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Functional Constraints and the Evolution of TTX-resistance in TTX-bearing Salamanders

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Advisor: Charles Hanifin -Department of Biology

Abstract

Tetrodotoxin (TTX) binds to voltage-gated sodium (Na_V) channels, blocking the initiation of action potentials and the activity of nerve and muscle fibers. Some salamanders use TTX as an anti-predator defense. These salamanders are resistant to TTX because they express TTX-resistant Na_V channels in their nerves and muscles. In vertebrates, Na_V channels are encoded by a multi-gene family. Different members of this gene family possess different functional properties and are expressed in different neuromuscular tissues. Cardiac muscle and the cardiac muscle channel (Na_V 1.5) of amphibians and many reptiles is TTX-sensitive while Na_V 1.5 in mammals (and some reptiles) is TTX-resistant. TTX-resistant channels (Na_V 1.4) have also been identified in the skeletal muscle of salamanders that possess TTX and snakes that eat TTX-bearing prey. TTX-resistance of Na_V 1.5 of mammals and reptiles results from changes in domain I of the channel, but TTX-resistance in Na_V 1.4 from salamanders and snakes results from amino acid substitutions in domains 3 and 4 suggesting that functional requirements play an important role in channel evolution. Cardiac muscle of TTX-bearing salamanders (amphibians) is TTX-resistant, but the molecular basis of resistance unknown. The goals of my project are to answer two questions:

1) Is Na_V 1.5 of TTX-bearing salamanders TTX-resistant?

2) Do the changes in Na_v 1.5 of salamanders occur in domain I of the protein or do they occur in domains 3 and 4?

Completing the goals of this project will improve our understanding of the dynamics of molecular evolution and the physiology of Na_V channels.

Literature Review

Voltage-gated sodium channels are a critical component of nerve and muscle function. These proteins are responsible for the initiation and propagation of actions potentials (APs) that form the basis of signal transmission in the neuromuscular system [1]. In vertebrates, these proteins are encoded by a multimember gene family with complex tissue-specific patterns of expression. One long-standing hypothesis is that differential functional properties of individual Na_v genes coupled with tissue-specific expression patterns underlies the complexity of vertebrate neuromuscular systems [1].

There are a number of natural product toxins that target voltage-gated sodium channels. One of the best studied of these is tetrodotoxin (TTX). Tetrodotoxin targets the pore region of the sodium channels. When Na_v channels open, the influx of sodium ions through the pore changes the electrochemical gradient of the cell and depolarizes the cell membrane. This depolarization causes nearby sodium channels to open leading to the dramatic change in membrane potential that then initiates an action potential (AP) [6]. Tetrodotoxin binds to the pore and blocks the influx of sodium ions. When Na_v channels are blocked by TTX no AP can occur and no signal is sent ultimately leading to paralysis and death in animals that are exposed to TTX [1].

Animals that use TTX as an anti-predator defense or are exposed to TTX through predation have evolved resistant to TTX [2, 3, 4, 5]. Some garter snakes of the genus *Thamnophis* are known to eat TTX-bearing newts and are also resistant to TTX. TTX-bearing salamanders

have also been shown to be highly resistant to TTX [5]. Resistance in these lineages results from the expression of TTX-resistant voltage-gated sodium channels. TTX-resistance in sodium channels results from adaptive substitutions in the pore regions of multiple members of the voltage-gated sodium channel gene family [2, 5, 7]. Adaptive substitutions associated with TTX-resistance have now been identified in the channel expressed in skeletal muscle (Na_V 1.4), cardiac muscle (Na_V 1.5), as well as two neuronal channel genes (Na_V 1.6, Na_V 1.7) of garter snakes.

Some voltage-gated sodium channel genes have evolved resistance to TTX independently of selective pressures of either eating TTX-bearing prey or using TTX as an anti-predator defense [1]. The lamprey heart channel (Na_v 1.5) is TTX-resistant suggesting that a TTX-resistant cardiac Na_v was present in an early ancestor of vertebrates [3]. However, TTX-resistance seems to have vanished since fish, amphibians and birds all have TTX-sensitive Na_v 1.5 channels. Resistance appears to have been lost through a single amino acid substitution in domain 1 of the heart channel pore. Surprisingly mammals and some reptiles have TTX-resistant cardiac muscle. It is unknown why mammals and some reptiles have TTX-resistant Na_v 1.5 but it most likely results from selective pressures associated with functional requirements of mammalian cardiac tissues that caused the reemergence of the substitution in domain 1[1].

The ancestor of modern salamanders likely possessed TTX-sensitive Na_V 1.5 channels but these channels must be resistant in modern TTX-bearing salamanders because of their exposure to TTX. This suggests that TTX-bearing salamanders have likely evolved resistant Na_V 1.5 channels independently of other vertebrate lineages. Functional requirements of Na_V 1.5 may, however, constrain its evolution in TTX-bearing salamanders. Evidence from mammals and reptiles (see above) suggests that substitutions in domain I are favored over changes in domains 3 and 4 in Na_V 1.5. It is unclear whether the functional requirements of salamander heart tissue are more similar to mammals or reptiles heart tissue or skeletal muscle and testing this possibility is a core element of this proposal.

The evolution of TTX-resistance in cardiac muscle tissue of TTX-bearing salamanders may be further complicated by patterns of gene expression. In contrast to skeletal muscle tissue, cardiac muscle tissue of amphibians expresses two channel isoforms, Na_v 1.4 and Na_v 1.5. Cardiac muscle resistance to TTX may have evolved simply because this tissue expresses the TTX-resistant isoform, Na_v 1.4 or TTX resistance may have evolved independently in Na_v 1.5. My project will test both possibilities.

Hypotheses

Taricha granulosa is resistant to its own toxin. I hypothesize that all of its individual Na_v channels including Na_V 1.5 will be resistant to TTX. Resistance in the skeletal muscle specific channel (Na_v 1.4) results from amino acid substitutions in domains 3 and 4. I predict that the functional requirements of cardiac tissue and the cardiac specific channel (Na_V 1.5) from this species is more similar to mammals and reptiles that possess TTX-resistant cardiac channels and the changes associated with TTX-resistance in NaV 1.5 of this species will occur in domain I of the channel rather than domains 3 and 4.

Ambystoma tigrinum does not posses TTX, as such I hypothesize that none of its Na_v channels will be resistant.

Project Plan

1. Extract total RNA from heart and skeletal muscle of *Taricha granulosa* and *Ambystoma tigrinum.* (completed)
2. Generate cDNA using rtPCR and Rapid Amplification of cDNA Ends (RACE) as well as Na_v channel specific mRNA primers. (in progress)
3. Use Na_v1.4 and Na_v 1.5 gene specific primers to amplify respective genes. (in progress)
4. Because I expect both Na_v 1.4 and Na_v 1.5 to be expressed in heart muscle tissue I will use recombinant cloning to isolate and amplify each ortholog present in the heart. (in progress)

Outcomes of *Taricha* sequence data

1. Changes present in Na_V 1.5 of *Taricha* will occur in domain 1 of the protein suggesting that cardiac tissue in these salamanders is under the same or similar functional constraints as mammals.
2. Changes present in Na_V 1.5 of *Taricha* will occur in domains 3 and 4 of the protein; and will similar to the substitutions seen in skeletal muscle channel of TTX-resistant salamanders. These results suggest that, the biophysical requirements of the two tissue types may not be different but the similar function requirements shape the adaptive evolution and physiology of these two channels in salamanders.
3. Changes present in Na_V 1.5 of *Taricha* will occur in neither domain 1 nor domains 3 or 4 demonstrating that other substitutions can impart TTX-resistance in Na_V 1.5 of salamanders and that the functional requirements of cardiac tissue in amphibians differs from the skeletal muscle of these species as well as the cardiac tissue of mammals.

Outcomes of *Ambystoma* sequence data

1. If there are no changes in *Ambystoma* cardiac channel sequence then the ancestor of TTX-resistant TTX-bearing salamanders did not have cardiac TTX resistance and that heart channel resistance evolved in modern salamanders.
2. If there are changes in the *Ambystoma* cardiac channel sequence then the ancestor of newts had resistant cardiac channels and that heart channel resistance preceded TTX-toxicity.

Budget

Total Request: $1000

From URCO: $500

From Utah State, Uintah Basin (Dean B. Edwards): $500

Materials and Supplies	Cost	Sponsor
Smarter RACE cDNA Amplification Kit 10 rxns (Clontech)	$734.00	Shared-URCO & USUBC
Salamander Na_V 1.5 gene specific RACE primers(Life Tech.)	$100.00	USUBC

Advantage Genomic LA Polymerase 100 rxns (Clontech)	$175.00	URCO

All other required supplies, equipment, and additional expenses will be covered by C. Hanifin, Assistant Professor in Biology at USUBC.

Timeline of Data Acquisition

Complete final sequence of Na_V 1.4 from *Ambystoma tigrinum*	November 1, 2013
Complete cDNA synthesis of *A. tigrinum* and *T. granulosa* Na_V 1.5	November 1, 2013
Amplify and purify Na_v 1.5 from both species using PCR	January 30, 2014
Sequence DNA and analyze data.	February, 2014

Presentation of Results

USU student showcase.	April, 2014
Uintah Basin Research Conference	May, 2014

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