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Hemolymph ionic regulation and adjustments in gill (Na^+, K^+)-ATPase activity during salinity acclimation in the swimming crab *Callinectes ornatus* (Decapoda, Brachyura)

Daniela P. Garçon [a], Douglas C. Masui [a], Fernando L.M. Mantelatto [b], Rosa P.M. Furriel [a], John C. McNamara [b], Francisco A. Leone [a,*]

[a] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-901, SP, Brasil
[b] Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-901, SP, Brasil

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ABSTRACT

We evaluate hemolymph osmotic and ionic regulatory abilities and characterize a posterior gill microsomal (Na^+, K^+)-ATPase from the marine swimming crab, *Callinectes ornatus*, acclimated to 21‰ or 33‰ salinity. *C. ornatus* is isosmotic after acclimation to 21‰ but is hyposmotic at 33‰ salinity; hemolymph ions do not recover initial levels on acclimation to 21‰ salinity but are anisoionic compared to ambient concentrations, revealing modest regulatory ability. NH_4^+ modulates enzyme affinity for K^+, which increases 187-fold in crabs acclimated to 33‰ salinity. The (Na^+, K^+)-ATPase redistributes into membrane fractions of different densities, suggesting that altered membrane composition results from salinity acclimation. ATP was hydrolyzed at maximum rates of 182.6 ± 7.1 nmol Pi min^{-1} mg^{-1} (21‰) and 76.2 ± 3.5 nmol Pi min^{-1} mg^{-1} (33‰), with little change in K_M values ($\approx$50 μmol L^{-1}). K^+ together with NH_4^+ synergistically stimulated activity to maximum rates of $\approx$240 nmol Pi min^{-1} mg^{-1}. K_I values for ouabain inhibition ($\approx$110 μmol L^{-1}) decreased to 44.9 ± 1.0 μmol L^{-1} (21‰) and 28.8 ± 1.3 μmol L^{-1} (33‰) in the presence of both K^+ and NH_4^+. Assays employing various inhibitors suggest the presence of mitochondrial F_0F_1-, and K^+- and V-ATPase activities in the gill microsomes.

1. Introduction

The (Na^+, K^+)-ATPase (E.C.3.6.1.37) or sodium pump is a member of the P_{2C} ATPase family and couples ATP hydrolysis to the transport of two K^+ ions into, and three Na^+ ions out of the cell, generating an electrochemical gradient that underlies cell osmotic equilibrium, establishes membrane resting potential, and determines cells' excitable properties (for review see Kaplan, 2002; Horisberger, 2004; Morth et al., 2007; Pedersen, 2007). Ion transport by the (Na^+, K^+)-ATPase across the plasma membrane involves cycling of the enzyme between at least two main conformations: a phosphorylated form E_1, with high-affinity for intracellular Na^+, and a dephosphorylated form E_2, to which extracellular K^+ ions bind with high-affinity (Kaplan, 2002; Horisberger, 2004; Morth et al., 2007).

Crustacean gills are multifunctional organs, performing respiratory gas exchange, hemolymph acid-base and osmo-ionic regulation, as well as the excretion of nitrogenous metabolites (for review see Lucu and Towle, 2003; Weihrauch et al., 2004; Tresguerres et al., 2008; Freire et al., 2008). Highly coordinated, complex mechanisms of ion transport across the gills maintain hemolymph osmotic and ionic equilibria and compensate for diffusive and urinary ion losses (Péqueux, 1995; Onken and Riestenpatt, 1998, 2002; Lucu and Towle, 2003; Kirschner, 2004). Covered by a thin cuticle, the gill epithelium constitutes a selective interface between the internal and the external media across which Na^+ and Cl^- are actively absorbed from dilute environments. Weak hyperosmoregulating crustaceans apparently possess a common set of ion transporters, including the (Na^+, K^+)-ATPase, together with K^+ and Cl^- channels, located in the basal membranes of the gill epithelial cells. In addition to these ion transporters and the (Na^+, K^+)-ATPase present in the basal membranes, Na^+ channels, V-type proton pumps and Cl^-/HCO_3^- exchangers present in the apical membranes underlie NaCl uptake by strong hyperosmoregulators (reviewed by Kirschner, 2004; Freire et al., 2008). However, while the action of the basal (Na^+, K^+)-ATPase constitutes an important driving force for ion uptake in both weak and strong hyperosmoregulators (Péqueux, 1995; Furriel et al., 2000; Lucu and Towle, 2003; Kirschner, 2004; Lovett et al., 2006; Jayasundara et al., 2007), the V-type ATPase seems to play an important role in Na^+ capture in strong hyperosmoregulators in dilute media (for review see Freire et al., 2008; Weihrauch et al., 2004).

Crustaceans are widely distributed throughout aquatic and terrestrial biotopes and show adaptations that underlie their ability to occupy different habitats. Aquatic crustaceans are ammoniotelic, excreting their nitrogenous end products across the gill epithelium

* Corresponding author. Tel.: +55 16 3602 3668; fax: +55 16 3602 4838.
E-mail address: fdaleone@ffclrp.usp.br (F.A. Leone).

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largely as ammonia ($NH_3 + NH_4^+$). At physiological pH, circulating hemolymph ammonia is almost 99% in the ionic form (Weihrauch et al., 1998, 1999, 2004). The substitution of K^+ by NH_4^+ in (Na^+, K^+)-ATPases from various origins has long suggested that this displacement might be related to ammonium transport from the hemolymph into the gill cell cytoplasm (Towle and Holleland, 1987). Later studies on isolated, perfused crab gills have revealed a ouabain-sensitive mechanism of ammonia excretion against strong inwardly-directed ammonia gradients across both the anterior and posterior gills (reviewed by Weihrauch et al., 2004).

The gill (Na^+, K^+)-ATPase from the blue crab *Callinectes danae* is synergistically stimulated by NH_4^+ and K^+, suggesting the presence of additional sites for NH_4^+ on the enzyme molecule (Masui et al., 2002) as seen in the freshwater shrimp *M. olfersi* (Furriel et al., 2004). Magnesium ions regulate this NH_4^+ extra-pumping activity, acting together with Na^+, displacing the equilibrium towards the E_1 conformation and exposing additional NH_4^+ sites, eliciting synergistic activation. These findings corroborate current models of ammonia excretion across the crustacean gill epithelium, since NH_4^+ transport from the hemolymph to the external medium appears to depend on the coordinated action of the basal (Na^+, K^+)-ATPase and other basolateral and apical transporters (reviewed by Weihrauch et al., 2004; Freire et al., 2008).

Callinectes ornatus Ordway, 1863 is a euryhaline, brachyuran swimming crab, amply distributed along the west coast of the Atlantic Ocean, from North Carolina to southern Brazil (Mantelatto and Fransozo, 2000). The crab plays a significant ecological role as scavenger, predator and food resource for other aquatic organisms and shore birds (Haefner, 1990). Although its life cycle and osmotic and ionic regulatory abilities are not known, *C. ornatus* constitutes about 60% of all brachyuran species in Ubatuba bay, Brazil, where it participates in the feeding dynamics of the shallow water megabenthos (Mantelatto and Fransozo, 2000). The crab is found in bay waters of 28 to 35‰ salinity (Mantelatto and Fransozo, 1999) usually buried in sand, mud or shell covered sediments, occurring from the intertidal zone to 75 m depth (Mantelatto and Christofoletti, 2001). This behavior may affect nitrogen excretion, since ambient ammonia levels can increase locally, reducing passive efflux or even leading to ammonia influx across the gill epithelium (Weihrauch et al., 1999). Thus, as seen in *C. danae* (Masui et al., 2002, 2005), the synergistic stimulation of the *C. ornatus* gill (Na^+, K^+)-ATPase by K^+ and NH_4^+ might afford outwardly-directed, active ammonia transport even at normal hemolymph K^+ concentrations (Weihrauch et al., 2004; Masui et al., 2002, 2005; Garçon et al., 2007).

In this study, we investigate hemolymph osmotic and ionic regulatory ability and perform an extensive kinetic characterization of the (Na^+, K^+)-ATPase expressed in the posterior gill tissue of *C. ornatus* acclimated to a moderately dilute salinity of 21‰ or acclimated to 33‰ salinity, close to that of its habitual medium. *C. ornatus* is isosmotic after acclimation to 21‰ but is hyposmotic at 33‰ salinity; major hemolymph ions do not recover initial levels on acclimation to 21‰ salinity but are anisoionic compared to ambient concentrations, characterizing modest ion regulatory capability. Enzyme activity is synergistically stimulated (»30%) by K^+ plus NH_4^+ in microsomes from crabs at both salinities, with 5- and 187-fold increases in the apparent affinity for K^+ in the 21‰- and 33‰-acclimated crabs, respectively. Apparently the (Na^+, K^+)-ATPase redistributes into membrane fractions of different density in response to salinity acclimation.

2. Materials and methods

2.1. Material

All solutions were prepared using Millipore MilliQ ultrapure, apyrogenic water and all reagents were of the highest purity commercially available. Imidazole, pyruvate kinase (PK), phosphoenolpyruvate (PEP), NAD^+, NADH, N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid (Hepes), lactate dehydrogenase (LDH), ouabain, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate (G3P), alamethicin, 3-phosphoglyceraldehyde diethyl acetal, ATP ditris salt, ouabain, ethacrynic acid, oligomycin, thapsigargin, bafilomycin A_1, sodium orthovanadate and alamethicin were purchased from Sigma Chemical Co (St. Louis, MO, USA). Dimethyl sulfoxide and triethanolamine were from Merck (Darmstadt, Germany). The protease inhibitor cocktail was from Calbiochem (Darmstadt, Germany). The alpha-5 monoclonal antibody against the a-subunit of the (Na^+, K^+)-ATPase (all isoforms) was purchased from Developmental Studies Hybridoma Bank (Iowa, IA, USA). Antimouse IgG, alkaline phosphatase conjugate was purchased from Promega Corporation (Madison, WI, USA).

2.2. Reagents

Glyceraldehyde-3-phosphate was prepared by hydrolysis of 3-phosphoglycer-aldehyde diethyl acetal with 150 µL HCl ($d = 1.18$ g mL^{-1}) in a boiling-water bath for 2 min, and neutralized with 50 µL triethanolamine. Sodium orthovanadate solution was prepared according to Furriel et al. (2000).

2.3. Depletion of ammonium ions from enzyme suspensions

Crystalline suspensions of LDH and PK were centrifuged at 20,000 ×*g* for 15 min, at 4 °C, in an Eppendorf Model 5810 refrigerated centrifuge. The pellet was resuspended in 300 µL of 50 mmol L^{-1} Hepes buffer (pH 7.5), transferred to an YM-10 Microcon filter and centrifuged 5 times in the same buffer until complete depletion of ammonium ions (tested with the Nessler reagent). Finally, the pellet was resuspended to the original volume. For PGK and GAPDH, the suspension was treated as above with 50 mmol L^{-1} triethanolamine buffer (pH 7.5), and containing 1 mmol L^{-1} dithiothreitol. When necessary, enzyme solutions were concentrated using Microcon filters.

2.4. Hemolymph sampling and gill excision

Adult intermolt specimens of *C. ornatus* were collected using double rig trawl nets from Ubatuba Bay (23° 26′ S, 45° 02′ W), São Paulo State (Brazil). The crabs were transported to the laboratory in seawater from the collection site (34‰ salinity) and were maintained in tanks containing 32 L aerated 33‰ seawater for two days. They were then acclimated in groups of 8 crabs each to 21‰ or 33‰ salinity for up to 15 days at 25 °C and were fed on alternate days with shrimp tails.

For hemolymph sampling, including fresh-caught crabs, at time = 0 h (crabs acclimated to 33‰ salinity for 10 days) and after 1, 5, 24, 120 or 360-h exposure to 21‰, individual 200-µL hemolymph samples were drawn into insulin syringes using #25-8 needles from the junction of the arthrodial membrane at the base of the last pereiopod from each of three different crabs ($N = 3$). Samples were stored at −20 °C until processing.

For each gill homogenate prepared, after 10 days acclimation at 21‰ salinity, 5 to 8 crabs were anesthetized by chilling at −20 °C and the entire carapace was quickly removed. Posterior gill pairs 6, 7, and 8 were rapidly excised and placed in 10 mL ice-cold homogenization buffer constituted by 20 mmol L^{-1} imidazole buffer (pH 6.8), containing 250 mmol L^{-1} sucrose, 6 mmol L^{-1} EDTA and the protease inhibitor cocktail (5 µmol L^{-1} leupeptin, 5 µmol L^{-1} antipain, 1 mmol L^{-1} benzamidine and 1 µmol L^{-1} pepstatin A).

2.5. Preparation of the gill microsomal fraction

The gills were rapidly diced and homogenized in homogenization buffer (20 mL/g wet tissue) using a Potter homogenizer. After

centrifugation of the crude extract at 20,000 ×*g* for 30 min at 4 °C, the supernatant was placed on crushed ice and the pellet was resuspended in an equal volume of homogenization buffer. After further centrifugation as described above, the two supernatants were gently pooled and centrifuged at 100,000 ×*g* for 2 h at 4 °C. The resulting pellet was resuspended in 20 mmol L^{-1} imidazole buffer (pH 6.8), containing 250 mmol L^{-1} sucrose (8 mL or 6 mL buffer/g wet tissue of 21‰- or 33‰-acclimated crabs, respectively). Finally, 0.5 mL aliquots were rapidly frozen in liquid nitrogen and stored at −20 °C. Under these conditions, no changes in (Na^+, K^+)-ATPase activity were seen after two-month's storage. When required, the aliquots were thawed, placed on crushed ice and used immediately.

2.6. Measurement of protein

Protein concentration was estimated according to Read and Northcote (1981), using bovine serum albumin as the standard.

2.7. Continuous-density sucrose gradient centrifugation

An aliquot (4 mg) of the (Na^+, K^+)-ATPase-rich microsomal fraction was layered into a 10–50% (w/w) continuous-density sucrose gradient in 20 mM imidazole buffer (pH 6.8), and centrifuged at 180,000 ×*g* and 4 °C for 2 h using a PV50T2 Hitachi vertical rotor. Fractions (0.5 mL) were collected from the bottom of the tube and were assayed for protein, total ATPase and ouabain-insensitive activities and refractive index.

2.8. Western blot analysis

SDS-PAGE was performed in 5–20% gels according to Garçon et al. (2007), using 3 µg and 60 µg protein/slot for protein staining and blotting analysis, respectively. After electrophoresis the gel was split, one half being stained with silver nitrate and the other electroblotted using a Hoefer SE200 system, employing nitrocellulose membranes according to Garçon et al. (2007). The nitrocellulose membrane was incubated for 1 h at 25 °C in a 1:10 dilution of the alpha-5 monoclonal antibody. After washing 3 times in 50 mmol L^{-1} Tris–HCl buffer (pH 8.0) containing 150 mmol L^{-1} NaCl and 0.1% Tween 20, the membrane was incubated for 1 h at 25 °C in a 1:7500 dilution of antimouse IgG, alkaline phosphatase conjugate. Specific antibody incorporation was developed in 100 mmol L^{-1} Tris–HCl buffer (pH 9.5) containing 100 mmol L^{-1} NaCl, 5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} NBT and 0.8 mmol L^{-1} BCIP. Immunoblots were scanned and imported as JPG files into a commercial software package (Kodak 1D 3.6) where the immuno-reaction densities were quantified and compared. Western blot analysis was repeated 3 times using different gill tissue preparations.

2.9. Measurement of ATPase activity

ATPase activity was assayed at 25 °C using a PK/LDH linked system in which ATP hydrolysis was coupled to NADH oxidation (Garçon et al., 2007). NADH oxidation was monitored at 340 nm ($\varepsilon_{340\ nm}$, pH 7.5 = 6200 mol^{-1} L cm^{-1}) in a Hitachi U-3000 spectrophotometer equipped with thermostatted cell holders. Standard conditions for estimation of enzyme activity in gill homogenates from 21‰-acclimated crabs were: 50 mmol L^{-1} Hepes buffer (pH 7.5) containing 0.5 mmol L^{-1} ATP (or 1.0 mmol L^{-1} for 33‰), 0.7 mmol L^{-1} $MgCl_2$ (or 2.0 mmol L^{-1} for 33‰), 5 mmol L^{-1} KCl (or 10 mmol L^{-1} for 33‰), and 50 mmol L^{-1} NaCl (both salinities), 0.14 mmol L^{-1} NADH, 2.0 mmol L^{-1} PEP, 82 µg PK (49 U) and 110 µg LDH (94 U) in a final volume of 1.0 mL. Alternatively, ATPase activity was estimated using the GAPDH/PGK linked system in which ATP hydrolysis was coupled to the reduction of NAD^+ at 340 nm. Standard conditions for estimation of enzyme activity in gill homogenates from 21‰-acclimated crabs were: 50 mmol L^{-1} triethanolamine buffer (pH 7.5), containing 0.5 mmol L^{-1} ATP (or 1.0 mmol L^{-1} for 33‰), 0.7 mmol L^{-1} $MgCl_2$ (or 2.0 mmol L^{-1} for 33‰), 5 mmol L^{-1} KCl (or 10 mmol L^{-1} for 33‰), and 50 mmol L^{-1} NaCl (both salinities), 1.0 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1.0 mmol L^{-1} G3P, 150 µg GAPDH (12 U) and 20 µg PGK (9 U) in a final volume of 1 mL. ATPase activity was also assayed at 25 °C after 10 min pre-incubation in alamethicin (1 mg/mg protein) to demonstrate the presence of leaky and/or disrupted vesicles. Controls without added enzyme were included in each experiment to quantify non-enzymatic substrate hydrolysis. Initial velocities were constant for at least 15 min provided that less than 5% of NADH (or NAD^+) was oxidized (reduced). The reaction rate for each modulator concentration was estimated in duplicate aliquots from the same microsomal preparation, and the mean values were used to fit the respective saturation curve. Each saturation curve was repeated using three different microsomal homogenates ($N = 3$). The two coupling systems gave equivalent results with a difference of less than 10%. ATP hydrolysis was also estimated in the presence of 3 mmol L^{-1} ouabain to access the ouabain-insensitive activity. The difference in measured activity in the absence (total ATPase activity) or presence of ouabain (ouabain-insensitive activity) was considered to represent the (Na^+, K^+)-ATPase activity.

2.10. Measurement of hemolymph osmolality and cation concentrations

Hemolymph osmolality was measured in 10-µL samples using a Wescor Model 5500 vapor pressure osmometer (Logan, Utah). Hemolymph Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were measured by emission spectroscopy using a Shimadzu Model AA-680 atomic absorption spectrophotometer (Kyoto, Japan) employing 10-µL hemolymph aliquots diluted 1:15,000 in Millipore MilliQ ultrapure apyrogenic water. Data are provided as the mean ± SD of three different hemolymph samples ($N = 3$) and were analyzed using a one-way analysis of variance (salinity or exposure time) followed by Student–Newman–Keuls multiple means testing. Effects and differences were considered significant at $P = 0.05$.

2.11. Estimation of kinetic parameters

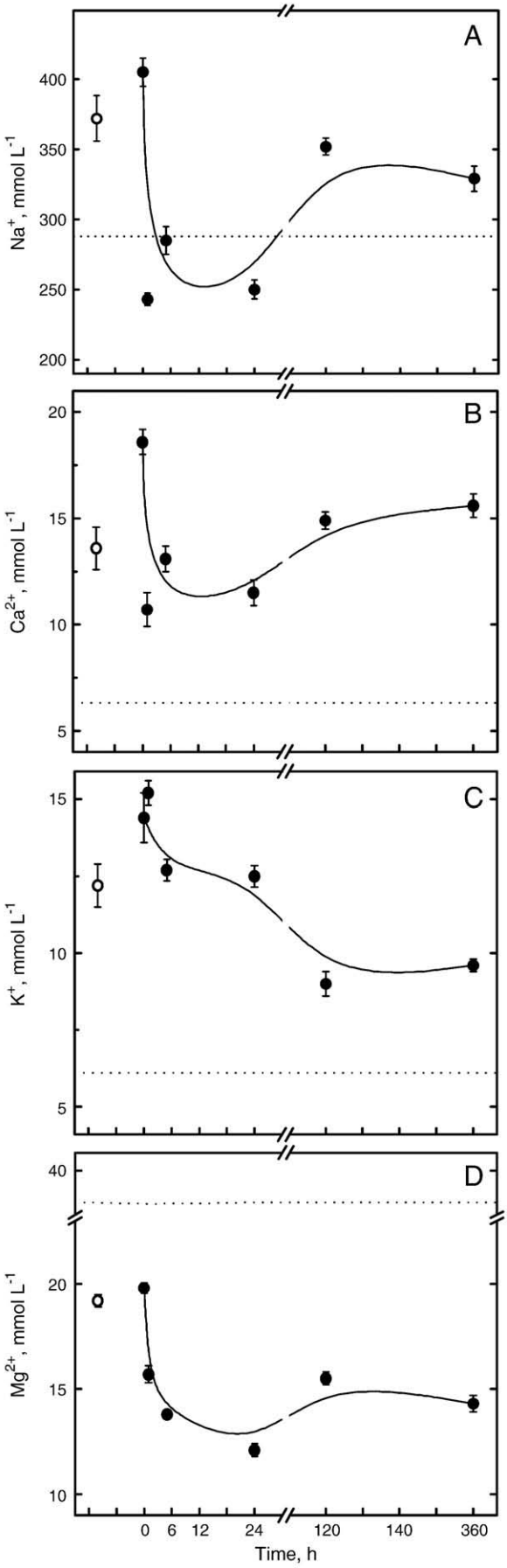
The kinetic parameters V (maximum velocity), $K_{0.5}$ (apparent dissociation constant), K_M (Michaelis–Menten constant) and the n_H value (Hill coefficient) for ATP hydrolysis were calculated using the software SigrafW as described by Leone et al. (2005a). The curves presented are those which best fit the experimental data. The apparent dissociation constant, K_I, of the enzyme-inhibitor complex was estimated as described by Furriel et al. (2000). The kinetic parameters provided in the tables are calculated values and are given as the mean ± SD from three different microsomal preparations ($N = 3$). Data were analyzed using a two-way analysis of variance (salinity and inhibitor) followed by Student–Newman–Keuls multiple means testing. Effects and differences were considered significant at $P = 0.05$.

3. Results

3.1. Hemolymph osmolality and cation concentrations

Hemolymph osmolality was similar ($P > 0.21$) in fresh-caught (853.7 ± 1.2 mOsm kg^{-1} H_2O) and 33‰-acclimated crabs (830.5 ± 30.8 mOsm kg^{-1} H_2O), declining to 681.8 ± 21.6 mOsm kg^{-1} H_2O in crabs acclimated to 21‰ for 15 days.

Hemolymph cation concentrations, however, were always significantly less (≈85%, $P < 0.001$) in fresh-caught crabs than in 33‰-acclimated crabs. During the time course of acclimation to 21‰ of crabs previously acclimated at 33‰, hemolymph Na^+ (405.0 ± 9.9 mmol L^{-1}) (Fig. 1A)



and Ca^{2+} (18.6 ± 0.6 mmol L^{-1}) (Fig. 1B) concentrations decreased rapidly to minimum values within 1 h, (243.0 ± 4.3 mmol Na^+ L^{-1}, 10.7 ± 0.8 mmol Ca^{2+} L^{-1}), remained roughly stable between 5 and 24 h, recovering partially to concentrations significantly less (≈80%, $P<0.001$) than initial values by 15 days (329.6 ± 8.8 mmol Na^+ L^{-1}, 15.6 ± 0.6 mmol Ca^{2+} L^{-1}). Hemolymph K^+ (14.4 ± 0.8 mmol L^{-1}) (Fig. 1C) was not regulated temporally and declined steadily to a minimum (9.6 ± 0.2 mmol L^{-1}) after 15-days acclimation while hemolymph Mg^{2+} (19.8 ± 0.3 mmol L^{-1}) (Fig. 1D) reached a minimum at 24 h (12.1 ± 0.3 mmol L^{-1}), recovering to a concentration (14.3 ± 0.4 mmol L^{-1}) significantly less (70%, $P<0.001$) than the initial value by 15 days. Final Na^+, Ca^{2+} and K^+ concentrations in 21‰ lay well above respective ambient concentrations while Mg^{2+} concentration was considerably lower.

3.2. Sucrose density gradient centrifugation analyses

The sucrose density gradient centrifugation analyses of gill microsomes from *C. ornatus* acclimated to 21‰ or 33‰ salinity for 10 days are shown in Fig. 2. Independently of acclimation salinity, two peaks of (Na^+, K^+)-ATPase activity coincident with the protein peaks were seen: a light fraction (I) at around 30% sucrose, and a heavier fraction (II) at about 42% sucrose. Although the maximum protein concentrations for peaks I and II were fairly similar in both salinities, there were striking differences in (Na^+, K^+)-ATPase activity. For peak I, the (Na^+, K^+)-ATPase activity in homogenates from 21‰-acclimated crabs (Fig. 2A) was »4-fold greater than that for the 33‰-acclimated crabs (Fig. 2B). In contrast, the ratio for peak II was only 2-fold (Fig. 2A and B). Further, in gill microsomes from 33‰-acclimated crabs the peak II/peak I activity ratio was around 1.4 but dropped to 0.7 in 21‰-acclimated crabs. These differences strongly suggest that acclimation to the different salinities induces the redistribution of enzyme molecules into membrane fractions of different density, possibly reflecting distinct lipid and/or protein compositions.

3.3. SDS-PAGE and Western blot analyses

SDS-PAGE and Western blot analyses of gill microsomes from crabs acclimated to 33‰ (lanes A and C) or 21‰ salinity for 10 days (lanes B and D) are shown in Fig. 3. Western blot data (lanes A and B) using the α-5 monoclonal antibody against the avian (Na^+, K^+)-ATPase α-subunit identified a single immunoreactive band of 105 kDa in microsomal preparations from both 21‰- and 33‰-acclimated crabs. The different SDS-PAGE profiles (lanes C and D) suggest altered protein expression in response to salinity acclimation. Quantitative image analysis revealed a 2.5-fold higher density for the (Na^+, K^+)-ATPase a-subunit in the microsomal fraction of 21‰-acclimated crabs.

3.4. Modulation by ATP of (Na^+, K^+)-ATPase activity

Modulation by ATP of (Na^+, K^+)-ATPase activity in gill microsomes from 21‰- and 33‰-acclimated crabs is shown in Fig. 4. As ATP concentration increases from 10^{-6} to 10^{-3} mol L^{-1} activity was stimulated obeying Michaelis–Menten kinetics ($n_H = 1.0$) to maximum values of $V = 182.6 \pm 7.1$ nmol Pi min^{-1} mg^{-1} for 21‰- and 76.2 ± 3.5 nmol Pi min^{-1} mg^{-1} for 33‰-acclimated crabs. K_M values were similar (50.0 ± 2.0 µmol L^{-1} and 46.0 ± 2.0 µmol L^{-1} for 21‰ and 33‰ salinity, respectively) showing that acclimation induces changes in reaction rate but not in apparent affinity of the enzyme for ATP. Ouabain-insensitive ATPase activity was stimulated to maximum

Fig. 1. Time course of alterations in major hemolymph ion concentrations in *Callinectes ornatus* during 15-days acclimation to 21‰ salinity on direct transfer from 33‰ salinity. A – Na^+ concentration. B – Ca^{2+} concentration. C – K^+ concentration. D – Mg^{2+} concentration. (○) – Fresh-caught crabs. (●) – Ion concentrations after directly transferring crabs acclimated for 10 days at 33‰ salinity (time = 0 h) to 21‰ salinity. Data are the mean ± SD ($N = 3$). Dotted lines indicate respective ion concentrations in 21‰ salinity.

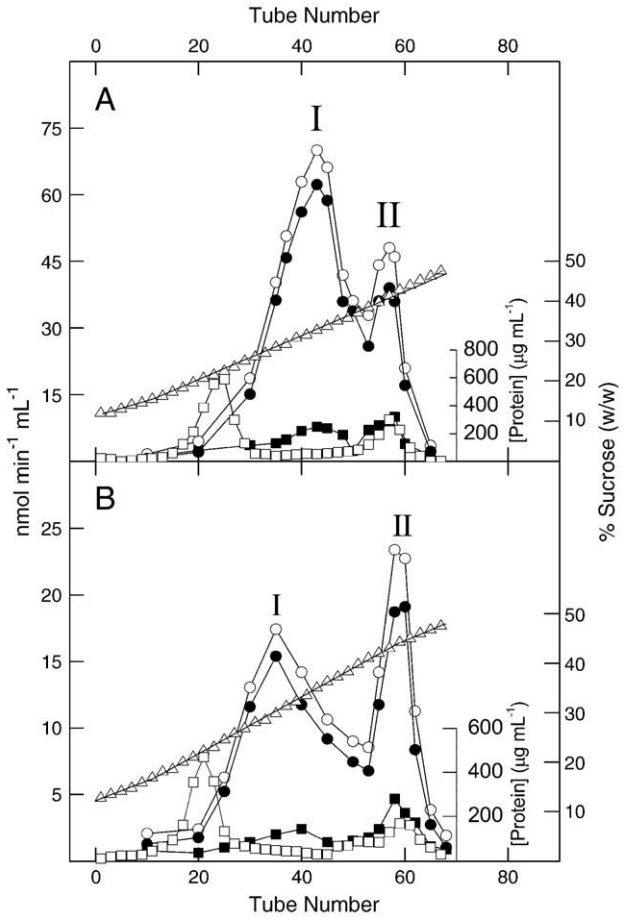


Fig. 2. Sucrose density gradient centrifugation of posterior gill tissue from *C. ornatus* acclimated to 21 or 33‰ salinity for 10 days. An aliquot containing 4 mg protein was layered into a 10 to 50% (w/w) continuous sucrose density gradient. ATPase activity was estimated in 50 mmol L^{-1} Hepes buffer (pH 7.5) under optimal conditions of substrate and ions, and fractions (0.5 mL) were collected from the bottom of the gradient and analyzed for total ATPase activity (○); (Na^+, K^+)-ATPase activity (●); ouabain-insensitive ATPase activity (■); protein concentration (□) and sucrose concentration (△). A – 21‰-acclimated crabs. B – 33‰-acclimated crabs. Three ($N=3$) different gill homogenates were used, and representative curves obtained from one homogenate are given.

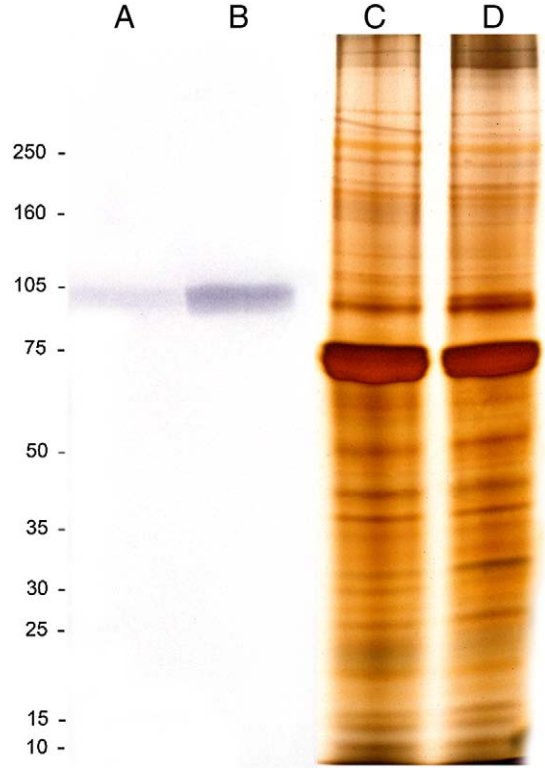


Fig. 3. SDS-PAGE and Western blot analyses of a microsomal fraction from the posterior gill tissue of *Callinectes ornatus* acclimated to 21 or 33‰ salinity for 10 days. Electrophoresis was performed in a 5–20% polyacrylamide gel. A and B: Western blot analysis using 60 µg protein from both gill microsome preparations. C and D: silver-stained protein using 3 µg protein/slot from both gill microsome preparations. A and C: 33‰-acclimated crabs. B and D: 21‰-acclimated crabs.

values of $V=24.9\pm1.2$ nmol Pi min^{-1} mg^{-1} for 21‰- and 11.1 ± 0.5 for 33‰-acclimated crabs over the same ATP concentration range. These activities correspond to about 13% of total ATPase activity and strongly suggest the presence of hydrolyzing ATPases other than the (Na^+, K^+)-ATPase.

3.5. Modulation by Na^+ and Mg^{2+} of (Na^+, K^+)-ATPase activity

Fig. 5 shows the modulation of *C. ornatus* gill (Na^+, K^+)-ATPase activity by Mg^{2+} and Na^+. In the range 10^{-4} to 10^{-3} mol Mg^{2+} L^{-1}, stimulation by Mg^{2+} of enzyme activity obeyed cooperative kinetics ($n_H>1.0$) attaining maximum rates of $V=178.2\pm6.7$ nmol Pi min^{-1} mg^{-1} for 21‰ salinity and 74.5 ± 3.1 nmol Pi min^{-1} mg^{-1} for 33‰ salinity (Fig. 5A). Like ATP, the $K_{0.5}$ values (270.0 ± 10.0 µmol L^{-1} for 21‰- and 330.0 ± 20.0 µmol L^{-1} for 33‰-acclimated crabs) were similar. Considerable Mg^{2+}-stimulated ouabain-insensitive ATPase activity was detected (23.3 ± 0.9 nmol Pi min^{-1} mg^{-1} and 11.6 ± 0.4 nmol Pi min^{-1} mg^{-1}, for 21‰- and 33‰-acclimated crabs, respectively) over the same Mg^{2+} concentration range (inset to Fig. 5A). Sodium ions stimulated the gill (Na^+, K^+)-ATPase from 21‰-acclimated crabs following cooperative kinetics to maximum rates of $V=175.9\pm6.3$ nmol Pi min^{-1} mg^{-1} (Fig. 5B); however, stimulation was 2-fold lower in 33‰-acclimated crabs ($V=76.4\pm3.0$ nmol Pi min^{-1} mg^{-1}). Again, $K_{0.5}$ values were similar (4.18 ± 0.16 mmol L^{-1} and 5.30 ± 0.20 mmol L^{-1}, for 21‰ and 33‰ salinity, respectively). The lack of stimulation of the ouabain-insensitive ATPase activity over the same Na^+ concentration range (not shown) excludes Na^+-ATPase activity in both microsomal preparations.

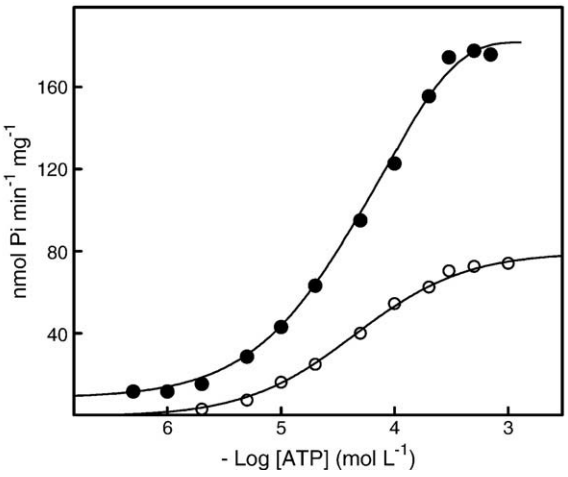


Fig. 4. Effect of ATP concentration on (Na^+, K^+)-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* acclimated to 21 or 33‰ salinity for 10 days. Duplicate aliquots containing 36.2 µg protein from three ($N=3$) different gill homogenates were used, and representative curves obtained from one homogenate are given. ATPase activity was estimated in 50 mmol L^{-1} Hepes buffer (pH 7.5) under optimal ionic conditions, and representative curves obtained from one homogenate are given. Crabs were acclimated for 10 days at 21‰ (●) or 33‰ (○) salinity.

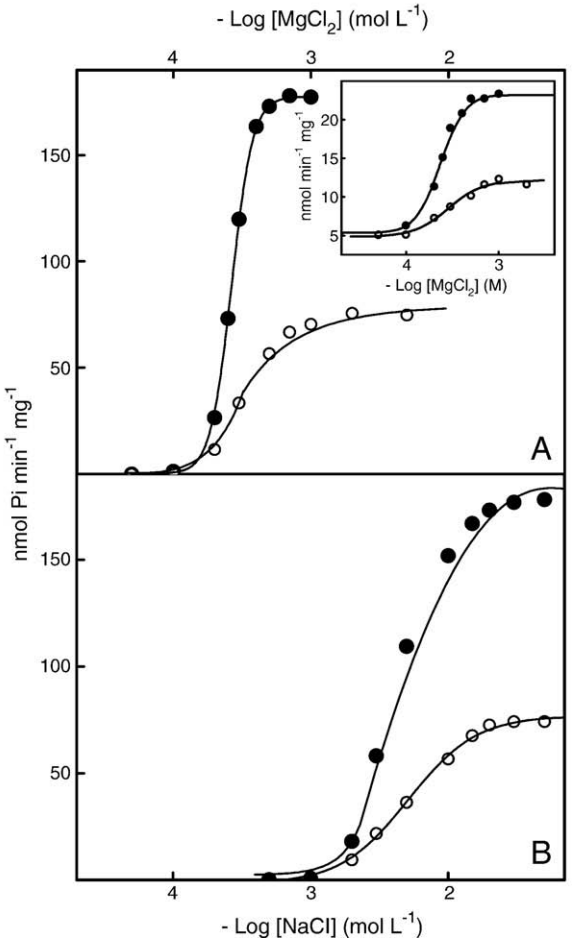


Fig. 5. Effect of magnesium and sodium ions on (Na^+, K^+)-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* acclimated to 21 or 33‰ salinity for 10 days. Duplicate aliquots containing 36.2 µg protein from three ($N=3$) different gill homogenates were used, and representative curves obtained from one homogenate are given. ATPase activity was estimated in 50 mmol L^{-1} Hepes buffer (pH 7.5). A – Mg^{2+}. B – Na^+. Inset to Fig. 4A: effect of Mg^{2+} concentration on ouabain-insensitive ATPase activity of 33‰- (○) or 21‰-acclimated (●) crabs.

3.6. Modulation by K^+ and NH_4^+ of (Na^+, K^+)-ATPase activity

The stimulation by K^+ or NH_4^+ of gill (Na^+, K^+)-ATPase activity in crabs acclimated to 21‰ or 33‰ is shown in Fig. 6. From 10^{-4} to 10^{-2} mol L^{-1}, increasing K^+ concentration stimulated the (Na^+, K^+)-ATPase activity of 21‰-acclimated crabs to a maximum value of $V=181.8\pm6.9$ nmol Pi min^{-1} mg^{-1}; for 33‰-acclimated crabs the maximum value was 76.4 ± 3.0 nmol Pi min^{-1} mg^{-1}. ATP hydrolysis obeyed cooperative kinetics ($n_H>1.0$) and $K_{0.5}$ values for K^+ were similar (0.89 ± 0.03 mmol L^{-1} and 1.03 ± 0.05 mmol L^{-1} for 21‰- and 33‰-acclimated crabs, respectively) (Fig. 6A). Stimulation of the ouabain-insensitive ATPase activity over the same K^+ concentration range gave maximum values of 23.1 ± 1.3 nmol Pi min^{-1} mg^{-1} and 10.8 ± 0.6 nmol Pi min^{-1} mg^{-1} for 21‰ and 33‰ salinity, respectively (inset to Fig. 6A), suggesting the presence of a K^+-stimulated ATPase. In the absence of K^+, NH_4^+ also stimulated the (Na^+, K^+)-ATPase obeying cooperative kinetics (Fig. 6B). Increasing NH_4^+ concentrations from 10^{-4} to 10^{-1} mol L^{-1} stimulated the (Na^+, K^+)-ATPase activity to maximum values of $V=240.7\pm11.2$ nmol Pi min^{-1} mg^{-1} (21‰ salinity) and 98.9 ± 3.9 nmol Pi min^{-1} mg^{-1} (33‰ salinity) with $K_{0.5}$ values around 4.0 mmol L^{-1}. Ouabain-insensitive ATPase activity was not stimulated by NH_4^+ over the same K^+ concentration range. Table 1 summarizes the kinetic parameters calculated for the stimulation of (Na^+, K^+)-ATPase activity in gill microsomes from 21‰- and 33‰-acclimated *C. ornatus*.

3.7. Synergistic stimulation by K^+ and NH_4^+ of (Na^+, K^+)-ATPase activity

When assayed in the presence of both K^+ and NH_4^+, (Na^+, K^+)-ATPase activity was stimulated synergistically (Fig. 7 and Table 2). Increasing K^+ concentrations in the NH_4^+-containing reaction media (1 to 50 mmol L^{-1}) synergistically stimulated (Na^+, K^+)-ATPase activity, maximum rates reaching $V=238.3\pm9.2$ nmol Pi min^{-1} mg^{-1} for 21‰ (Fig. 7A) and 98.8 ± 3.9 nmol Pi min^{-1} mg^{-1} for 33‰ salinity (Fig. 7B). However, while the apparent dissociation constant $K_{0.5}$ for 21‰-acclimated crabs decreased 5.5-fold (from 0.89 ± 0.03 to 0.16 ± 0.01 mmol L^{-1}) in the presence of increasing NH_4^+ concentrations, the $K_{0.5}$ for 33‰-acclimated crabs decreased 187-fold (from 1.03 ± 0.05 mmol L^{-1} to 0.0055 ± 0.0001 mmol L^{-1}). Similarly, the addition of increasing NH_4^+ concentrations to the K^+-containing reaction media (1 to 10 mmol L^{-1}) gave maximum rates of around 240 nmol Pi min^{-1} mg^{-1} for 21‰- (Fig. 8A) and 100 nmol Pi min^{-1} mg^{-1} for 33‰-acclimated (Fig. 8B) crabs. Table 2 shows that $K_{0.5}$ values were altered mainly with change in NH_4^+ concentration. Enzyme catalytic efficiency (V/K) was greater in crabs acclimated either to 21‰ or 33‰, in response to increasing NH_4^+ concentrations. However, increasing K^+ concentrations had little effect on V/K values. Synergistic stimulation by NH_4^+ and K^+ of the gill (Na^+, K^+)-ATPase activity obeyed cooperative kinetics ($n_H>1.0$) under all conditions assayed.

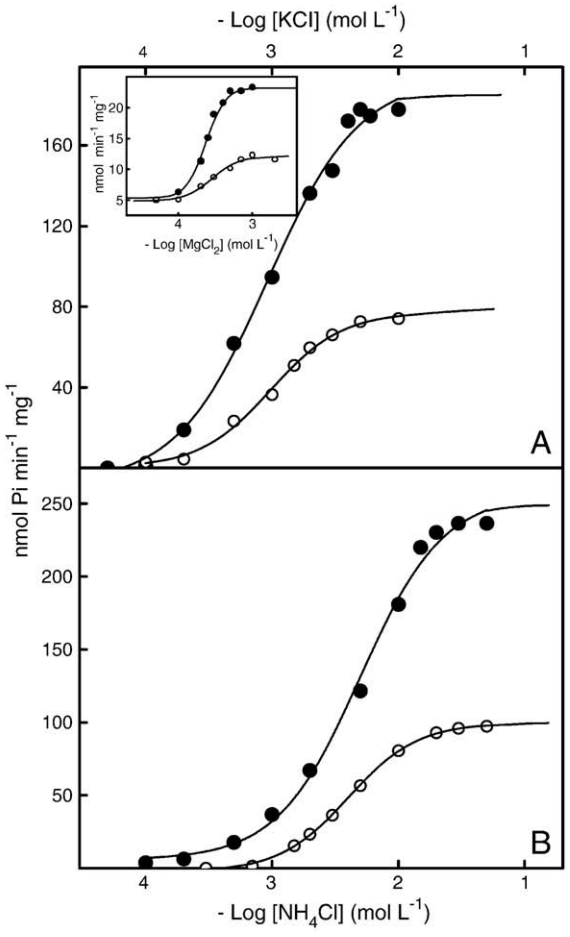


Fig. 6. Effect of potassium and ammonium ions on (Na^+, K^+)-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* acclimated to 21 or 33‰ salinity for 10 days. Duplicate aliquots containing 36.2 µg protein from three ($N=3$) different gill homogenates were used, and representative curves obtained from one homogenate are given. ATPase activity was estimated in 50 mmol L^{-1} Hepes buffer (pH 7.5). The modulation of enzyme activity by each effector was evaluated under optimal concentrations of the other. A – K^+. B – NH_4^+. Inset to Fig. 4A: effect of K^+ concentration on ouabain-insensitive ATPase activity of 33‰- (○) and 21‰-acclimated (●) crabs.

Table 1
Kinetic parameters for the stimulation by Na^+, K^+, Mg^{2+}, NH_4^+ and ATP of (Na^+, K^+)-ATPase activity in gill tissue from *Callinectes ornatus* acclimated to 21‰ or 33‰ salinity for 10 days.

Effector	V (nmol min^{-1} mg^{-1})		$K_{0.5}$ or K_M (mmol L^{-1})		n_H		V/K	
	21‰	33‰	21‰	33‰	21‰	33‰	21‰	33‰
ATP	182.6 ± 7.1	76.2 ± 3.5	0.050 ± 0.002	0.046 ± 0.002	1.0	1.1	3652	1656
Na^+	175.9 ± 6.3	76.4 ± 3.0	4.18 ± 0.16	5.30 ± 0.20	2.5	1.9	42	14
Mg^{2+}	178.2 ± 6.7	74.5 ± 3.1	0.27 ± 0.01	0.33 ± 0.02	5.8	3.0	660	226
K^+	181.8 ± 6.9	76.4 ± 3.0	0.89 ± 0.03	1.03 ± 0.05	1.5	1.7	204	74
NH_4^+	240.7 ± 11.2	98.9 ± 3.9	4.44 ± 0.17	4.10 ± 0.20	1.4	1.7	54	24

Assays were performed in 50 mmol L^{-1} Hepes buffer (pH 7.5) in a final volume of 1.0 mL. The effect of each agent was evaluated under optimal concentrations of the others. Data are the mean ± SD from three ($N = 3$) different microsomal preparations.

3.8. Inhibition studies

Ouabain inhibition of total ATPase activity in gill microsomes from *C. ornatus* acclimated to 21 or 33‰ salinity, in the absence and presence of NH_4^+ is shown in Fig. 9. At saturating ATP, Mg^{2+}, Na^+ and K^+ concentrations, a simple ouabain inhibition profile was seen, independently of NH_4^+. Ouabain concentrations up to 3 mmol L^{-1} did not completely inhibit ATPase activity, and ouabain-insensitive activities corresponding to around 25 nmol Pi min^{-1} mg^{-1} and 11 nmol Pi min^{-1} mg^{-1} of total ATPase activity were found for 21‰- and 33‰-acclimated crabs, respectively. In the absence of NH_4^+, apparent inhibition constants of 110.0 ± 5.0 μmol L^{-1} and 114.9 ± 5.6 μmol L^{-1} were calculated for 21‰- and 33‰-acclimated crabs, respectively, (inset to Fig. 9A). Substantially lower K_I values (44.9 ± 1.0 μmol L^{-1} in 21‰ and 28.8 ± 1.3 μmol L^{-1} in 33‰) were found in the presence of 50 mmol L^{-1} NH_4^+ (inset to Fig. 9B).

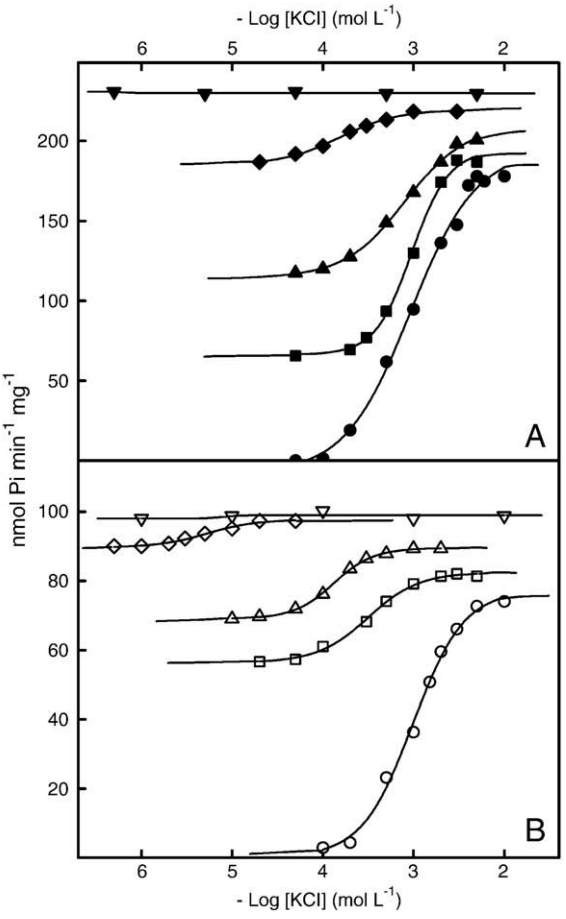


Fig. 7. Effect of ammonium ion concentration on the modulation by potassium ions of (Na^+, K^+)-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* acclimated to 21 or 33‰ salinity for 10 days. Duplicate aliquots containing 36.2 μg protein from three ($N = 3$) different gill homogenates were used, and representative curves obtained from one homogenate are given. For 21‰-acclimated crabs activity was assayed in 50 mmol L^{-1} triethanolamine buffer (pH 7.5), containing 0.5 mmol L^{-1} ATP (or 1.0 mmol L^{-1} for 33‰), 0.7 mmol L^{-1} $MgCl_2$ (or 2.0 mmol L^{-1} for 33‰), and 50 mmol L^{-1} NaCl (both salinities), 1.0 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1.0 mmol L^{-1} G3P, 150 μg GAPDH (12 U) and 20 μg PGK (9 U) in a final volume of 1 mL. The modulation of enzyme activity by each effector was evaluated under optimal concentrations of the other. A – 21‰-acclimated crabs. Fixed ammonium ion concentrations used were: (●) 0 mmol L^{-1}, (■) 1 mmol L^{-1}, (▲) 5 mmol L^{-1}, (♦) 10 mmol L^{-1}, (▼) 30 mmol L^{-1}. B – 33‰-acclimated crabs. Fixed ammonium ion concentrations used were: (○) 0 mmol L^{-1}, (□) 5 mmol L^{-1}, (△) 10 mmol L^{-1}, (◊) 30 mmol L^{-1}, (▽) 50 mmol L^{-1}.

To identify putative enzymes accounting for the ouabain-insensitive activity, various inhibitors were assayed together with ouabain (Table 3). The absence of further inhibition of ouabain-insensitive ATPase activity by theophylline, orthovanadate, thapsigargin and EGTA suggests that both microsomal preparations are free of phosphatases, P-ATPases other than (Na^+, K^+)-ATPase, and Ca^{2+}-ATPase. In contrast, additional inhibition by oligomycin and aurovertin B suggests the presence of mitochondrial F_0F_1-ATPase in the microsomal fractions from crabs acclimated to both salinities. Inhibition by ethacrynic acid of ouabain-insensitive ATPase activity (4% for both salinities), together with stimulation by K^+ (see inset to Fig. 6A) also suggests K^+-ATPase activity. The additional inhibition by bafilomycin A_1 or concanamycin A suggests some V-ATPase activity.

4. Discussion

In this investigation, we accompany alterations in hemolymph osmolality together with the time course of changes in major hemolymph ion concentrations during direct acclimation of the marine swimming crab *C. ornatus* to 21‰ from 33‰ salinity. We also provide an extensive kinetic characterization of the (Na^+, K^+)-ATPase expressed in the posterior gill tissue of the crab when acclimated to salinities of 21‰ or 33‰.

C. ornatus hyporegulates hemolymph osmolality when acclimated for 10 days to 33‰ ($\Delta = 192$ mOsm kg^{-1} H_2O) but is essentially isosmotic after 15-days acclimation to 21‰ salinity. Further, hemolymph Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations do not recover initial values seen in 33‰ ($\Delta = 65$–80%) after 15-days acclimation to 21‰. These data reveal that *C. ornatus* is a limited osmotic and ionic regulator in dilute medium, particularly with regard to K^+. However, hemolymph Na^+, K^+ and Ca^{2+} are held well above, and Mg^{2+} well below, respective ambient ion concentrations after 15-days acclimation to 21‰ salinity, revealing moderate overall anisoionic regulatory ability. Thus, despite its isosmoticity, *C. ornatus* seems to be physiologically capable of penetrating dilute media for extended periods. The increased gill (Na^+, K^+)-ATPase activity seen in 21‰-acclimated crabs apparently underlies this ability.

Two novel findings concerning the (Na^+, K^+)-ATPase are evident. Firstly, the enzyme from 33‰-acclimated crabs exhibits a 187-fold increase in apparent affinity for K^+ in the presence of increasing NH_4^+ concentrations. Secondly, the enzyme molecules are distributed into membrane fractions of different density, suggesting altered membrane composition, depending on acclimation salinity.

Table 2
Kinetic parameters for the stimulation by both K^+ and NH_4^+ of (Na^+, K^+)-ATPase activity in gill tissue from *Callinectes ornatus* acclimated to 21‰ or 33‰ salinity for 10 days.

[KCl] (mmol L^{-1})	[NH_4Cl] (mmol L^{-1})	V (nmol min^{-1} mg^{-1})		$K_{0.5}$ (mmol L^{-1})		n_H		V/K	
		21‰	33‰	21‰	33‰	21‰	33‰	21‰	33‰
Variable	0	181.8 ± 6.9	76.4 ± 3.0	0.89 ± 0.03	1.03 ± 0.05	1.5	1.7	205	71
Variable	1	191.6 ± 8.5	–	0.92 ± 0.03	–	2.2	–	208	–
Variable	5	203.6 ± 9.2	82.5 ± 3.3	0.75 ± 0.02	0.31 ± 0.01	1.6	1.5	271	266
Variable	10	218.9 ± 9.6	89.5 ± 3.5	0.16 ± 0.01	0.13 ± 0.01	1.5	2.0	1368	688
Variable	20	–	97.6 ± 3.9	–	0.0055 ± 0.0001	–	1.8	–	17,745
Variable	30	238.3 ± 9.2	–	–	–	–	–	–	–
Variable	50	–	98.8 ± 3.9	–	–	–	–	–	–
0	Variable	240.7 ± 11.2	98.9 ± 3.9	4.4 ± 0.2	4.1 ± 0.2	1.4	1.7	55	24
1	Variable	240.6 ± 9.6	99.9 ± 4.0	4.8 ± 0.3	6.0 ± 0.2	1.6	1.6	50	16
3	Variable	238.3 ± 9.2	98.5 ± 3.8	4.0 ± 0.2	6.4 ± 0.2	1.9	1.7	59	16
5	Variable	237.0 ± 9.0	–	4.9 ± 0.3	–	2.0	–	48	–
10	Variable	–	99.7 ± 3.9	–	6.7 ± 0.3	–	1.7	–	15

Assays were performed using 36.2 μg protein in 50 mmol L^{-1} triethanolamine buffer (pH 7.5) containing 0.5 mmol L^{-1} ATP (or 1.0 mmol L^{-1} for 33‰), 0.7 mmol L^{-1} $MgCl_2$ (or 2.0 mmol L^{-1} for 33‰), and 50 mmol L^{-1} NaCl (both salinities), in a final volume of 1.0 mL. The effect of each agent was evaluated under optimal concentrations of the other. Data are the mean ± SD from three ($N = 3$) different microsomal preparations.

Sucrose density gradient centrifugation of the gill microsomal fraction from 21‰- and 33‰-acclimated *C. ornatus* revealed two peaks of (Na^+, K^+)-ATPase activity, one in a light fraction around 30% sucrose, the other in a heavier fraction at about 42% sucrose. This contrasts with fresh-caught crabs in which peak I, appearing between 25 and 35% sucrose, is coincident with the major protein peak, while peak II, appearing between 39 and 45% sucrose, has lower activity and negligible protein content (Garçon et al., 2007). These findings unequivocally show that the gill (Na^+, K^+)-ATPase redistributes into membrane fractions of different density in response to salinity acclimation. A similar redistribution of the gill enzyme into membrane fractions of different densities also occurs in *Macrobrachium amazonicum* (Belli et al., 2009). The phenomenon may derive from various factors: firstly, more gill (Na^+, K^+)-ATPase copies are expressed in the 21‰- compared to 33‰-acclimated crabs as clearly shown in Fig. 1A and B. Secondly, the expression of osmoregulatory proteins other than the (Na^+, K^+)-ATPase is greater in 21‰-compared to 33‰-acclimated crabs (see Fig. 1C and D). Thirdly, altered membrane lipid composition consequent to acclimation in the different salinities may result in membrane fractions of different densities. The lipid composition of *M. amazonicum* gill microsomes is strikingly altered in response to 21‰-acclimation salinity, resulting in 3-fold greater phospholipid/total protein and cholesterol/total protein ratios (N. M. Belli, personal communication). However, further studies are necessary to establish causality. Proteoma and lipid analyses of different microsomal fractions prepared from salinity-acclimated crabs are in progress in our laboratories.

With regard to ATP hydrolysis, the specific activity ($V = 76.2 \pm 3.5$ nmol Pi min^{-1} mg^{-1}) of the (Na^+, K^+)-ATPase in gill tissue from 33‰-acclimated *C. ornatus* lies within the range (35 to 225 nmol Pi min^{-1} mg^{-1}) for gill homogenates of other *Callinectes* species (Lovett and Watts, 1995; Lucu et al., 2000). The 2.4-fold increase in specific activity seen in the 21‰-acclimated crabs confirms many studies in various crustaceans demonstrating increased gill (Na^+, K^+)-ATPase activity in response to acclimation to dilute media (Holliday, 1985; D'Orazio and Holliday, 1985; Harris and Bayliss, 1988; Corotto and Holliday, 1996; Henry et al., 2002; Lucu and Towle, 2003; Kirschner, 2004; Lucu et al., 2008). A remarkable exception is the gill enzyme from *C. sapidus* that exhibits similar specific activities over an ample salinity range (Neufeld et al., 1980; Lovett and Watts, 1995; Lucu et al., 2000; Lucu and Towle, 2003).

Although high- (K_M between 0.1 and 1.0 μmol L^{-1}) and low-affinity (K_M between 0.01 and 0.2 mmol L^{-1}) ATP hydrolyzing sites are known for the vertebrate (Na^+, K^+)-ATPase (Glynn, 1985; Ward and Cavieres, 1998; Neufeld and Levy, 1969; Kanazawa et al., 1970; Robinson, 1976), their characterization is still contentious (Beaugé et al., 1997; Martin and Sachs, 2000). We have demonstrated crustacean gill (Na^+, K^+)-ATPase isoforms showing high- and low-affinity sites for ATP (Masui et al., 2002, 2008; Santos et al., 2007; Gonçalves et al., 2006). That only a single site of ATP hydrolysis is evident for the *C. ornatus* gill (Na^+, K^+)-ATPase may be characteristic of this specific enzyme, as reported for *Cancer pagurus* (Gache et al., 1977), fresh-caught *M. olfersi* (Furriel et al., 2000) and *C. sapidus* (Wheatly and Henry, 1987).

In the absence of magnesium ions, the *C. ornatus* gill (Na^+, K^+)-ATPase does not hydrolyze ATP, as known for the enzyme from other sources (Furriel et al., 2000; Glynn, 1985; Masui et al., 2002; Gonçalves et al., 2006). Further, the affinity of the (Na^+, K^+)-ATPase from 21‰- and 33‰-acclimated crabs for Mg^{2+} is salinity independent and similar to *C. danae* (Masui et al., 2002) and the intertidal hermit crab *Clibanarius vittatus* (Gonçalves et al., 2006), but less than that of several other crustaceans (Neufeld et al., 1980; Holliday, 1985; D'Orazio and Holliday, 1985; Corotto and Holliday, 1996; Furriel et al., 2000; Santos et al., 2007).

Different α-subunit isoforms of the vertebrate (Na^+, K^+)-ATPase show distinct affinities for Na^+ and K^+ (Levenson, 1994; Therien et al., 1996; Blanco and Mercer, 1998; Sweeney and Klip, 1998; Crambert et al., 2000; Segall et al., 2001; Lopez et al., 2002), and the same α-subunit isoform can exhibit diverse kinetic properties when associated with different β-subunit isoforms (Blanco et al., 1998; Therien et al., 1996; Geering, 2001). These differences in (Na^+, K^+)-ATPase affinity for Na^+ and K^+ are also tissue and species-specific, suggesting that membrane and/or post-translation factors may participate in enzyme regulation (Sweadner, 1989; Levenson, 1994; Therien et al., 1996; Lopez et al., 2002). The apparent affinity of the *C. ornatus* gill enzyme ($K_{0.5} = 4.18 \pm 0.16$ mmol L^{-1} for 21‰ and 5.3 ± 0.2 mmol L^{-1} for 33‰ salinity) for Na^+ lies in the range for various euryhaline crabs (Specht et al., 1997), *C. danae* (Masui et al., 2002) and *M. olfersi*, a diadromous freshwater shrimp (Furriel et al., 2000). The enzymes from *Carcinus maenas* and freshwater species exhibit Na^+ affinities 10 to 20-fold higher than marine or estuarine species (Harris and Bayliss, 1988). However, our findings, which show very similar Na^+ affinities for species from a broad range of habitats including the diadromous, freshwater palaemonid shrimps *M. olfersi* (Furriel et al., 2000) and *M. amazonicum* (Santos et al., 2007), the marine swimming crabs *C. danae* (Masui et al., 2002) and *C. ornatus* (present data), the intertidal hermit crab *Clibanarius vitattus* (Gonçalves et al., 2006) and the marine penaeid shrimp *Xyphopenaeus kroyeri* (Leone et al., 2005b) do not support this idea.

C. ornatus exhibited monophasic inhibition by ouabain in contrast to the biphasic response reported for *Uca pugnax* (Holliday, 1985), *Potamon potamios* (Tentes and Stratakis, 1991) and *C. sapidus* (Neufeld et al., 1980). While the K_I values (110.0 ± 5.0 μmol L^{-1} and 114.9 ± 5.6 μmol L^{-1} for 21‰- and 33‰-acclimated crabs, respectively) are

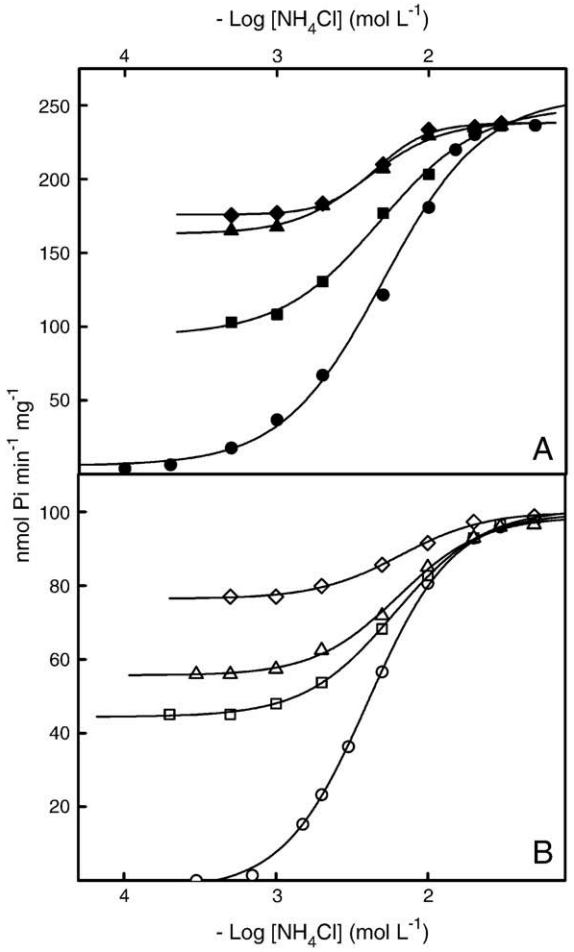


Fig. 8. Effect of potassium ion concentration on the modulation by ammonium ions of (Na^+, K^+)-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* acclimated to 21 or 33‰ salinity for 10 days. Duplicate aliquots containing 36.2 µg protein from three ($N=3$) different gill homogenates were used, and representative curves obtained from one homogenate are given. For 21‰-acclimated crabs activity was assayed in 50 mmol L^{-1} triethanolamine buffer (pH 7.5), containing 0.5 mmol L^{-1} ATP (or 1.0 mmol L^{-1} for 33‰), 0.7 mmol L^{-1} $MgCl_2$ (or 2.0 mmol L^{-1} for 33‰), and 50 mmol L^{-1} NaCl (both salinities), 1.0 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1.0 mmol L^{-1} G3P, 150 µg GAPDH (12 U) and 20 µg PGK (9 U) in a final volume of 1 mL. The modulation of enzyme activity by each effector was evaluated under optimal concentrations of the other. A – 21‰-acclimated crabs. Fixed potassium ion concentrations used were: (●) 0 mmol L^{-1}, (■) 1 mmol L^{-1}, (▲) 3 mmol L^{-1}, (♦) 5 mmol L^{-1}. B – Crabs acclimated to 33‰. Potassium ion concentrations: (○) 0 mmol L^{-1}, (□) 1 mmol L^{-1}, (△) 3 mmol L^{-1}, (◊) 10 mmol L^{-1}.

some 100 to 5000-fold (0.02 µmol L^{-1} to 1.0 µmol L^{-1}) higher than those for the vertebrate enzyme (Sweadner, 1989), they are similar to that for the enzyme from *C. sapidus* gill tissue (Neufeld et al., 1980), certain crabs (Holliday, 1985; D'Orazio and Holliday, 1985; Tentes and Stratakis, 1991; Corotto and Holliday, 1996) and other crustaceans (Horiuchi, 1997; Furriel et al., 2000). Given that in the presence of NH_4^+, the K_I values for ouabain inhibition were about 2 to 4-fold lower (44.9 ± 1.0 µmol L^{-1} and 28.8 ± 1.3 µmol L^{-1} for 21‰- and 33‰-acclimated crabs, respectively) than those in the absence of NH_4^+, a likely explanation is that the E_1–E_2 conformational equilibrium favors the ouabain-sensitive E_2 conformation. While the mechanistic basis for alterations in apparent K_I values is complex, various phosphoenzyme intermediates between E_1P and E_2P (Yoda and Yoda, 1988) and other subconformations of the E_2P phosphorylated enzyme form (Fedosova et al., 1998) may account for such alterations.

Acclimation of *C. ornatus* to 21‰ induced higher V-ATPase specific activity compared to 33‰ salinity while K^+-ATPase activity was unaltered. This finding corroborates data showing ATPase activity other than (Na^+, K^+)-ATPase in gill homogenates (Wheatly, 1999; Proverbio et al., 1991; Lovett et al., 1994; Furriel et al., 2000; Gonçalves et al., 2006), suggesting that such ouabain-insensitive activities may play a role in osmoregulation. However, the present data for salinity-acclimated *C. ornatus* contrast with those for fresh-caught *C. danae* in which total ATPase activity of the gill microsomal fraction is apparently due to (Na^+, K^+)-ATPase alone (Masui et al., 2002).

Many studies have examined the role of the crustacean gill (Na^+, K^+)-ATPase in NH_4^+ transport (Holliday, 1985; Towle et al., 1976; Towle and Weihrauch, 2001; Masui et al., 2002; Lucu and Towle, 2003; Furriel et al., 2004; Weihrauch et al., 2002, 2004; Gonçalves et al., 2006; Garçon et al., 2007; Santos et al., 2007). Compared to K^+, the stimulation by NH_4^+ of gill (Na^+, K^+)-ATPase activity in both 21‰- and 33‰-acclimated *C. ornatus* is about 30% higher and similar to that for fresh-caught *C. danae* (Masui et al., 2002). However, this stimulation is significantly lower than that for the enzyme from fresh-caught *C. ornatus* (Garçon et al., 2007) and *C. vitattus* (Gonçalves et al., 2006). The $K_{0.5}$ value for NH_4^+ stimulation of 21‰-acclimated *C. ornatus* is similar to those for the (Na^+, K^+)-ATPases of crustaceans inhabiting different biotopes (Masui et al., 2002; Furriel et al., 2004; Gonçalves et al., 2006; Garçon et al., 2007). The present data suggest that K^+ displaces NH_4^+ from its binding site, while NH_4^+ binding to the

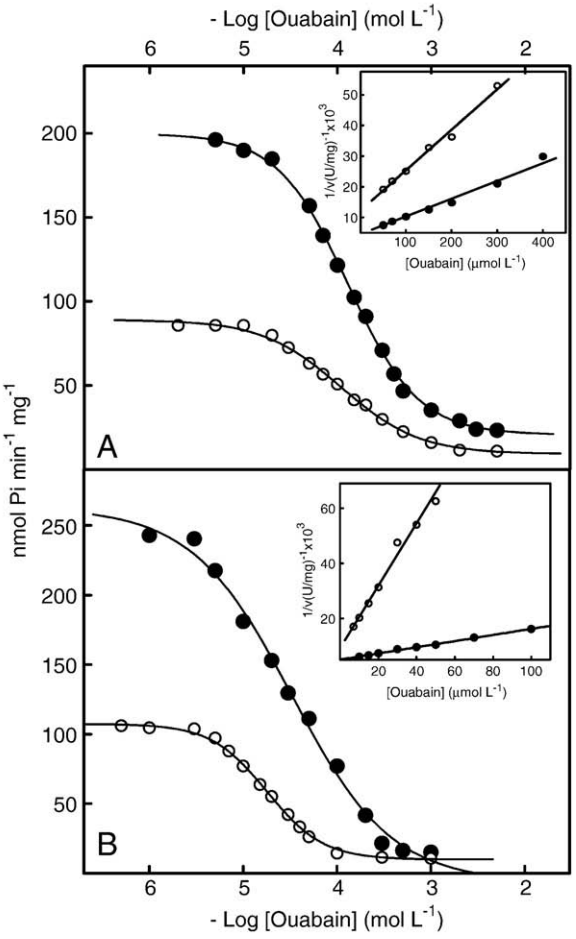


Fig. 9. Effect of ouabain on total ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* acclimated to 21 or 33‰ salinity for 10 days. Duplicate aliquots containing 36.2 µg protein from three ($N=3$) different gill homogenates were used, and representative curves obtained from one homogenate are given. ATPase activity was estimated in 50 mmol L^{-1} Hepes buffer (pH 7.5) under optimal conditions of substrate and ions in the absence (A) or presence (B) of ammonium ions using gill microsomal fractions from 21‰-acclimated (●) or 33‰-acclimated (○) crabs. Insets: Dixon plot for the estimation of K_I, the enzyme-inhibitor complex dissociation constant, in which v_c is the reaction rate corresponding to (Na^+, K^+)-ATPase activity alone.

Table 3
Effect of various inhibitors on ATPase activity in a microsomal fraction from the gill tissue from *Callinectes ornatus* acclimated to 21‰ or 33‰ salinity for 10 days.

Inhibitor	Residual activity (%)		V (nmol min^{-1} mg^{-1})	
	21‰	33‰	21‰	33‰
None (control)	100	100	207.5 ± 9.6	87.7 ± 4.3
Ouabain (3 mmol L^{-1})	11.6	12.7	24.0 ± 1.0	11.1 ± 0.5
Orthovanadate (0.1 mmol L^{-1})	11.3	12.7	23.4 ± 1.0	11.1 ± 0.5
Ouabain (3 mmol L^{-1}) + Orthovanadate (0.1 mmol L^{-1})	11.5	12.7	23.8 ± 1.1	11.1 ± 0.5
Ouabain (3 mmol L^{-1}) + Theophylline (5 mmol L^{-1})	11.3	12.7	23.4 ± 1.0	11.1 ± 0.5
Ouabain (3 mmol L^{-1}) + Ethacrynic Acid (2 mmol L^{-1})	8.8	6.8	18.3 ± 0.8	6.0 ± 0.3
Ouabain (3 mmol L^{-1}) + Oligomycin (1 μg mL^{-1})	6.9	8.5	14.3 ± 0.6	7.5 ± 0.3
Ouabain (3 mmol L^{-1}) + Thapsigargin (0.5 μmol L^{-1})	11.9	12.7	24.7 ± 1.1	11.1 ± 0.5
Ouabain (3 mmol L^{-1}) + Bafilomycin A_1 (0.4 μmol L^{-1})	8.2	11.8	17.0 ± 0.7	10.3 ± 0.5
Ouabain (3 mmol L^{-1}) + Aurovertin B (10 μmol L^{-1})	6.6	8.4	13.7 ± 0.5	7.4 ± 0.3
Ouabain (3 mmol L^{-1}) + Concanamycin A (1.0 μmol L^{-1})	9.4	11.0	19.5 ± 0.9	9.7 ± 0.4
Ouabain (3 mmol L^{-1}) + EGTA (1.0 mmol L^{-1})	11.6	12.7	24.0 ± 1.0	11.1 ± 0.5

Assays were performed using 36.2 μg protein in 50 mmol L^{-1} Hepes (or triethanolamine) buffer, pH 7.5, containing 0.5 mmol L^{-1} ATP (or 1.0 mmol L^{-1} for 33‰), 0.7 mmol L^{-1} $MgCl_2$ (or 2.0 mmol L^{-1} for 33‰), 5 mmol L^{-1} KCl (10 mmol L^{-1} for 33‰ salinity) and 50 mmol L^{-1} NaCl (both salinities), in a final volume of 1.0 mL. Data are the mean ± SD from three ($N = 3$) different microsomal preparations. All means for ATPase activity (V) between 33‰- and 21‰-acclimated crabs for control conditions (no inhibitor) and for each inhibitor/combination tested are significantly different (two way ANOVA [$P < 0.001$], Student–Newman–Keuls multiple means comparison [$P < 0.05$]).

newly exposed sites apparently induces conformational changes that increase enzyme affinity for K^+. As the enzyme becomes fully saturated with NH_4^+ (50 mmol L^{-1}), K^+ does not displace NH_4^+ in the range from 10^{-6} to 10^{-2} mol L^{-1} K^+ as already shown in *C. vitattus* (Gonçalves et al., 2006) and fresh-caught *C. ornatus* (Garçon et al., 2007), although in contrast to *C. danae* (Masui et al., 2002) and *M. amazonicum* (Santos et al., 2007) in which NH_4^+ and K^+ bind to different sites.

The capability of aquatic crustaceans to excrete ammonia across their gill epithelia is well known (Cameron and Batterton, 1978; Kormanik and Cameron, 1981; Regnault, 1987; Weihrauch et al., 1998; 1999; 2004) and ammonia diffusion across epithelial cells together with active NH_4^+ transport by the (Na^+, K^+)-ATPase has been amply discussed (Péqueux, 1995; Lucu and Towle 2003; Weihrauch et al., 2004; Kirschner, 2004; Leone et al., 2005b; Gonçalves et al., 2006; Garçon et al., 2007; Santos et al., 2007). However, cation and NH_4^+ fluxes across the gill cuticle of *C. maenas* are dose-dependently inhibited by amiloride (Onken and Riestenpatt, 2002; Weihrauch et al., 2002). Thus, earlier findings on apical, amiloride inhibitable, Na^+-dependent transport in intact or perfused gills should be interpreted cautiously since Na^+ absorption and ammonia excretion may not be directly correlated given this limiting cuticular component, particularly relevant to NH_4^+ excretion (Weihrauch et al., 2004).

The synergistic stimulation by K^+ and NH_4^+ of gill (Na^+, K^+)-ATPase activity in 21‰-acclimated *C. ornatus*, also seen for fresh-caught *C. danae* (Masui et al., 2002) and other crustaceans (Gonçalves et al., 2006; Garçon et al., 2007; Santos et al., 2007) suggests a significant role for the gill (Na^+, K^+)-ATPase in nitrogen excretion. Our findings suggest that different mechanisms stimulating (Na^+, K^+)-ATPase activity by K^+ and NH_4^+ might underlie such active nitrogen excretion. Stimulation of (Na^+, K^+)-ATPase activity in 33‰- and 21‰-acclimated *C. ornatus* by K^+ in the presence of NH_4^+ resulted in 5-fold, 62-fold and 37-fold increases in apparent affinity of the enzyme for K^+ compared to fresh-caught *C. ornatus* (Garçon et al., 2007) and *C. danae* (Masui et al., 2002) and 15‰-acclimated *C. danae* (Masui et al., 2009), respectively. Synergistic stimulation of the *C. danae* gill (Na^+, K^+)-ATPase activity by K^+ and NH_4^+ led us to complement the current model for ammonia excretion by the gill epithelia (Weihrauch et al., 2004) with an additional (Na^+, K^+)-ATPase pumping activity stimulated by NH_4^+ and inhibited by Mg^{2+} (Masui et al., 2005).

Finally, as a benthic crab, *C. ornatus* buries in bottom sediment (Mantelatto and Fransozo, 1999) and may be subject to elevated ammonia concentrations of at least 3 mmol L^{-1} (Weihrauch et al., 1999; Rebelo et al., 1999). Considering the elevated ammonia permeability of gills in estuarine and marine crustaceans (Weihrauch et al., 1999, 2004), this exposure may lead to increased hemolymph ammonia as a consequence of ammonia influx, possibly attaining lethal levels (Rebelo et al., 1999; Masui et al., 2002, 2003). However, the presence of an efficient NH_4^+ excretion mechanism by the (Na^+, K^+)-ATPase, directly modulated by excess NH_4^+, may reduce hemolymph ammonia contributing to nitrogen homeostasis in burrowing, shallow-water benthic crustaceans. The present data corroborate our previous studies suggesting a role for the (Na^+, K^+)-ATPase in ammonium excretion (Furriel et al., 2004; Masui et al., 2005; Garçon et al., 2007; Gonçalves et al., 2006).

Concluding, NH_4^+ excretion is vital to the aquatic Crustacea, and our findings suggest that the gill (Na^+, K^+)-ATPase participates in species-specific mechanisms of NH_4^+ excretion that have evolved in response to particular environmental conditions. However, the dramatic, salinity-induced increase in enzyme affinity for K^+ via putative alterations in membrane composition remains to be elucidated.

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