Characterization of a Na^+-dependent cation-chloride cotransporters (CCCs) in *Aedes aegypti* larvae by RNA interference

Jiayu Chen '17, Christopher M. Gillen
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Abstract

Aedes aegypti (Yellow fever mosquito) is a vector for various diseases such as dengue fever, chikungunya and yellow fever. Pesticides are currently the most common ways to control mosquitoes. However, because mosquitoes develop resistance to pesticides and several pesticides are deleterious to environment, there is demand for new methods. One possible target is osmoregulation (salt and water balance). At different stages of development, mosquitoes face various osmoregulatory stresses. Most mosquito larvae live in freshwater and encounter osmotic water influx and ion loss. Therefore, larvae must absorb ions. We hypothesize that the putative Na^+-dependent cation-chloride cotransporter (aeCCC2) contributes to ion absorption in mosquito larvae. To evaluate the function of aeCCC2, we exposed larvae to double-stranded RNA to knock down the expression of aeCCC2. The hemolymph ion concentrations in larvae were strongly altered. Mean total ammonia concentration of hemolymph was increased five-fold, and Na^+ concentration was increased by approximately 20%. There was no significant change in K^+ concentration. Even though the precise function of aeCCC2 is not clear, these findings suggest a physiological role for aeCCC2 in osmoregulation and ammonia balance of mosquito larvae.

Introduction

The osmoregulatory tissues of mosquitoes includes midgut, Malpighian tubules (MTs), hindgut and anal papillae (in larvae) (Bradley, 1987). There are three putative Na^+ dependent CCCs in mosquitoes: aeCCC1, aeCCC2 and aeCCC3. Phylogenetic analysis indicated that aeCCC1 is a bumetanide-sensitive Na^+, K^+, $2Cl^-$ cotransporter (NKCC), whereas aeCCC2/3 diverged from aeCCC1 specialized for ion absorption. Piermarini *et al*. (2011) and Sun *et al*. (2010) showed that aeCCC2/3 are more closely related to Na^+ coupled CCCs. Our preliminary data also showed that aeCCC2 is highly expressed in hindguts, which is an absorptive tissues (Akuma, 2014). Therefore, we hypothesized that RNAi knockdown of aeCCC2 would lead to decrease of expression of aeCCC2 mRNA and decrease of cation concentrations.

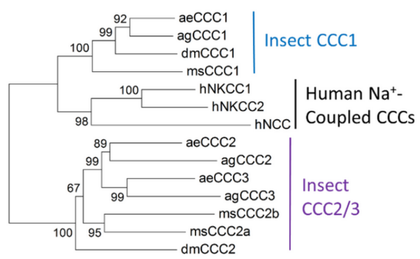

Figure 1. Phylogenetic analysis of amino acid sequences of CCC family in predicted transmembrane domains using Clustal W alignment and neighbor-joining method (Mega 4 program)

Methods

Mosquito rearing: Mosquito eggs (Liverpool strain) were hatched in fresh water in a beaker under low oxygen concentration in a vacuum chamber. Once they became larvae, they were transported to another deep container (~1.5 inch) with FW at 28 °C, 80% RH and 12hr light/day diurnal cycle. Larvae were fed with Total Goldfish® (Secaucus, New Jersey) *ad libitum* and water was changed when too cloudy.

RNA isolation and cDNA synthesis: RNA was isolated by TRIzol® reagent followed the manufacturer's instruction (Invitrogen, Carlsbad, CA). Isolated RNA were quantified by NanoDrop-1000 (Thermo Scientific, Wilmington, DE). Five µg of each RNA sample was purified by the TURBODNA-free kit (Ambion/Applied Biosystems, Austin, TX) and further purified with the RNA Clean & ConcentratorTM-25 kit (Zymo Research Corp., Irvine, CA). The resulting purified sample was reverse-transcribed to cDNA by Taqman® Reverse Transcription Reagents kit following the instruction by the manufacturer. The resultant cDNA products were further amplified by PCR with T7 promoters on each end. Negative controls were reactions without Taqman® reverse transcriptase.

RNA inference: Double strand RNA (dsRNA) was used for RNAi. Double strand RNA was synthesized by MEGAscript® RNAi kit (life technology) following manufacturer's protocol. First instar larvae were exposed to 0.3 – 0.7 µg/ µL 400 - 500 base pair aeCCC2 dsRNAs for 2 hours. Negative controls were no dsRNA or a dsRNA without sequence homology to *A. aegypti* genes.

Quantitative PCR: Expression level of aeCCC2 was quantified using the fluorescent marker SYBR Green (Applied Biosystems, Austin, TX) and the ribosomal protein S5 (aeRpS5) was used as internal control (Ribeiro *et al*., 2007). Quantitative PCR was run in triplicate on a 96-well plate. Gene expression levels were quantified by threshold cycle differences (dCT) on an ABI prism 7500 sequence detector.

Methods

Ion Concentrations in Hemolymph: Hemolymph was collected when the larvae reached 4th instar to determine the dsRNA knockout effect. Under compound microscope, the larvae's cuticle was broken by forceps, and 50.6 nL hemolymph was collected by glass capillary needle filled with mineral oil integrated with Nanoject II microinjector (Drummond Scientific, Broomall, PA). The original hemolymph was diluted in 150 µL deionized water and was stored in 4˚C refrigerator until cation chromatography. The concentrations of ions were measured by cation chromatography system. The outcome was further analyzed by Dionex PeakNet®-PA software v 5.21 (Dionex Corp., Sunnyvale, CA).

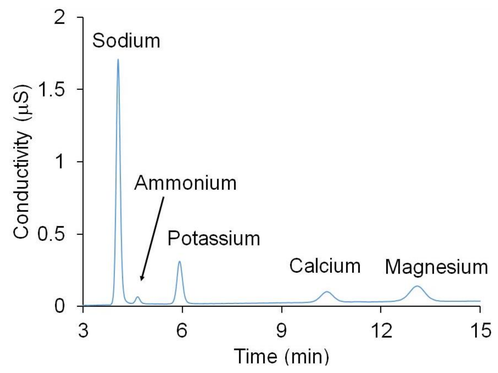

Figure 2. Chromatograph of a 3000-fold dilution of hemolymph collected from a 4th instar *Ae. aegypti* larva.

Results

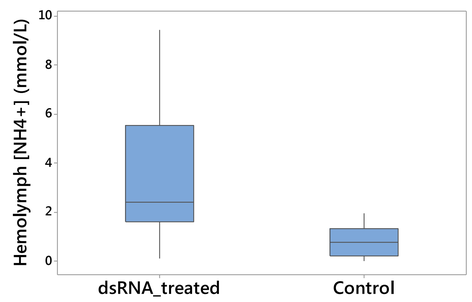

Figure 3. Hemolymph $NH4^+$ concentration of 4th instar *A. aegypti* larvae after treated with aeCCC2 dsRNA compared with the control groups. N(dsRNA) = 46; N(control)=36. One-way ANOVA, F = 35.02, P=0.000.

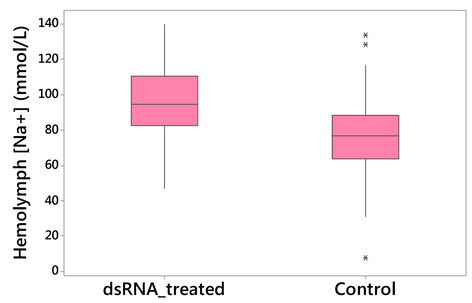

Figure 4. Hemolymph Na^+ concentration of 4th instar *A. aegypti* larvae after treated with aeCCC2 dsRNA compared with the control groups. N(dsRNA) = 46; N(control)=36. One-way ANOVA, F = 18.09, P = 0.000.

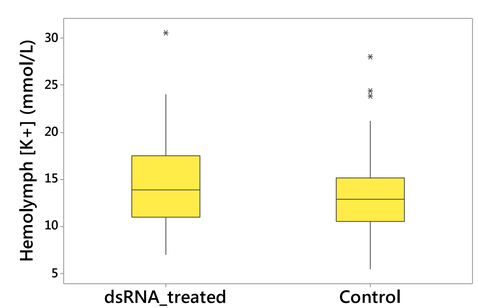

Figure 5. Hemolymph K^+ concentration of 4th instar *A. aegypti* larvae after treated with aeCCC2 dsRNA compared with the control groups. N(dsRNA) = 46; N(control)=36. One-way ANOVA, F = 0.42, P =0.519.

Results

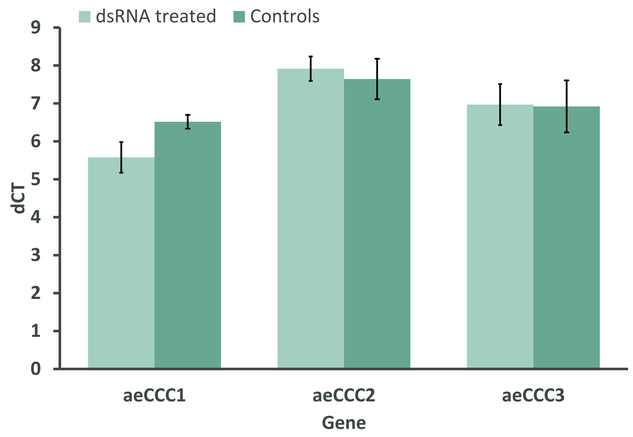

Figure 6. CCC expressions in 4th instar *A. aegypti* larvae after treatments with aeCCC2 dsRNA compared with the control groups. Higher dCT means the lower expression. Ribosomal RNA "Rps5" was used as internal control. N(dsRNA) = 26; N(control)=19. Error bars = SEM. Two-sample t test: aeCCC1: t = -2.11, P = 0.079; aeCCC2: t = 0.43, P = 0.674; aeCCC3: t = 0.06, P = 0.957.

Summary

- **Knockdown of aeCCC2 caused five-fold increase of ammonia concentration, and approximately 20% increase of sodium concentration in hemolymph (Fig. 3 and Fig. 4).**
- **There was no significant difference of potassium concentration in hemolymph after RNAi treatment between experimental and control groups (Fig.5).**
- **RNAi did not affect the mRNA expressions for all of three gene in whole body experiment (Fig. 6).**

Conclusion and future studies

- **The physiological results suggested that aeCCC2 may play an important role of balancing ammonia concentration, and possibly sodium concentration in mosquito larvae.**
- **Quantitative PCR results indicated that measurement of gene expression in specific tissue is necessary.**
- **These results also suggested that RNAi treatment may not affect mRNA level (transcription), but could inhibit protein level (translation). Therefore, immunohistochemistry experiments should be conducted using antibodies targeting specific transporter.**

Acknowledgement

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