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V-type H^+-ATPase and Na^+,K^+-ATPase in the gills of 13 euryhaline crabs during salinity acclimation

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Summary

Because of their diverse habitats, crabs are excellent experimental species to study owing to the morphological changes and physiological adaptation that occur during their terrestrial invasion. Their hemolymphic osmoregulation in brackish water is crucial for a successful terrestrial invasion. Crabs can actively uptake or excrete ions upon salinity change, and the gills play a major role among the osmoregulatory organs. Several enzymes are involved in the osmoregulatory process, including Na^+,K^+-ATPase and V-type H^+-ATPase (V-H^+-ATPase). Na^+,K^+-ATPase is the driving force in establishing an ion gradient across the epithelial cell membrane in marine crabs. It has been reported that the osmoregulatory mechanisms in freshwater crabs are different from those in marine ones, suggesting that the driving force may come from V-H^+-ATPase by generating the H^+ ion gradient to facilitate the ion flow. Thirteen crab species from two families were used in this study. These crabs lived in five different habitats, including marine, intertidal, bimodal, freshwater and terrestrial habitats. The distribution of V-H^+-ATPase in the 13 euryhaline crabs was revealed by histochemistry. V-H^+-ATPase was localized in the apical region in crabs that could survive in the freshwater environment. We found that the freshwater and terrestrial crabs with stable Na^+,K^+-ATPase activity during salinity changes tended to have an apical V-H^+-ATPase, whereas the intertidal ones with varying Na^+,K^+-ATPase activity showed a cytoplasmic V-H^+-ATPase distribution. Finally, in *Uca formosensis*, a crab that had stable Na^+,K^+-ATPase activity, a significant difference in V-H^+-ATPase activity between salinities was found. In conclusion, the hypothesis that V-H^+-ATPase plays a crucial role in the freshwater adaptation of crabs is supported by our systemic investigation on 13 euryhaline crabs.

Key words: V-type H^+-ATPase, Na^+,K^+-ATPase, ion regulation, freshwater adaptation, euryhaline crabs, crustacean, gills, immunolocalization, salinity acclimation.

Introduction

Intertidal crustaceans that have evolved a diversity of regulatory mechanisms to live between aquatic and aerial media are either hyper-osmoregulators or hyper-hypo-osmoregulators. In all of them, the gills are the site of various morphological and physiological modifications (Péqueux, 1995). Many organs are involved in osmoregulation, including the gills, the antennal gland, part of the intestine and the hepatopancreas. The gills are one of the most important osmoregulatory organs in aquatic crustaceans (Péqueux, 1995; Lucu and Towle, 2003; Chung and Lin, 2006). In osmoregulatory crabs, there are some functional differentiations among/within the gills. The anterior gills have thin epithelia (2–4 μm) and are the site for respiration. The posterior gills, which contain numerous mitochondria and membrane foldings for active ion regulation, are thicker (10–20 μm) and are responsible for osmoregulation (Barra et al., 1983; Péqueux, 1995; Takeda et al., 1996; Luquet et al., 2000; Lin et al., 2002). The posterior gill also has functional differentiation between thin and thick epithelium for uptake of different ions (Goodman and Cavey, 1990; Onken and McNamara, 2002).

There are several enzymes are responsible for ion transport in crustacean gills, including Na^+,K^+-ATPase, V-type H^+-ATPase (V-H^+-ATPase), carbonic anhydrase, Cl^-/HCO_3^- exchanger, and Na^+/H^+ exchanger. The relative importance of each enzyme in the osmoregulatory mechanism differs among crabs. Na^+,K^+-ATPase (NKA) and V-H^+-ATPase (a vacuolar-type H^+-ATPase, HA) are two of the most studied proteins. They are considered the key enzymes during the transition from a marine environment to land (Morris, 2001; Weihrauch et al., 2004). In marine crabs, NKA is abundant in the basolateral regions of the epithelial cells. It is a primary transporter and maintains the body fluid osmolality by actively pumping Na^+

to the hemolymph (Lucu et al., 2000). For example, in the marine crab *Carcinus maenas*, NKA is the driving force for Na^+ uptake in dilute seawater (Weihrauch et al., 2002). The osmoregulatory mechanism in freshwater crabs is different from that in marine ones. In freshwater crabs, not only NKA, but also HA plays an important role in the osmoregulatory process (Weihrauch et al., 2004).

HA has been reported to be responsible for acid–base balance and nitrogen excretion (Weihrauch, 2001). It is a protein that is highly conserved in eukaryotes, which includes yeast, plants and animals (Weihrauch et al., 2001). There are three types of proton-translocating enzymes: A-, F- and V-ATPases. All of then have a membrane-bound $A_0/F_0/V_0$ domain, central connecting stalk and peripheral $A_1/F_1/V_1$ domain (Grüber et al., 2001). Proton pump was thought to be a housekeeping enzyme in the endosomal membrane. However, there have been reports on the role of HA in generating the ion flow in freshwater and extremely diluted environments (Ehrenfeld and Klein, 1997; Klein et al., 1997; Wieczorek et al., 1999; Jensen et al., 2002; Weihrauch et al., 2004). The NKA can only partly supply the driving force for Na^+ absorption from a diluted medium. Instead, V-type H^+-ATPase participates in ion regulation by actively transporting protons out to the media: the apically located HA generates a transmembrane electrical potential difference which, in turn, allows Na^+ to flow in *via* Na channels and an Na^+/H^+ exchanger and is more efficient than generating the Na^+ gradient by NKA (Onken and Riestenpatt, 1998; Kirschner, 2004). Therefore, it has been suggested that HA is involved in the osmoregulatory process when it is localized in the apical membrane of the epithelial cells in crabs (Wieczorek et al., 1999; Weihrauch et al., 2001; Jensen et al., 2002).

Furthermore, apical HA may play an important role in freshwater osmoregulation of crabs from the marine environment onto land (Weihrauch et al., 2004; Genovese et al., 2005). Several recent studies have focused on the osmoregulatory role of HA in some euryhaline or freshwater-acclimated crabs, such as *Carcinus maenas* (Weihrauch et al., 2002), *Dilocarcinus pagei* (Weihrauch et al., 2004), *Chasmagnathus granulatus* (Genovese et al., 2005) and *Eriocheir sinensis* (Onken and Putzenlechner, 1995; Riestenpatt et al., 1995). Nevertheless, only a few studies have measured HA activity. Most of them determined the functions of HA using electrophysiological methodology (Onken and McNamara, 2002; Genovese et al., 2005) or mRNA expression (Luquet et al., 2005). A direct measurement of HA activity and its cytological distribution in the gill would be powerful support for the correlation of function, distribution and adaptation. Therefore, it is important to investigate NKA and HA simultaneously when considering the role of HA in freshwater adaptation.

The purposes of this study were to conduct a systemic examination of the distribution of HA among 13 species of euryhaline and freshwater crabs and to test whether the distribution is correlated to the variation in the NKA in the gills during their salinity acclimation. Three experiments were conducted. First, we examined the NKA activities in the gills of 13 species after acclimation in 5 p.p.t. and 35 p.p.t. salinity for 7 days. In the preliminary experiment, NKA activity reached a stable state within 7 days and it was not significantly different from the level after 14-day acclimation treatment. *E. sinensis* was acclimated at 5 and 35 p.p.t. for comparison of the NKA activity in the gills. These two salinities partly reflect the fluctuation confronted daily by the rest of the 12 species. As for the diadromous *E. sinensis*, individuals survived in both salinities and were in good condition during the acclimation. An average enzyme-specific activity (ESA) value for each species was obtained at both treated salinities. The ratio of the average NKA activity in 5 p.p.t. divided by that in 35 p.p.t. was defined as the response-to-salinity ratio (RSR). Next, we used immunohistochemical methods to illustrate the distribution of HA among these 13 species, in order to test the hypothesis that an apical distribution of HA is involved in the osmoregulatory process (Wieczorek et al., 1999; Weihrauch et al., 2001; Jensen et al., 2002). According to the distribution of HA, these 13 species were classified into three groups for further analyses on the RSRs of NKA. An upregulation of NKA is an indication of the individual's dependence on NKA to overcome hypotonic stress (Genovese et al., 2005). If the crab maintains stable NKA activity, HA activity is expected to change. Lastly, we chose *Uca formosensis*, known to have stable NKA activity, and measured its HA activity and protein abundance after salinity challenges. A change of HA in enzyme activity would thus be evidence for its osmoregulatory role.

Materials and methods

Animals and acclimation

This study included 13 crab species from marine to terrestrial and freshwater habitats and they varied in their degree of freshwater tolerance. They were, respectively, marine (M), intertidal (IT), bimodal (BI), terrestrial (T) and freshwater crabs (FW) and belonged to three different families: Portunidae, Ocypodidae and Grapsidae (Table 1). All the crabs were in the intermolt stage. Animals were kept in plastic containers with artificial brackish water (25 p.p.t. salinity) or freshwater and fed 3–4 times per week with frozen brine shrimp (*Artemia salina*) and commercial fish diet. Temperature was kept at 28°C (except for *Eriocheir sinensis,* which was kept below 20°C) with a 14 h:10 h L:D photoperiod. After acclimation for 1 week, these crabs were subjected to 5 p.p.t. diluted seawater (DSW) and 35 p.p.t. full-strength seawater (SW), respectively, for later experiments. The artificial seawater was made with Coralife Scientific Grade Marine Salt (Anaheim, CA, USA).

Protein extraction

After 7 days in 5 p.p.t. diluted seawater, the crabs were sacrificed on ice and the sixth gills (gill 6) were homogenized in a homogenizing medium that contained the protease inhibitor cocktails (3.31 mmol l^{-1} Antipain (Sigma, St Louis, MO, USA), 2.16 mmol l^{-1} Leupeptin (Sigma), 63.86 mmol l^{-1} Benzamidine (Sigma) in Aprotinin saline solution [(5–10

Table 1. *The Na^+,K^+-ATPase (NKA) activity in 13 euryhaline crabs in different salinities*

Species	Habitat	NKA (μmol mg^{-1} protein h^{-1}) 5 p.p.t.	*N*	35 p.p.t.	*N*	RSR	*t*-test
Portunidae							
Scylla paramamosain†	M	11.37±8.44	5	3.31±1.06[b]	6	3.44	0.02
Ocypodidae							
Macrophthalmus banzai	IT	10.69±1.47	6	3.42±2.66	7	3.12	<0.01
Macrophthalmus abbreviatus	IT	7.81±0.25	3	1.46±0.20	3	5.34	<0.01
Uca lactea	IT/BI	28.63±4.49	5	16.62±5.38[b]	5	1.72	0.04
Uca formosensis	BI/T	16.53±5.62	16	13.09±3.48	17	1.26[ns]	0.51
Ocypode stimpsoni	T	4.17±2.62	9	5.88±3.14	10	0.71[ns]	0.22
Grapsidae							
Chasmagnathus convexus	BI/T	4.85±1.52	10	3.93±2.20	6	1.23[ns]	0.34
Helice formosensis	BI/T	8.04±3.22	3	9.21±1.33	3	0.87[ns]	
	BI/T	4.81±3.28[a]	9	3.62±1.12	9	1.33[ns]	0.40
Eriocheir sinensis	FW	7.17±1.62	4	12.66±6.25	2	0.57[ns]	0.14
Hemigrapsus sanguineus	IT/BI	5.59±2.31	5	6.81±3.62	4	0.82[ns]	0.56
Hemigrapsus penicillatus	IT/BI	4.08±2.42	5	2.48±2.34	3	1.65[ns]	0.39
Perisesarma bidens	IT/BI	12.6±1.14	4	11.26±0.28	3	1.12[ns]	0.11
Chiromantes dehaani	BI/T	4.35±3.04	7	3.30±1.81	6	1.32[ns]	0.48

M, euryhaline marine crab; IT, intertidal crab; BI, bimodal crab; T, terrestrial crab; FW, freshwater crab.
Values are means ± s.d.
The response-to-salinity ratio (RSR) of NKA was calculated by dividing the mean activity in 5 p.p.t. over that in 35 p.p.t. salinity.
[a]Crabs acclimated in 3 p.p.t. salinity; [b]crabs acclimated in 45 p.p.t. salinity; [ns]no significant difference between salinities in that species.
†(from Chung and Lin, 2006).

trypsin inhibitor unit ml^{-1} (Sigma; catalog no. A 6279)]. For the homogenizing medium (containing 25 mmol l^{-1} Tris-HCl, 0.25 mmol l^{-1} sucrose, 20 mmol l^{-1} EDTA, 0.4% sodium deoxycholate), an ultrasonic processor (Sonics, Newton, CT, USA) was used and pH was adjusted to 7.4. The gills were first centrifuged at 4°C, 6000 ***g*** for 15 min and then centrifuged at 4°C, 20 160 ***g*** for 20 min. The crude homogenate was used for measurements of enzymatic activity. For *U. formosensis*, the suspension was used for NKA and HA activities measurement immediately (i.e. fresh protein extracts) and part of the protein extracts were stored at –78°C for later western blot detection. NKA activity was measured for all of the 13 species but HA activity and western blotting were only done for *Uca formosensis.* The protein concentration assay followed the method described previously (Chung and Lin, 2006). Protein concentration was determined using a detergent compatible kit (BioRad, Hercules, CA, USA, Cat. 500-01210) and absorbance was read at 695 nm using a spectrophotometer (U-2001 Spectrophotometer, Hitachi, Tokyo, Japan).

Enzyme activity

Na^+,K^+-ATPase activity was assayed by adding the supernatant to 400 μl reaction medium (ouabain-free group: 20 mmol l^{-1} imidazole, 100 mmol l^{-1} NaCl, 30 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, pH 7.4; ouabain group: 20 mmol l^{-1} imidazole, 130 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} ouabain, pH 7.4). The reason the group with ouabain had no KCl was to ensure that no K^+ was available for pumping before NKA had been fully inhibited by ouabain. This protocol was modified from Holliday (Holliday, 1985). The reaction was run by adding 100 μl ATP stock solution (25 mmol l^{-1} Na_2ATP), followed by incubation at 30°C for 15 min, before it was stopped by adding 200 μl ice-cold TCA stock solution (30% trichloroacetic acid). After centrifugation at 1640 ***g*** at 4°C for 10 min, an aliquot of 500 μl supernatant was taken and measured using the colorimetric method for the inorganic phosphate concentration by adding 1 ml ice-cold Boting's color reagent (560 mmol l^{-1} H_2SO_4, 8.10 mmol l^{-1} ammonium molybdate and 176 mmol l^{-1} $FeSO_4$). The color was allowed to develop in a water bath at 20°C for 20 min. The concentration was measured at 700 nm (U-2001 Spectrophotometer). The ESA of NKA was defined as the difference between the inorganic phosphates liberated in the presence and absence of ouabain in the reaction medium (Holliday, 1985; Lin et al., 2002; Chung and Lin, 2006).

Similarly, HA activity was assayed by adding the protein extract to 400 μl reaction medium (bafilomycin-free group: 20 mmol l^{-1} imidazole, 130 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} ouabain, 1 mmol l^{-1} sodium azide, 1 mmol l^{-1} sodium ortho-vanadate, 10 μl DMSO, pH 7.4; bafilomycin group: 20 mmol l^{-1} imidazole, 130 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} ouabain, 1 μmol l^{-1} bafilomycin A_1 (in DMSO), 1 mmol l^{-1} sodium azide, 1 mmol l^{-1} sodium ortho-vanadate, 10 μl DMSO, pH 7.4). The reaction was run by adding 100 μl ATP stock solution (30 mmol l^{-1} Na_2ATP), followed by incubation at 30°C for 15 min, and then stopped by adding 200 μl ice-cold TCA stock

solution (30% trichloroacetic acid). The rest of the procedure was the same as that for NKA activity. The ESA of HA was defined as the difference between the inorganic phosphates liberated in the presence and absence of bafilomycin A1 in the reaction medium. Although a concentration as high as 10 μmol l^{-1} bafilomycin A1 was used in a study of frog skin (Klein et al., 1997), we used 1 μmol l^{-1} in our study, which was the same concentration as that used in other studies (Breton et al., 1998; Wieczorek et al., 1991).

Antibodies

A mouse monoclonal antibody (α5) against the α-subunit of the avian sodium pump was purchased from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA). The antibody of HA was a mouse monoclonal antibody against the yeast vacuole-type H^+-ATPase B-subunit (Molecular Probes, Eugene, OR, USA). We used β-actin as an internal control for western blotting (β-actin monoclonal antibody purchased from Sigma, USA). An anti-mouse IgG with horseradish peroxidase conjugate antibodies (Jackson Immunoresearch, West Grove, PA, USA) was used in the western blotting for detecting each of the primary antibodies.

Immunohistochemical localization of V-H⁺-ATPase B-subunit in gill lamellae

Crabs acclimated in 5 p.p.t. DSW for at least 4 days were sacrificed on ice and the posterior gills (the 6th, 7th and 8th pairs) were removed and immersed in 4% paraformaldehyde and 5% glutaraldehyde (P_4G_5) in 0.1 mol l^{-1} phosphate buffer (0.1 mol l^{-1} NaH_2PO_4 and 0.1 mol l^{-1} Na_2HPO_4) fixative solution for 15–18 h at 4°C. The gills were washed in phosphate buffer solution (136.9 mmol l^{-1} NaCl, 2.68 mmol l^{-1} KCl, 10.15 mmol l^{-1} Na_2HPO_4, 1.76 mmol l^{-1} NaH_2PO_4, pH 7.4), dehydrated in a graded ethanol series, then embedded in paraffin. We included the afferent vessel in the sectioning to ensure the orientation of the specimen and the location of ionocytes. In each species, a longitudinal section of each posterior gill was prepared. Series sections of 5 μm were mounted on poly-L-lysine coated glass slides. These sections were stained with the monoclonal antibody to HA B-subunit (Molecular Probes) and then stained with a commercial kit containing a 2nd antibody HRP/Fab polymer conjugate and aminoethyl carbazole (AEC) single solution chromogen (PicTure-Plus™, Zymed, South San Francisco, CA, USA). By detecting the red deposition, HA can be localized. The negative control experiments used the PBS instead of the primary antibody and showed no non-specific staining in our gill samples. In addition, we stained these sections with the primary antibody of NKA α-subunit to confirm their location on the basolateral membrane (data not shown).

Western blotting

To identify the relative abundance of the HA B-subunit during salinity changes, the supernatant obtained from fresh protein extraction was prepared for western blotting. An aliquot of 20 μg protein extract was mixed with the same volume of sample buffer and heated at 100°C for 4 min. Two copies were prepared for different primary antibody staining. Samples were run on 5% upper and 12% lower polyacrylamide gels. After SDS-PAGE, samples were electroblotted to PVDF membrane (NEN Life Science, Boston, MA, USA). After blocking with 5% non-fat milk powder, one copy was incubated with a mouse monoclonal antibody against yeast vacuolar HA B-subunit monoclonal antibody (Molecular Probes) and the other with a β-actin monoclonal antibody for 15 h at 4°C. Goat anti-mouse IgG with horseradish peroxidase conjugate and western blot chemiluminescence reagent plus system (NEN Life Science) were used to indicate HA and actin. The relative protein abundance was estimated by an Intelligent Dark Box II with Fujifilm LAS-1000 digital camera and analyzed by Image Gauge 4.0 (Fujifilm). The relative protein abundance was shown by HA B-subunit/β-actin in each set of treatments.

Statistical analysis

All the results from this study are expressed as mean values ± s.d. Comparison of the NKA and HA activity differences and protein abundance between the two salinity treatments was calculated by *t*-test. We compared the RSRs of NKA among the three groups that had different HA distribution patterns using a one-way ANOVA.

Results

Na⁺,K⁺-ATPase activity

Among the 13 species investigated, nine of them did not differ significantly in their NKA activities between the 5 and 35 p.p.t. treatments. That is, they had relatively stable NKA activity during salinity change. The RSRs, the ratios of the average NKA in 5 p.p.t. to that in 35 p.p.t., ranged from 0.57 in *E. sinensis* to 1.65 in *H. penicillatus*. The other three crab species had higher NKA activity in 5 p.p.t. than in 35 p.p.t. The RSRs were from 1.72 in *U. lactea* to as high as 9.27 in *M. banzai*. Finally, the crabs from Ocypodidae seemed to have higher NKA activity than those from Grapsidae and Portunidae (Table 1).

Immunohistochemical localization of H⁺-V-ATPase

Cytoplasmic distribution of HA was defined where HA was evenly stained throughout the epithelial cells (Fig. 1A) and apical distribution was where a denser stain was found in the apical region than in the cytoplasmic regions of the epithelial cells (Fig. 1C). The negative control showed no cross reaction between the 2nd antibody and the epithelial cells (Fig. 1B,D). These 13 crabs were classified into three groups according to the results of immunohistochemical staining of HA in each of the three posterior gills (Table 2). Four of the crab species had a cytoplasmic distribution of HA among the three posterior gills and were classified into group 1, which included *S. paramamosain*, *M. benzai*, *M. abbreviatus* and *U. lactea*. All gills of the crabs in group 2 had an apical HA distribution; these were *U. formosensis*, *O. stimpsoni*, *C. convexus*, *H.*

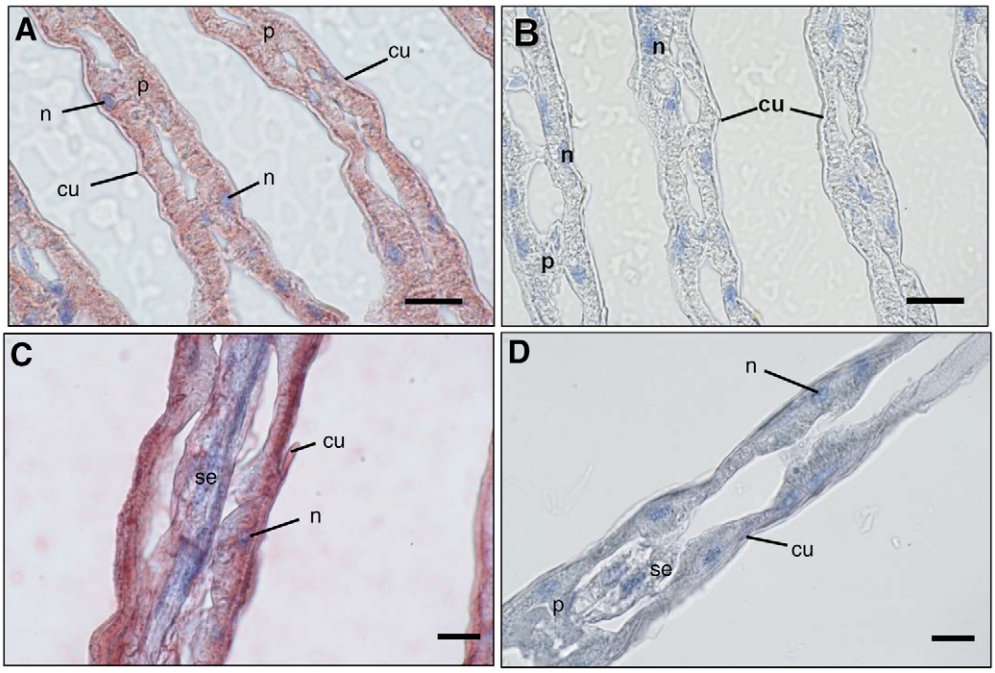

Fig. 1. Typical immunohistochemical staining in gill epithelia. (A) *Macrophthalmus abbreviatus*; red deposits represent a cytoplasmic HA distribution. (B) *Macrophthalmus abbreviatus*; negative control. (C) *Eriocheir sinensis*; red deposits represent an apical HA distribution. (D) *Eriocheir sinensis*; negative control. A, apical region. C, cytoplasm. cu, cuticle. n, nucleus. p, pillar cell. se, septum. Bars, 20 μm.

Table 2. *The distribution of V-type H^+-ATPase in the posterior gills of the 13 euryhaline crabs*

Species	Habitat	Gill 6	Gill 7	Gill 8	*N*	Group
Portunidae						
Scylla paramamosain	M	C	C	C	4	1†
Ocypodidae						
Macrophthalmus banzai	IT	C	C	C	3	1†
Macrophthalmus abbreviatus	IT	C	C	C	3	1†
Uca lactea	IT/BI	C	C	–	4	1†
Uca formosensis	BI/T	A	A	–	4	2
Ocypode stimpsoni	T	A	A	–	2	2
Grapsidae						
Chasmagnathus convexus	BI/T	A	A	A	4	2
Helice formosensis	BI/T	A	A	A	4	2
Eriocheir sinensis	FW	A	A	A	4	2
Hemigrapsus sanguineus	IT/BI	A<C	C	×	4	3
Hemigrapsus penicillatus	IT/BI	C	A>C	×	4	3
Perisesarma bidens	IT/BI	A	C	×	2	3
Chiromantes dehaani	BI/T	C	A	A	3	3

M, euryhaline marine crab; IT, intertidal crab; BI, bimodal crab; T, terrestrial crab; FW, freshwater crab.

In these 13 crabs, gills 6–8 were all posterior gills. C, the V-H^+-ATPase was located in the cytoplasmic region; A, the V-H^+-ATPase was located mainly in the apical region; –, this gill does not exist in this species; ×, the gill was not tested; A<C, most epithelial cells had the cytoplasmic V-H^+-ATPase; A>C, most epithelial cells had the apical V-H^+-ATPase.

The crabs were separated into three groups according to the localization of V-H^+-ATPase. Group 1 had cytoplasmic V-H^+-ATPase in all posterior gills. Group 2 had apical V-H^+-ATPase in all posterior gills. Group 3 had non-uniform distribution of V-H^+-ATPase either among all posterior gills or in a single gill lamella.

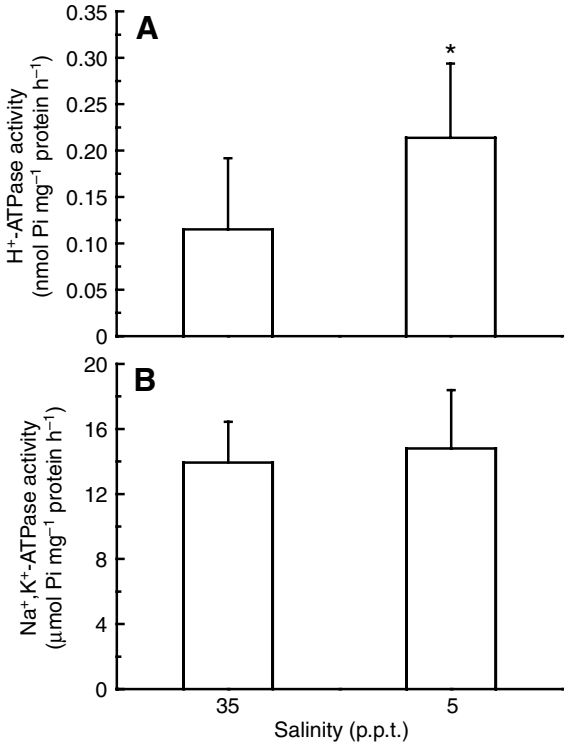

Fig. 2. (A) *Uca formosensis*. V-type H^+-ATPase activity in 5 p.p.t. and 35 p.p.t. salinity. **P*=0.01. (B) *U. formosensis*. Na^+,K^+-ATPase activity in 5 p.p.t. and 35 p.p.t. salinity. There was no significant difference between salinities.

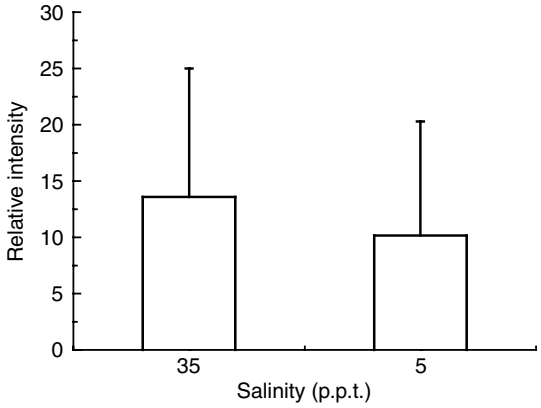

Fig. 3. Relative protein abundance of V-type H^+-ATPase in *U. formosensis*. There was no statistical difference between salinity treatments.

formosensis and *E. sinensis*. Lastly, some crabs (*H. sanguineus*, *H. penicillatus*, *P. bidens* and *C. dehaani*) did not have a consistent and uniform distribution of HA either within a gill lamella or among the three posterior gills (not shown) and they were classified into the third group.

For the three groups classified according to immunolocalization of HA, a significant difference was found in the RSRs of NKA (one-way ANOVA, $F_{2,11}$=7.28, P=0.01), and the species in group 1 with a cytoplasmic distribution of HA had a significantly higher RSR than those in the other two groups (Duncan's Multiple Range Test, P<0.05). No difference was found between groups 2 and 3.

V-H⁺-ATPase activity and protein abundance

Uca formosensis, the species that had stable NKA activity during salinity fluctuation, had higher HA activity in 5 p.p.t. (0.22±0.04, N=11) than in 35 p.p.t. salinities (0.07±0.03, N=13) (t=2.18, P=0.007). The NKA activity was simultaneously measured using the same protein extract and no difference was found between 5 p.p.t. (11.36±1.64, N=16) and 35 p.p.t. (10.04±1.13, N=17) (t=0.49, P=0.51). However, the relative protein abundance (HA/actin) was not significantly different between salinities (t=0.558, P=0.59, N=6). That is, the difference in relative HA protein abundance was not as significant as that of HA activity.

Discussion

In euryhaline crabs, there is evidence for functional differentiation of the gills (Péqueux, 1995). The anterior gills play a role in gas exchange, whereas the posterior ones have a role in ion balance (Péqueux, 1995; Lin et al., 2002; Lucu and Towle, 2003). Indeed, to a different extent, all the crabs in the present study had functional differentiation between the anterior and posterior gills and the posterior gills had thickened epithelial cells, responsible for osmoregulation. Furthermore, ionocytes in the gill lamellae of the posterior gills were mainly in the afferent area (Taylor and Taylor, 1992). Therefore, to ensure we had found most of the ionocytes, only the afferent side of the gills was sectioned and prepared for immunohistological examination in this study.

It has been reported that *D. pagei* has asymmetrical lamella epithelia (Onken and McNamara, 2002; Weihrauch et al., 2004). That is, one epithelial layer is significantly thinner than the other side of the gill lamella. According to the different responses analyzed in the electrophysiological part of their study, Onken and McNamara (Onken and McNamara, 2002) concluded that different parts of lamellae (i.e. the thin and thick epithelial cells in the asymmetric gills) respond differently for different functions; the authors reported that the thin epithelium participates in Cl^- uptake, whereas the thick one absorbs the Na^+ ion. This finding suggests that the functional differentiation in crab gills may not only be between anterior/posterior gills, but also within gill lamellae. In the present study, the non-uniform orientation of HA in gill epithelium cells in the species of group 3 may also imply the functional differentiation among gills. The other explanation for this non-uniform orientation is physiological regulation of HA by reversible assembly/disassembly of the V_1 and V_0 domains forming the HA-containing vesicles in the cytoplasmic region (Brown and Breton, 1996; Forgac, 1998). In the Pacific spiny dogfish *Squalus acanthias*, HA was found to be located on the vesicle membranes and its cellular relocation was under physiological regulation (Tresguerres et al., 2005; Tresguerres et al., 2006). In the present study, the

two *Uca* species examined had different H^+-ATPase distribution. *Uca lactea*, a widely distributed species in the mid- to high-tidal regions, has cytoplasmic HA distribution. In contrast, *Uca formosensis*, which inhabits only the uppermost tidal region, has an apical HA distribution. Further acclimation studies are needed to clarify this regulation of protein distribution in crabs from different habitats.

In the past, NKA was considered the only driving force in osmoregulation and its activity in the posterior gills of marine and intertidal crabs increased as the external salinity decreased (Péqueux, 1995; Lucu and Towle, 2003). The results of NKA activities in the present study indicate that crabs responded differently to salinity acclimation. The NKA activity in some euryhaline crabs that could not survive in freshwater (*S. paramamosain*, *M. abbreviatus*, *M. banzai*, *U. lactea*) was higher than that in diluted seawater. The seawater NKA activity in *S. paramamosain*, *M. abbreviatus*, *M. banzai*, *U. lactea*, which could not survive in a freshwater environment, was significantly higher than that in diluted seawater. On the other hand, for those crabs that could tolerate extremely diluted seawater or freshwater, NKA activity was not significantly different between salinities. This implies a possible involvement of other enzymes in osmoregulation. In the bimodal crab, *C. granulatus*, HA was more important than NKA in a DSW environment (Genovese et al., 2005). This was also supported by research on the other two freshwater crabs, *Dilocarcinus pagei* (Onken and McNamara, 2002) and *Eriocheir sinensis* (Onken and Putzenlechner, 1995; Morris, 2001). From the HA location, our results suggest that crabs in groups 2 and 3 may have different strategies or compensatory mechanisms to overcome osmotic stress. In group 3 crabs, the discrepancy in HA distribution may imply that these crabs have physiological plasticity and may be in the transition state from a marine environment to land.

An increase in NKA specific activity of gills is expected in aquatic Crustacea during acclimation to dilute seawater or freshwater (for reviews, see Péqueux, 1995; Lucu and Towle, 2003). A positive correlation between NKA specific activity and hemolymph–medium difference was obtained in 31 species of both osmoconforming and regulating crabs (Lucu and Towle, 2003). *E. sinensis* was not included in the analysis. Although there are several electro-physiology studies on the gill epithelia and split gill lamellae (for a review, see Onken and Riestenpatt, 1998), we cannot find information about the NKA specific activity of *E. sinensis* in the literature, although Whiteley et al. (Whiteley et al., 2001) mentioned little change of NKA activity in posterior gills as an unpublished observation. In the present study, the NKA activity in gills of *E. sinensis* is not different in 5 p.p.t. and 35 p.p.t. salinity. This seems to be contradictory to earlier findings (Lucu and Towle, 2003). Further study is needed on the effects of the full spectrum of salinity acclimation by this diadromous crab.

We propose that those crabs with an apical distribution of HA employ a mechanism similar to the freshwater mechanism in aquatic animals, including fish (for a review, see DeRenzis and Bornancin, 1984), amphibia (Ehrenfeld et al., 1985) and crustaceans (Weihrauch et al., 2001), to overcome osmotic stress (Wieczorek et al., 1999; Morris, 2001; Kirschner, 2004). In our study, five of the 13 species examined had an apical distribution of HA, implying that HA in these crabs is more responsive to salinity than NKA, and HA in these crabs participates in the osmoregulatory function. In contrast, *S. paramamosain, M. abbreviatus* and *M. banzai* are species that cannot survive in freshwater; they had cytoplasmic HA, which presumably plays a lesser role in osmoregulation. In previous studies on the distribution of HA, the marine crab *Carcinus maenas* was found to have a cytoplasmic HA, which participated mainly in acid–base regulation and nitrogen excretion (Weihrauch et al., 2001), and HA locations may be under physiological control by the assemble/disassemble of the V_1 domain. In this study, the apical HA was shown to be involved in ion regulation. Similar suggestions have already been proposed in studies of the gills of *Eriocheir sinensis* and *Uca tangeri* (Drews and Graszynski, 1987; Krippeit-Drews et al., 1989; Onken and Putzenlechner, 1995).

The proton-motive force generated by HA has been indicated in freshwater crabs such as *E. sinensis*, *Dilocarcinus maenas* and *C. granulatus* (Onken and Putzenlechner, 1995; Onken and McNamara, 2002; Weihrauch et al., 2004; Genovese et al., 2005). In the present study, we used *Uca formosensis* to demonstrate an apically distributed HA in gill epithelia and an upregulation in HA activity but stable NKA activity upon a low salinity challenge. Based on the results of both the enzyme activity and the distribution of HA, we conclude that the apical distribution of HA is one of the characteristics of freshwater adaptation in crustaceans. It would be interesting to investigate some true freshwater species in order to further examine their HA distribution in the gill epithelia.

List of abbreviations

AEC	aminoethyl carbazole
BI	bimodal
DSW	diluted seawater
ESA	enzyme-specific activity
FW	freshwater
HA	vacuolar-type H^+-ATPase
IT	intertidal
M	marine
NKA	Na^+,K^+-ATPase
P_4G_5	4% paraformaldehyde and 5% glutaraldehyde
RSR	response-to-salinity ratio
T	terrestrial

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