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A novel mutation from gene splicing of a voltage-gated sodium channel in a marine copepod and its potential effect on channel function

Lihua Chen [a,1], Huan Zhang [a], Michael Finiguerra [a,2], Yuriy Bobkov [b], Christelle Bouchard [b], David E. Avery [a,3], Peter A.V. Anderson [b], Senjie Lin [a], Hans G. Dam [a,*]

[a] Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340, United States
[b] The Whitney Laboratory for Marine Bioscience, University of Florida, 9505 Ocean Shore Blvd., St. Augustine, FL 32080, United States

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ABSTRACT

The saxitoxins (STX), a group of potent neurotoxins produced by some marine algae (dinoflagellates and cyanobacteria), block voltage-gated sodium channels, inhibiting nerve-signal transmission in consumers of STX-bearing prey. Populations of grazers (clams and copepods) persistently exposed to the STX-bearing dinoflagellate *Alexandrium fundyense* are less susceptible to STX than naïve ones. Adaptation to STX in clams is linked to a point mutation at the STX-binding site in the sodium channel, which dramatically lowers the sensitivity to the toxin (STX resistance). The present study tested if a similar mechanism of STX resistance occurs in the copepod *Acartia hudsonica*. Our cloning and sequencing results indicate that two full-length cDNA variants (*AhNa$_V$1* and *AhNa$_V$2*) of the sodium channel exist in *A. hudsonica*, which result from alternative splicing of the single coding gene. Both variants have identical nucleotide sequences except that *AhNa$_V$1* (the putative mutant isoform) contains a three-amino-acid (GRD) insertion and a single adjacent aa-substitution (A to V) close to the inactivation gate on the cytoplasmic linker between domains III and IV of the sodium channel. All individuals express both *AhNa$_V$1* and *AhNa$_V$2* in varying proportions. The functional consequences of the mutation were studied by inserting the three-amino acid codons into a rat (rNa$_V$1.2) sodium channel expressed in both *Xenopus* oocytes and HEK cells. Currents carried by construct rNa$_V$1.2 bearing the GRD insertion did not inactivate as completely, and recovered faster from inactivation than rNa$_V$1.2. These two rNa$_V$1.2 constructs were, however, equally sensitive to STX, suggesting that the GRD variation does not confer STX resistance on the rat sequence of Na$_V$1.2. These results render unlikely the hypothesis that this novel mutation is responsible for the adaptation (via resistance) of *A. hudsonica* to STX-bearing prey.

1. Introduction

Understanding the mechanisms that confer resistance or tolerance to toxic prey in predators remains a fundamental challenge to biologists. The marine dinoflagellate genus *Alexandrium* has a widespread distribution in coastal areas (Anderson, 1997; Yamaguchi et al., 2002). Similarly to cyanobacteria, several species in the genus *Alexandrium* produce a suite of neurotoxins collectively known as the saxitoxins (herein referred as STX) (Shimizu, 1993). STX can be transferred throughout the food web and accumulated in marine animals, including clams (Bricelj et al., 2005), crustaceans (Wekell et al., 1996), mollusks (Hwang et al., 2007), fish (Galvao et al., 2009), and mammals (Batoreu et al., 2005). Toxic *Alexandrium* spp. blooms represent a threat to public health, to the fishery industry, and to the marine food web (Shumway, 1990).

STX bind to the voltage-gated sodium channels in metazoan cells and block the movement of sodium ions across nerve and muscle cell membranes (Denac et al., 2000), inhibiting action potentials, nerve transmission, and ultimately muscle contractions. Thus, consumption of contaminated shellfish and fish is known as paralytic shellfish poisoning (PSP). Symptoms of PSP in humans include numbness, paralysis, disorientation, and even death (Lehane, 2001; Ritchie and Rogart, 1977). In marine animals, effects of ingestion of STX include diminished performance manifested as inability to burrow in clams (Bricelj et al., 2005), and decreased ingestion and reproduction (Colin and Dam, 2002, 2007), respiration (Colin and Dam, 2003), and survival (Colin and Dam, 2004) of coastal copepods. It has been demonstrated, nonetheless, that a mutation in the binding site of STX in the voltage-gated sodium channel confers resistance to PSP toxins in clams. That is, individual clams that carry the mutation have dramatically lower

* Corresponding author. Tel.: +1 860 4059098.
E-mail address: hans.dam@uconn.edu (H.G. Dam).
[1] Current address: Department of Pharmacology, Center for Compulsive Behaviors and Addiction, Rush University Medical Center, 1735 W. Harrison Street, Cohn Research Building, Chicago, IL 60612, United States.
[2] Current address: Department of Ecology and Evolutionary Biology, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340, United States.
[3] Current address: Department of Arts and Sciences, Maine Maritime Academy, 54 Pleasant St., Castine, ME 04420, United States.

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sensitivity to STX than susceptible ones. The mutation allows nerve transmission to occur normally in resistant animals even under toxic food conditions (Bricelj et al., 2005); resistant clams outcompete susceptible individuals and predominate in areas where toxic *Alexandrium* spp. occur (Bricelj et al., 2010; Connell et al., 2007). Similar mutations confer resistance to tetrodotoxin, a toxin that also blocks sodium channels, in snakes (Geffeney et al., 2002) and pufferfish (Venkatesh et al., 2005). Mutations in the sodium channel also confer resistance to insecticides in mosquitoes (Ranson et al., 2000) and scorpion toxins in mice (Rowe et al., 2013).

Adaptation of the copepod *Acartia hudsonica* to STX-producing *Alexandrium* spp. prey has also been observed. Populations of *A. hudsonica*, which have persistently co-occurred with toxic *Alexandrium* spp., had higher ingestion and egg production rates, and population fitness than those naïve to *Alexandrium* spp. (Colin and Dam, 2002, 2003, 2004, 2007). Adaptation is unlikely to result exclusively by avoiding ingestion of toxic cells; i.e., *A. hudsonica* can select against toxic *A. fundyense* cells, but nonetheless consumes those toxic cells at high rates even in the presence of alternative prey (Colin and Dam, 2003; Sent-Batoh et al., 2015; Teegarden et al., 2003). Genetic selection experiments showed that adaptation of *A. hudsonica* to toxic *Alexandrium* is heritable, and that adaptation in a naïve population is manifested in less than three generations (Colin and Dam, 2004). A rapid loss of adaptation was observed when exposure to toxic *Alexandrium* stopped in this population (Avery and Dam, 2007). This suggested that adaptation involves a simple genetic system in *A. hudsonica*, and that it may be similar to the mechanism of adaption in clams, pufferfish, and garter snakes; i.e., a mutation in the sodium channel.

The present study reports, for the first time, the sequence for the alpha-subunit of the voltage-gated sodium channel of the copepod *A. hudsonica*. The cDNA of the sodium channel gene from *A. hudsonica* was cloned and sequenced and two cDNA variants were found. In the process of characterizing the intron/exon structure of the coding gene, however, only one sequence was found, leading to the discovery that alternative splicing is responsible for the expression of the two mRNA isoforms. In addition, reverse transcription quantitative PCR was used to determine the relative expression levels of the two variants for individual copepods. Another purpose of the study is to report the results of electrophysiological experiments to determine the potential consequences of the two isoforms on the sodium channel function.

2. Materials and methods

2.1. Cloning and sequencing of copepod sodium channel alpha subunit

2.1.1. RNA isolation

Approximately one thousand *A. hudsonica* individuals of mixed copepodid (C4–C6) stage were fixed in 5 ml of Trizol Reagent (Invitrogen) to isolate total RNA using the "Modified Qiagen" method, according to the recommendations of Zhang et al. (2013). Briefly, copepods were pelleted by centrifugation, homogenized by bead-beating, extracted with multiple phenol: chloroform (5:2; v:v) additions, and purified using a Qiagen RNeasy mini-kit column and reagents (Qiagen, Valencia, CA). Poly(A) mRNA was further isolated from 100 μg of the total RNA by using PolyA Tract mRNA Isolation System IV (Promega, Madison, WI). The RNA samples were stored at −80 °C.

2.1.2. Construction of cDNA libraries

The full-length cDNA encoding the sodium channel is about 7 kb in other organisms, and a similar length was expected for the copepod channel. Consequently, to maximize coverage and obtain the full-length sequence of the copepod sodium channel, four kinds of *A. hudsonica* first-strand cDNAs were synthesized: 1) the first kind of cDNA pool was expected to contain fragments with intact 5′end, coding region and 5′UTR. It was generated using a modified reverse transcription template-switching method (Chenchik et al., 1998). Random hexamers were used as the primers to synthesize the first strand cDNA. Then a modified DNA oligo R-SOligo-1 (CGACTGGAGCACGAGG ACACTGACATGGACTGAAGGAGTAGGG) was used for the template switch; 2) the second kind of cDNA pool was expected to contain fragments downstream of the 5′end of the cDNA. Random hexamers were used as the primers to synthesize the 1st strand cDNA; 3) the third kind of cDNA pool was expected to contain fragments which cover the middle region of the sodium channel as well as fragments upstream of the 3′ extremity of the channel transcript. A standard oligo-dT_{16} was used to prime mRNAs; and 4) the last, fourth, 1st strand cDNA pool was synthesized using GeneRacer™ Oligo dT (Invitrogen, Carlsbad, CA, USA) to resolve the sequence of the 3′ extremity of the channel including 3′-UTR.

For the first cDNA pool, 1 μl of oligo R-SOligo-1 (10 μM), 1 μl of random hexamers (50 ng/μl), 1 μl of dNTP (10 mM) and 7 μl of mRNA (~70 ng) were added to a reaction tube, and the manufacturer's protocol for Improm II reverse transcriptase (Promega Corporation, Fitchburg, WI) for synthesizing random hexamer 1st strand cDNA was followed. The 1st strand cDNAs from "2, 3, and 4" were synthesized using ~500 ng total RNA as the template, basically following the manufacturer's protocol.

2.1.3. Primer design, PCR and sequencing

Degenerate primers (Table 1) were designed based on the conserved region of sodium channels from insects (*Drosophila melanogaster*, *Blattella germani*, *Pediculus humanus*) and scorpion (*Mesobuthus martensiia*). These primers were used in various combinations in PCR with ExTaq DNA polymerase (TaKaRa Bio Inc., Madison, WI, USA) and a touch-up PCR program: 94 °C for 1 min; 94 °C 20 s, 52 °C 30 s, 72 °C 40 s for 5 cycles; 94 °C 20 s, 56 °C 30 s, 72 °C 40 s for 30 cycles; 72 °C for 10 min. Several fragments of the copepod sodium channel were successfully amplified and sequenced. Based on the sequence of these fragments, specific primers (Table 2) for the sodium channel of *A. hudsonica* were designed and

Table 1
Degenerate primers designed for sodium channel alpha-subunit of the copepod *Acartia hudsonica*.

Primer name	Primer sequence	Application
SC_F1	GACATHTTYTGYGTNTGGGAYTGYTG	cDNA cloning
SC_F2	TTCTTCACCGCYACYTTYGCNATHGARGC	cDNA cloning
SC_F3	TTCATCTTCGCTGTNATGGGNATGCA	cDNA cloning
SC_F4	ATGATTGTTTTCCGAGTBCTCTGYGGNGARTGG	cDNA cloning
SC_F5	TACTTTACYAAYGCNTGGTGYTGG	cDNA cloning
SC_F6	CAAGTCGCTACTTTYAARGGNTGGAT	cDNA cloning
SC_F7	GGTGGTTCTYTSGARATGTTYATG	cDNA cloning
SC_F8	TACTACAACGCNATGAARAARATGGG	cDNA cloning
SC_F9	ATGTTCATCTTYGCNATHTTYGGNATG	cDNA cloning
SC_F10	ATGTCCACTTCTGCNGGNTGGGA	cDNA cloning
SC_F11	CAGGCTACCGAAGAYGTNCARGARGG	cDNA cloning
SC_F12	GACTACGACATGTAYTAYGARATHTGG	cDNA cloning
SC_R1	GATGAAGTCGAARATRTTCCANCCYTC	cDNA cloning
SC_R2	TTCTTGCCGAASARYTGCATNCCCAT	cDNA cloning
SC_R3	CCACAGAGVACTCGRAANACDATCAT	cDNA cloning
SC_R4	ACAATGACGAARTCSARCCARCACCA	cDNA cloning
SC_R5	CCAGCAAAGAGYTGNACNCCCAT	cDNA cloning
SC_R6	TTCTTCTGGTCYTCNGTCATRAACAT	cDNA cloning
SC_R7	TTGTAGTACTTCTTYTGRTCYTCNGTCAT	cDNA cloning
SC_R8	CATACCGAAGATNGCRAADATRAACAT	cDNA cloning
SC_R9	TTCATCGAGGATGACNGCDATRTACAT	cDNA cloning
SC_R10	TCGAATTGTTGCCADATYTCRTARTACAT	cDNA cloning
SCN_F1	GAAGAGGAGAGGAGNCTNTTYAGRCCNTTYAC	cDNA cloning
SCN_R1	GCAGACAAYCTRAARATRTCYTTNCC	cDNA cloning
SCN_R2	ATCACAACRAARTCNARCCARTTCCA	cDNA cloning
SCN_R3	GCAAGATTWCCAAGNTCDATNCCCAT	cDNA cloning
SCN_R4	TTCTTAACAGACTCAATNACNGCNCC	cDNA cloning
SCN_R5	CACTTCTGRGTNAGNACNCCCAT	cDNA cloning
SCN_R6	ATGAAAAARAGCATRTGCCANGGNCC	cDNA cloning

Note: F: forward primer; R: reverse primer.