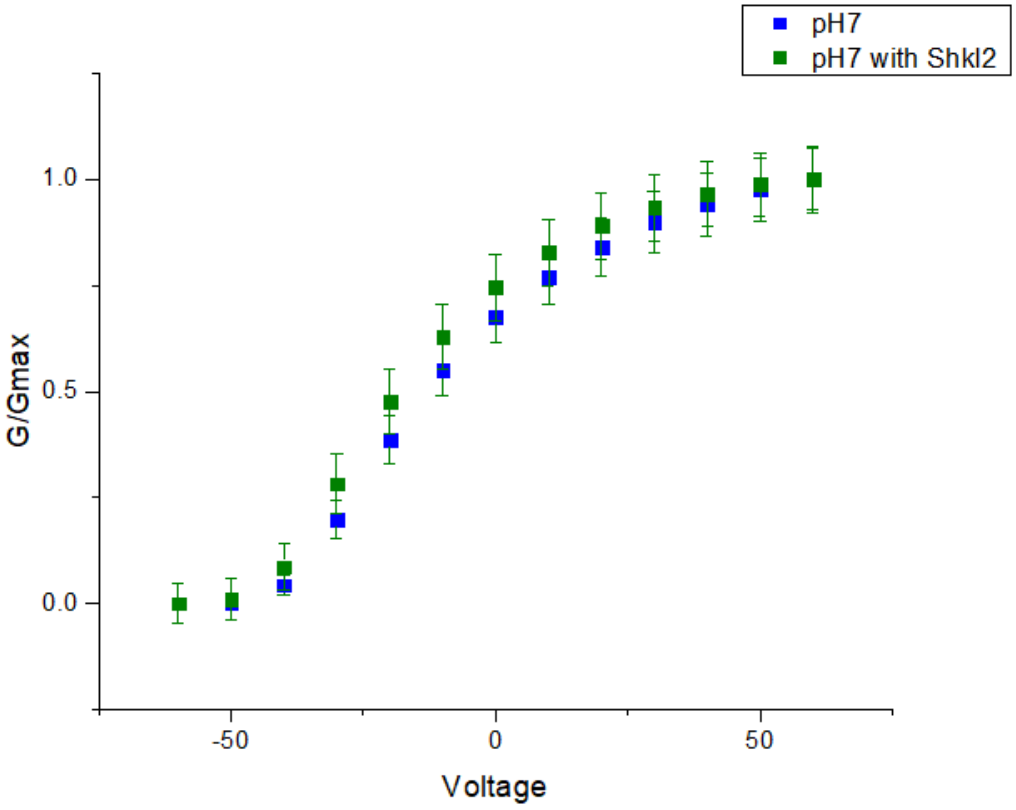

Figure 1. The voltage activation curve for NvShak1 ΔN comparing exposure to pH7 and pH7 with Shkl2. Activation of the current was achieved by depolarization voltage steps of 100ms from a holding potential of -60mV. Isochronal tail currents were used to measure conductance upon repolarization to -40mV.

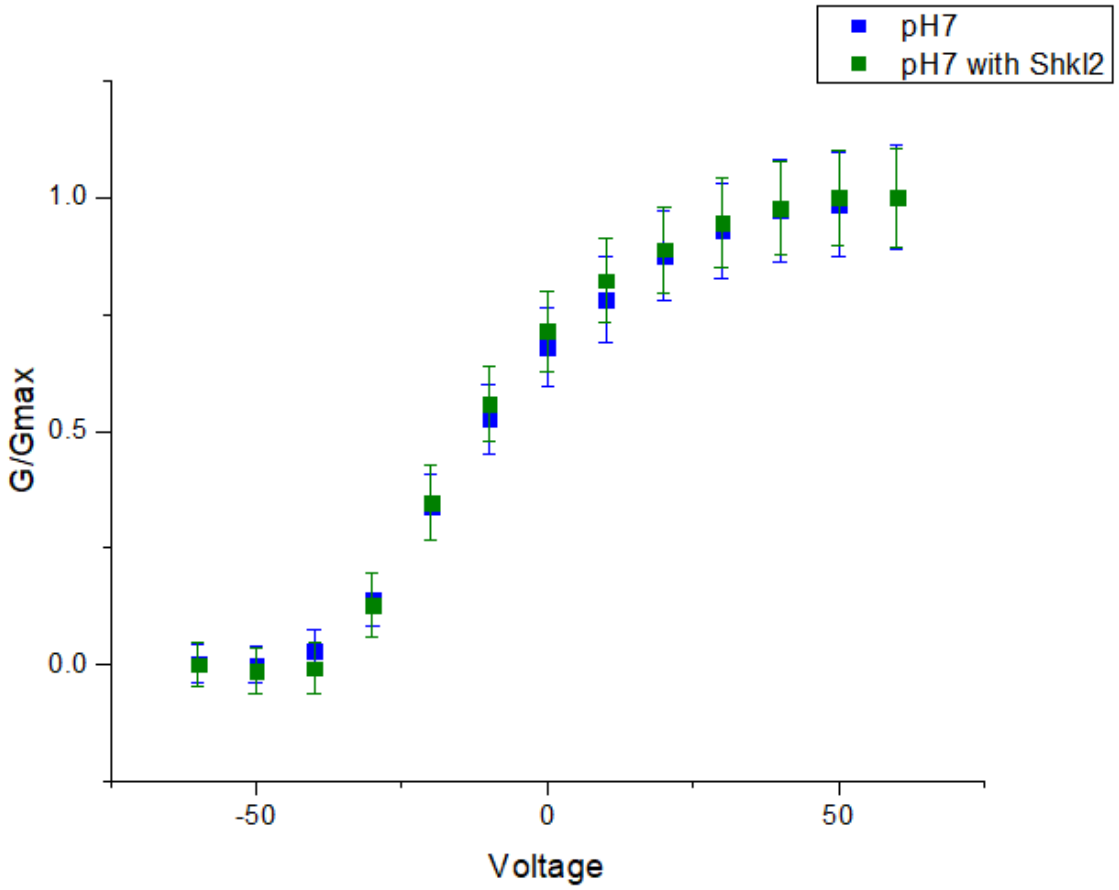

Figure 2. The voltage activation curve for NvShak5 ΔN comparing exposure to pH7 and pH7 with Shkl2. Activation of the current was achieved by depolarization voltage steps of 100ms from a holding potential of -60mV. Isochronal tail currents were used to measure conductance upon repolarization to -40mV.

Table 1. These values represent the parameter values of the voltage activation curves for NvShak1 ΔN and NvShak2 ΔN with and without exposure to Shkl2. G_v is the voltage activation curve. The V_{50} and G_v slope values are recorded in mV. All the values are average with standard error ranging from +/- 0.1-2.1.

Shaker Channel cDNA	GV V50	GV Slope
NvShak1ΔN pH7	-14.8 +/- 2.1	16.1 +/- 1.9
NvShak1ΔN Shkl2 with pH7	-20.0 +/- 1.4	15.6 +/- 2.1
NvShak5ΔN pH7	-12.2 +/- 1.4	14.1 +/- 0.1
NvShak5ΔN Shkl2 with pH7	-13.2 +/- 0.6	12.3 +/- 0.5

Table 2. Results of two tailed t-tests comparing the average V_{50} values for NvShak1 ΔN with exposure to pH7 and pH7 with Shkl2. A two tailed t-test was also conducted for NvShak5 ΔN comparing the same values. P-value significance level is measured at $\alpha \leq 0.05$.

Shaker Channel cDNA	NvShak1ΔN	NvShak5ΔN
P-value	0.063277	0.560344
SE Mean	1.994104	0.75914

Discussion

Upon observation the half activation curves for NvShak1 ΔN and NvShak5 ΔN do not appear to have a statistical difference when they are exposed to Shkl2 compared to when they are not. Subsequent two tailed t-tests for the V_{50} values of NvShak1 ΔN with and without exposure to toxin showed no statistical significance at a p-value ≤ 0.05 (p-value = 0.063277, SE Mean = 1.994104). The t-test for NvShak5 ΔN was done with similar values and showed far more statistical insignificance. (p-value = 0.560344 and SE = 0.75914). It is important to note that results for the two tailed t-test for NvShak1 ΔN may show statistical significance upon reexamination considering how close the p-value is to $\alpha \leq 0.05$. However, these results suggest that NvShak1 ΔN NvShak5 ΔN may be resistant to Shkl2. Since NvShak5 ΔN showed potentially notable differences in conductance of current sizes between the control and the experimental samples, a two-tailed t-test was conducted to compare peak current sizes between both samples.

Similar neurotoxic structures like Shk toxin found in *Strichodactyla helianthus* have been commonly used to block Kv1.3 channels found in human memory effector T cells (Chi et al. 2012). Shk has also been found to block similar Shaker channels like Kv1.1 and Kv1.2 (Castañeda and Harvey 2009). However, the results show evidence for resistance of Shkl2 toxin in NvShak1 ΔN and NvShak5 ΔN. Since Shkl2 is found in the venom of *Nematostella* resistance (Sachkova et al. 2020) of this neurotoxin down to the level of the ion channels may suggest coevolutionary adaptation of the *Nematostella* neurotoxin interaction with their ion channels. Similar studies have looked at Tetrodotoxin (Na_v channel toxin) resistance in various animals. A notable example is that of the *Fugu pardalis* (pufferfish) which is resistant to TTX and is a TTX containing animal. Specifically, a nonaromatic amino acid at position 401 in its skeletal muscle $Na_v1.4a$ is largely responsible for this resistant (Soong and Venkatesh 2006). A similar amino acid substation was identified in epibatidine resistance of frogs that contain these toxins (Tarvin et al. 2017). A similar coevolutionary response may be responsible for NvShak1 ΔN and NvShak5 ΔN channel resistance in *Nematostella* although more research is needed to look at this possibility. Finally,

Future work may look at possible resistance in other NvShak subfamily genes to provide more insight into this possibility. To further validate this idea future work should compare the structure of NvShak subfamily genes with other sensitive Kv1 subfamily to determine a potential structural resistance of NvShak subfamily genes. Overall, these results suggest a co-evolution of neurotoxins and target channels in anthozoans.

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