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Molecular characterization of a cDNA encoding $Na^+/K^+/2Cl^-$ cotransporter in the gill of mud crab (*Scylla paramamosain*) during the molt cycle: Implication of its function in osmoregulation

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ABSTRACT

Although iono-regulatory processes are critical for survival of crustaceans during the molt cycle, the mechanisms involved are still not clear. The $Na^+/K^+/2Cl^-$ cotransporter (NKCC), a SLC12A family protein that transports Na^+, K^+ and $2Cl^-$ into cells, is essential for cell ionic and osmotic regulation. To better understand the role of NKCC in the molt osmoregulation, we cloned and characterized a *NKCC* gene from the mud crab, *Scylla paramamosain* (designated as *SpNKCC*). The predicted *Sp*NKCC protein is well conserved, and phylogenetic analysis revealed that this protein was clustered with crustacean NKCC. Expression of *SpNKCC* was detected in all the tissues examined but was highest in the posterior gills. Transmission electron microscopy revealed that posterior gills had a thick type of epithelium for ion regulation while the anterior gills possessed a thin phenotype related to gas exchange. During the molting cycle, hemolymph osmolality and ion concentrations (Na^+ and Cl^-) increased significantly over the postmolt period, remained stable in the intermolt and premolt stages and then decreased at ecdysis. Meanwhile, the expression of *SpNKCC* mRNA was significantly elevated (26.7 to 338.8-fold) at the ion re-establishing stages (postmolt) as compared with baseline molt level. This pattern was consistent with the coordinated regulation of Na^+/K^+-ATPase α-subunit (*NKA α*), carbonic anhydrase cytoplasmic (*CAc*) isoform and Na^+/H^+ exchanger (*NHE*) genes in the posterior gills. These data suggest that *SpNKCC* may be important in mediating branchial ion uptake during the molt cycle, especially at the postmolt stages.

1. Introduction

Molting, a phenomenon of the periodic shedding the old cuticle and subsequent reconstruction of a new rigid exoskeleton, occurs in all crustaceans and is essential for growth, metamorphosis and reproduction (Ghanawi and Saoud, 2012; Li et al., 2015). Generally, the molt cycle in decapod crustaceans is composed of different stages, including preecdysis (premolt, D stage), ecdysis (E stage), postecdysis (postmolt, A and B stages) and intermolt (C stage) stages (Drach and Tchernigovtzeff, 1967). At ecdysis, the shedding of the restrictive cuticle and the size increase in crustaceans are achieved by the enhanced water absorption (Perry et al., 2001; Wilder et al., 2009). Such increase in water uptake may lead to a simultaneous decrease in hemolymph osmolality and ion concentrations in crustaceans. Hence, osmoregulation is directly affected by the molting process.

Crustaceans do have to keep hemolymph osmolality and major ionic concentrations (Na^+, Cl^-) within the range of physiological homeostasis through absorption and excretion of Na^+ and Cl^- across the gills (Freire et al., 2008; McNamara and Faria, 2012). A suite of ion pumps, ion transporters and ion transport-related proteins in the gills are involved in the active uptake of ions from the external media into the hemolymph. In general, the electrogenic basal Na^+/K^+-ATPase (NKA) drives Na^+ transport to the hemolymph sustained by apical Na^+ uptake possibly through the Na^+/H^+ exchanger (NHE) (Towle et al., 1997a; Chung and Lin, 2006; Leone et al., 2015). The carbonic anhydrase (CA) is believed to provide support for the overall transport mechanisms through the catalyzed hydration of respiratory CO_2, which produces H^+ and HCO_3^- needed to support Na^+/H^+ and HCO_3^-/Cl^- exchange, respectively (Henry, 1988; Serrano and Henry, 2008; McNamara and Faria, 2012). Studies have indicated that $Na^+/K^+/2Cl^-$ cotransporter (NKCC) may also serve as a key ion transporter for marine invertebrates (Riestenpatt et al., 1996; Luquet et al., 2005; Havird et al., 2014).

The $Na^+/K^+/2Cl^-$ cotransporter (NKCC), is a member of the cation-chloride cotransporter (CCC) family that plays an essential role in the osmoregulatory processes in the gills of aquatic species by transporting Na^+, K^+ and Cl^- into animal cells simultaneously (Gagnon et al., 2002; Gamba, 2005). In vertebrates, two distinct isoforms of NKCC cotransporters (NKCC1 and NKCC2) have been identified (Markadieu

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Table 1
Primers used in the present study.

Primer name	Sequence 5′→3′	Amplicon size (bp)	Annealing temperature (°C)
SpNKCC-F	TCAACTTCTCCTGYTTCCAC	1029	53
SpNKCC-R	TGCCC TTCTTCTGYTTCCTC		
Universal primer mix (UPM)			
Long primer	CTAATACGACTCACTATAGGGC AAGCAGTGGTATCAACGCAGAGT		
Short primer	CTAATACGACTCACTATAGGGC		
SpNKCC-5′	TGCCTCGCTGTCTGTTCCATCTTG CGTG	2469	68
SpNKCC-3′ outer	TTTCAACACCCTGCATGAGGCTCT GGAC	1093	70
SpNKCC-3′ inner	CAACCAGCTCACGCAAGATGGAA CAGAC	1743	68
SpNKCC real-F	CCTCTGATCTACGCTGGGTG	169	55
SpNKCC real-R	GGACATAACCACGCACAGG		
β-actin real-F	GCCCTTCCTCACGCTATCCT	185	58
β-actin real-R	GCGGCAGTGGTCATCTCCT		
NKA real-F	GGACCTACCACGACCGCAAGATA	113	58
NKA real-R	GGAGTTACGGCGGGTCTTACAAA		
NHE-F	GTGTYTGGGCAAGATTGGBTTCCAC	1630	58
NHE-R	GGCTTGTARTCVTTGGAGAGSAGA		
NHE real-F	GGACAACATCATGGCAGTGGACAT	112	58
NHE real-R	TGGTGACGAAGGCAGTGAGGAA		
CAc-F	CAGGGGTGGTACGACYTGAGTC	633	60
CAc-R	GGGCAGCAAGCAGTCCCCYATTRA		
CAc real-F	GCCGTGCTGGGAATGTTCCTG	120	56
CAc real-R	AAGGAGGCGTGGTGAGAGAGC		
AQP-1-F	TTCGCTGTCGGCATTATTGTC	333	50
AQP-1-R	ACATCGTTGCGTCGCTCGTCA		
AQP-1 real-F	TTCGCTGTCGGCATTATTGTC	185	58
AQP-1 real-R	AGACCCTTCAGGATGGCAGAG		

and Delpire, 2014). In contrast, a unique NKCC has been identified from crustaceans, including *Callinectes sapidus* (Towle et al., 1997b), *Carcinus maenas*, *Chasmagnathus granulatus* (Luquet et al., 2005) and *Halocaridina rubra* (Havird et al., 2014). *NKCC* exhibits a salinity-sensitive expression pattern, and its transcription level increases when crustaceans are exposed to low salinity water (Spanings-Pierrot and Towle, 2004; Luquet et al., 2005; Havird et al., 2014). The NKCC was presumably involved in the initial step of ion transport from the ambient medium to the gill. In the green shore crab *C. maenas*, electrophysiological evidence has been presented for the participation of an apical NKCC in this process (Riestenpatt et al., 1996). Although there is a growing number of evidence implicating the involvement of crustacean NKCC activity in ionic homeostasis in different salinities, very little is known about whether NKCC is involved in osmoregulation during the molt processes in crustaceans. Therefore, the aim of the present study was to gain new insights into the characterization of NKCC and its role in osmoregulation in different molt stages of mud crab (*Scylla paramamosain*), a euryhaline species that was found to molt at least 18 times after metamorphosis (Ong, 1966; Chung and Lin, 2006). Here, we report the molecular cloning of a NKCC from gills of *S. paramamosain*. Further, hemolymph osmolality, ion concentration as well as the mRNA expression patterns of *NKCC*, Na^+/K^+-ATPase α-subunit (*NKA α*), carbonic anhydrase cytoplasmic (*CAc*) isoform, *NHE* and aquaporin-1(*AQP-1*) in the gills of various molt stages were determined. In addition, gill architecture of the intermolt animals has also been studied to assess its possible role as a site for osmoregulation. These findings are brought together to elucidate the biochemical, transcriptional and ultrastructural underpinnings of ion regulation in crustacean during the molt cycle.

2. Materials and methods

2.1. Animal collection and maintenance

The experimental animals, *Scylla paramamosain*, measuring 40.05 ± 6.87 g in weight, were obtained from Wenzhou, Zhejiang Province, China. All individuals were cultured individually in plastic trays (0.3 × 0.2 × 0.2 m) with running seawater of approximately 17 ppt salinity as their original culture water at 29 ± 1 °C. Crabs were fed with live clams every day until 2 days before sampling.

The molt stages were determined by observing the setae on the maxlliped exopodite on the animals under a light microscope (Xu et al., 2015). A and B stages were designated as postmolt stages, C as intermolt, D_0 as early premolt, D_1 as mid premolt, D_2 as late premolt, and E as ecdysis, based on the established criteria (Drach and Tchernigovtzeff, 1967).

2.2. Tissue preparation

Crabs ($n = 7$) in each stage of the molt cycle were sampled. Briefly, hemolymph samples were withdrawn from the infrabranchial sinus at the base of the fifth pereiopod using a 22-gauge needle and 1 mL syringe. The samples were frozen at −20 °C until osmolality determination and ion analysis. For dissection, crabs were anesthetized on crushed ice for 10 min and then anterior (G1), posterior (G6) gills and other tissues (gills, muscle, hepatopancreas, gut, thoracic ganglia, hypodermis, hemocytes, antennal gland and heart) were dissected out and immediately placed in liquid nitrogen and kept at −80 °C until required. Only specimens in the intermolt stage were used for tissue distribution analysis, histological and electron microscopic study.

2.3. Growth metrics

The quantitative method was used as previously described (Chang et al., 2011). At the beginning of the study, crabs (n = 7) from the late premolt stage D_2 were selected and measured individually for carapace parameters (width and length) and weight to the nearest 0.1 mm and 0.01 g, respectively. The crabs were held as described above and the general molting state of these crabs was observed frequently. After ecdysis, carapace parameters and weight of each crab (stage A) were measured within 1 h following the molt stage. The parameters were calculated as follows:

$$\text{Percent weight gain (WG, \%)} = 100\% \times (W_A - W_{LD})/W_{LD}$$

where W_A is the final body weight from early postmolt stage after ecdysis; W_{LD} is the body weight from the late premolt stage.

2.4. Hemolymph osmolality and ion concentrations

Hemolymph samples were thawed on ice, sonicated for 15 s, and centrifuged at 14,000 gravity unit for 1 min to separate out clot material. Osmolality was then measured on 10 μL samples using a vapor pressure osmometer (Wescor 5600, Logan, UT, USA).

Hemolymph Cl^- analysis was determined by ion chromatography (Dionex ICS 2100, Sunnyvale, CA, USA). For measurements of Na^+ and K^+ concentrations, hemolymph (0.2 mL) was diluted and injected to an Inductively Coupled Plasma of Atomic Emission Spectroscopy technique (ICP-AES) (Optima 8000, Perkin Elmer, Boston, USA). The osmolality of the seawater was also measured following hemolymph sampling.

Fig. 1. Nucleotide and deduced amino acid sequence of *S. paramamosain* NKCC. The start and stop codons (asterisk) are in boldface. The polyadenylation signal (AATAAA) depicts in dotted line. A GT-repeat motif is underlined with bold line. The SLC12A domain (residues: 117-631) and the C-terminal tail domain (residues: 640-1053) are marked with wavy line and double-underline.