J Membrane Biol (2011) 244:9–20
DOI 10.1007/s00232-011-9391-5

ORIGINAL ARTICLES

Na^+,K^+-ATPase Activity in the Posterior Gills of the Blue Crab, *Callinectes ornatus* (Decapoda, Brachyura): Modulation of ATP Hydrolysis by the Biogenic Amines Spermidine and Spermine

Daniela P. Garçon · Malson N. Lucena · Juliana L. França · John C. McNamara · Carlos F. L. Fontes · Francisco A. Leone

Received: 13 May 2011 / Accepted: 11 August 2011 / Published online: 5 October 2011

Abstract We investigated the effect of the exogenous polyamines spermine, spermidine and putrescine on modulation by ATP, K^+, Na^+, NH_4^+ and Mg^{2+} and on inhibition by ouabain of posterior gill microsomal Na^+,K^+-ATPase activity in the blue crab, *Callinectes ornatus*, acclimated to a dilute medium (21‰ salinity). This is the first kinetic demonstration of competition between spermine and spermidine for the cation sites of a crustacean Na^+,K^+-ATPase. Polyamine inhibition is enhanced at low cation concentrations: spermidine almost completely inhibited total ATPase activity, while spermine inhibition attained 58%; putrescine had a negligible effect on Na^+,K^+-ATPase activity. Spermine and spermidine affected both *V* and *K* for ATP hydrolysis but did not affect ouabain-insensitive ATPase activity. ATP hydrolysis in the absence of spermine and spermidine obeyed Michaelis–Menten behavior, in contrast to the cooperative kinetics seen for both polyamines. Modulation of *V* and *K* by K^+, Na^+, NH_4^+ and Mg^{2+} varied considerably in the presence of spermine and spermidine. These findings suggest that polyamine inhibition of Na^+,K^+-ATPase activity may be of physiological relevance to crustaceans that occupy habitats of variable salinity.

Keywords Na^+,K^+-ATPase modulation · Posterior gill · Polyamine · *Callinectes ornatus* · Kinetic analysis

D. P. Garçon · F. A. Leone (✉)
Departamento de Química, Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo, Ribeirão Preto, Avenida Bandeirantes, 3900, Ribeirão Preto, SP 14040-901, Brazil
e-mail: fdaleone@ffclrp.usp.br

M. N. Lucena · J. L. França
Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, SP 14040-901, Brazil

J. C. McNamara
Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo, Ribeirão Preto, SP, Brazil

C. F. L. Fontes
Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil

Introduction

The gills of euryhaline crustaceans provide various physiological and biochemical capabilities that aid in exploiting habitats of variable salinity, among them the ability to hyperregulate hemolymph osmotic and ionic concentrations, hemolymph pH regulation (Mantel and Farmer 1983; Péqueux 1995) and ammonia excretion (Péqueux 1995; Weihrauch et al. 2004). In crabs, the anterior gill lamellae exhibit thin epithelia and are specialized in gas exchange. The posterior gill lamellae are characterized by thick ionocytes that show extensive membrane foldings associated with mitochondria and are involved in active ion uptake from dilute external media (for review, see Péqueux 1995; Towle and Weihrauch 2001; Freire et al. 2008). The anterior and posterior gills may also harbor different Na^+,K^+-ATPase isoforms that exhibit different kinetic properties (Jayasundara et al. 2007).

Euryhaline crabs may be classified generally as "strong" or "weak" hyperosmoregulators (Kirschner 2004). Strong regulators spend most of their life cycle in freshwater; weak regulators inhabit dilute media but cannot survive long in freshwater. Briefly, Na^+ uptake across the gill epithelium to the hemolymph of strong hyperosmoregulators proceeds through apical amiloride-sensitive

Na^+ channels and a basally located Na^+,K^+-ATPase. Cl^- absorption via apical Cl^-/HCO_3^- exchangers and basal Cl^- channels is driven by a V-type H^+ pump that hyperpolarizes the cells causing trans-apical Na^+ entry and Cl^- exit across the basal membrane (Onken and Riestenpatt 1998). In the gills of weak hyperosmoregulators from dilute media, coupled NaCl absorption depends on an apical $Na^+/K^+/2Cl^-$ cotransporter, K^+ channels and Cl^-/HCO_3^- and Na^+/H^+ exchangers, together with the basally located Na^+,K^+-ATPase and K^+ and Cl^- channels (for review, see Towle and Weihrauch 2001; Freire et al. 2008). In both cases, hydration of CO_2 by cytoplasmic carbonic anhydrase provides the H^+ and HCO_3^- counter-ions. Na^+,K^+-ATPase-specific activity increases in euryhaline crabs acclimated to dilute media (Lucu and Towle 2003; Genovese et al. 2004; Mañanes et al. 2002; Garçon et al. 2009) and may result from augmented enzyme activity and/or de novo synthesis of pump protein (Lucu and Flik 1999; Lucu and Towle 2003; Masui et al. 2005; Luquet et al. 2005; Garçon et al. 2009).

The sodium–potassium ATPase (E.C. 3.6.1.37) is a ubiquitous, ATP-powered ion pump that establishes strong Na^+ and K^+ concentration gradients across the plasma membrane by exchanging cytoplasmic Na^+ for K^+ from the extracellular fluid (Glynn 1993; Kaplan 2002). These gradients are used in generating membrane electrical potential, cell volume regulation, active transepithelial salt and passive water movement as well as Na^+/glucose/amino acid/nucleotide cotransport (Jorgensen and Pedersen 2001; Martin 2005; Schoner and Scheiner-Bobis 2007). Na^+,K^+-ATPase is a P_{2c}-type ATPase that forms a phosphorylated intermediate by transfer of the γ-phosphate from ATP to a conserved aspartate residue in the α-chain P-domain (Axelsen and Palmgren 1998; Pedersen 2007). Conformational changes of the E_1P to the E_2P state lead to the active extrusion of bound Na^+; on K^+ binding, dephosphorylation of E_2P is stimulated. Dissociation and release of the phosphate together with ATP binding at regulatory sites stimulates K^+ deocclusion and release, modifying the enzyme from the E_2 to the E_1 state, completing the catalytic cycle (Jorgensen and Pedersen 2001).

The biologically active polyamines putrescine, spermidine and spermine are ubiquitous, small, positively charged molecules that are widespread among all prokaryotic and eukaryotic cells and directly or indirectly essential for normal cellular function and proliferation (for review, see Kalac 2009; Iarashi and Kashiwagi 2010; Pegg 2009). The diamine putrescine is the precursor of the triamine spermidine and the tetramine spermine (Jantaro et al. 2003; Pegg 2009). Polyamines are closely associated with phospholipids (Toner et al. 1988) and negatively charged proteins through cytoplasmic amino-terminal domain glutamate residues (Lin et al. 2006; Tassoni et al. 1996). Besides increased ornithine decarboxylase levels, increased polyamine content constitutes one of the earliest signs of cell replication since these polycations bind to DNA molecules with high affinity, regulating DNA synthesis (Wu et al. 2007). Spermine and spermidine increase notably in plant cells under salt stress and appear to be associated with mechanisms of salt tolerance (Krishnamurthy and Bhagwat 1989).

Some information on polyamines in crustaceans is available. In *Artemia* sp. nauplii, putrescine increases with decreasing external salinity (Watts et al. 1994). Putrescine levels increase in gill pairs 6 and 7 on exposure of *Callinectes sapidus* to seawater (35‰ salinity), while spermidine increases in 10‰-acclimated *C. sapidus* (Lovett and Watts 1995). Na^+,K^+-ATPase activity also decreases with increased polyamine level (Lee and Watts 1994), and increasing putrescine, spermine and spermidine concentrations inhibited *Callinectes danae* gill Na^+,K^+-ATPase by 40% (Silva et al. 2008). At low Na^+ concentrations, spermine and spermidine block Na^+,K-ATPase ATP-driven phosphorylation, while at 100 mmol Na^+ l^{-1} the phosphorylated E-P intermediate increases markedly (Silva et al. 2008). Exogenously added polyamines affect the synergistic stimulation by both NH_4^+ and K^+ of microsomal *C. danae* gill Na^+,K^+-ATPase activity (Silva et al. 2008). Anterior and posterior gills also exhibit differences in polyamine concentration (Péqueux et al. 2002).

Callinectes ornatus Ordway 1863 is a euryhaline, brachyuran swimming crab, widely 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 a scavenger and predator and as a food resource for other aquatic organisms and shorebirds (Haefner 1990). In Ubatuba Bay, Brazil, *C. ornatus* constitutes about 60% of all brachyuran species, participating in the feeding dynamics of the shallow-water megabenthos (Mantelatto and Fransozo 2000); its life cycle and osmotic and ionic regulatory abilities are not well known. While C. *ornatus* occurs in dilute media (9‰ salinity) (Brues 1927), the crab is usually found at 28–35‰ salinity (Mantelatto and Fransozo 1999), buried in sand, mud or shell-covered sediments and ranging 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). NH_4^+ concentration in unpolluted seawater does not exceed 5–7 μM (Weihrauch et al. 1999), but benthic crabs may encounter ambient ammonia concentrations above their normally low NH_4^+ hemolymph concentrations, leading to passive NH_4^+ influx (Masui et al. 2002, 2005). *C. ornatus* can be encountered in sediments that may contain ammonia

concentrations as high as 2–3 mM (Rebelo et al. 1999). Thus, like *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).

Despite the plethora of information showing that salt and osmotic stress can affect polyamine concentration in crustacean gill tissue, information is scarce as to a putative role in modulating Na^+,K^+-ATPase activity. Here, we examine the effect of putrescine, spermine and spermidine on the modulation by ATP, Na^+, K^+, NH_4^+ and Mg^{2+} on *C. ornatus* gill Na^+,K^+-ATPase activity.

Materials and Methods

Materials

All solutions were prepared using Millipore (Bedford, MA) MilliQ ultrapure, apyrogenic water; and all reagents were of the highest purity commercially available. Dithiothreitol, 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), ouabain, alamethicin, phosphoglycerate kinase (PGK), 3-phosphoglyceraldehyde diethyl acetal and ATP ditris salt were purchased from Sigma (St. Louis, MO). The protease inhibitor cocktail was from Calbiochem (Darmstadt, Germany). Triethanolamine and HCl were from Merck (Darmstadt, Germany).

Reagents

Glyceraldehyde-3-phosphate (G3P) was prepared by hydrolysis of the barium salt of 3-phospho-glyceraldehyde diethyl acetal with 150 µl HCl ($d = 1.18$ g/ml) in a boiling-water bath for 2 min, after removal of the barium salt with Dowex $50H^+$ resin, as recommended by the manufacturer (see Sigma product information for product number G5376). Final pH was adjusted to 7.0 with 50 µl triethanolamine just before use.

Depletion of Ammonium Ions from Enzyme Suspensions

Crystalline suspensions of LDH and PK were centrifuged at $20{,}000\times g$ for 15 min, at 4°C, in an Eppendorf (Hamburg, Germany) model 5810 refrigerated centrifuge. The pellet was resuspended in 300 µl 50 mM HEPES buffer (pH 7.5), transferred to a YM-10 Microcon filter (Millipore) and centrifuged five times in the same buffer until completely depleted of ammonium ions (tested with Nessler reagent). Finally, the pellet was resuspended in the original volume. For PGK and GAPDH, the suspension was treated as above with 50 mM triethanolamine buffer (pH 7.5) containing 1 mM dithiothreitol. When necessary, enzyme solutions were concentrated using YM-10 Microcon filters.

Gill Excision

Adult intermolt specimens of *C. ornatus* were collected using double rig trawl nets from Ubatuba Bay, São Paulo, Brazil. Crabs were transported to the laboratory in seawater from the collection site (34‰ salinity), where they were maintained in tanks containing 32 l aerated seawater (33‰ salinity, 25°C) for 2 days. To stimulate gill Na^+,K^+-ATPase activity, the crabs were then acclimated to 21‰ salinity for up to 10 days, in groups of eight each, and fed on alternate days with shrimp tails. For each homogenate prepared, crabs were anesthetized by chilling on crushed ice for 5 min. The carapace was quickly removed, the crabs were killed by destroying the cerebral and ventral ganglionic masses and posterior gill pairs 6, 7 and 8 were excised and homogenized in 10 ml of ice-cold homogenization buffer, consisting of 20 mM imidazole (pH 6.8) plus 250 mM sucrose, 6 mM EDTA and a proteinase inhibitor cocktail (1 mM benzamidine, 5 µM antipain, 5 µM leupeptin, 1 µM pepstatin A and 5 µM phenylmethylsulfonyl fluoride).

Preparation of the Gill Microsomal Fraction

Gills were immediately diced with small scissors and homogenized in homogenization buffer (20 ml/g wet tissue). After centrifugation of the crude homogenate at $20{,}000\times g$ for 35 min at 4°C, the supernatant was removed and held in an ice bath; the pellet was resuspended in an equal volume of homogenization buffer, and after further centrifugation as above, the two supernatants were pooled and centrifuged at $100{,}000\times g$ for 2 h at 4°C. The pellet was then homogenized in 20 mM imidazole buffer (pH 6.8) containing 250 mM sucrose (8 ml buffer/g wet tissue). Finally, 0.5-ml aliquots were frozen in liquid nitrogen and stored at −20°C until measurement of ATPase activity; no appreciable loss of activity was observed even after 3 months' storage. When required, aliquots were thawed, placed on crushed ice and used immediately.

Steady-State Measurement of ATPase Activity

ATPase activity was assayed at 25°C using a PK/LDH coupling system described by Rudolph et al. (1979) in which ATP hydrolysis was coupled to NADH oxidation

according to Garçon et al. (2009). NADH oxidation was monitored at 340 nm ($\varepsilon_{340\ nm}$, pH 7.5, 6,200 M^{-1} cm^{-1}) in a Hitachi (Tokyo, Japan) U-3000 spectrophotometer equipped with thermostatted cell holders. Standard conditions for estimation of enzyme activity in the gill homogenates were as follows: 50 mM HEPES buffer (pH 7.5) containing 0.2 mM ATP, 0.7 mM $MgCl_2$, 5 mM KCl and 50 mM NaCl; 0.14 mM NADH; 2.0 mM PEP; 82 μg PK (49 U); and 110 μg LDH (94 U) in a final volume of 1.0 ml. To examine modulation of the enzyme by K^+, we also used a GAPDH/PGK coupling system in which ATP hydrolysis was coupled to the reduction of NAD^+ at 340 nm according to Garçon et al. (2009) since neither enzyme is K^+-dependent. For the GAPDH/PGK linked system, standard conditions for estimation of enzyme activity in gill homogenates were as follows: 50 mM triethanolamine buffer (pH 7.5) containing 0.2 mM ATP, 0.7 mM $MgCl_2$, 5 mM KCl and 50 mM NaCl; 1.0 mM NAD^+; 0.5 mM sodium phosphate; 1.0 mM G3P; 150 μg GAPDH (12 U); and 20 μg PGK (9 U) in a final volume of 1.0 ml. The two coupling systems gave equivalent results with a difference of <10%. ATPase activity was also assayed after 10 min preincubation at 25°C in alamethicin (1 mg/mg protein), as a control for leaky and/or disrupted vesicles. The initial velocities were constant for at least 15 min provided that <5% NADH (NAD^+) was oxidized (reduced); such measurements are considered to have been obtained under steady-state conditions. For each microsomal preparation, assay linearity was checked using samples containing 5–50 μg protein, and total microsomal protein added to the cuvette always fell well within the linear range of the assay. Neither NADH, PEP, LDH, PK, NAD^+, G3P, PGK nor GAPDH was rate-limiting over the initial course of the assay; and no activity could be measured in the absence of NADH or NAD^+. Controls without added enzyme were also included in each experiment to quantify nonenzymatic substrate hydrolysis. 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. The figures show a representative curve from a single experiment. ATP hydrolysis was also estimated in the presence of 3 mM ouabain to evaluate 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. The effect of exogenous polyamines on Na^+,K^+-ATPase activity was examined as above, the enzyme being preincubated at 25°C for 10 min with increasing concentrations of spermine (from 1 μM to 10 mM) or spermidine (from 0.5 mM to 10 mM). One enzyme unit (U) is defined as the amount of enzyme that hydrolyzes 1.0 nmol of ATP per minute at 25°C, and specific activity is given as nanomoles per minute per milligram protein.

Measurement of Protein

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

Estimation of Kinetic Parameters

The kinetic parameters V_m (maximum velocity), $K_{0.5}$ (apparent dissociation constant of the enzyme–modulator complex), K_M (Michaelis–Menten constant) and the n_H value (Hill coefficient) for ATP hydrolysis were calculated using SigrafW software (Leone et al. 2005). 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 Marks and Seeds (1978). The kinetic parameters V_m, K_M and $K_{0.5}$ are calculated values and are given as the mean ± SD from three different microsomal preparations. Data were analyzed using a one-way analysis of variance (inhibitor), followed by Student-Newman–Keuls multiple means testing. Effects and differences were considered significant at $P \leq 0.05$. SigrafW software can be obtained from http://portal.ffclrp.usp.br/sites/fdaleone/downloads.

Results

Effect of Polyamines on Total ATPase Activity

Figure 1 shows the effect of spermidine and spermine on total ATPase activity of *C. ornatus* gill microsomal preparations with and without ouabain. As spermidine concentration increased from 5×10^{-4} to 5×10^{-2} M, total ATPase activity decreased from 207.0 ± 6.2 to less than 10.3 ± 0.4 nmol Pi/min/mg (Fig. 1a). Spermine was less effective, inhibiting the total ATPase activity (120.1 ± 4.8 nmol Pi/min/mg) by 57.9% over the concentration range 10^{-6}–10^{-2} M (Fig. 1b). Putrescine had a negligible effect on Na^+,K^+-ATPase activity (not shown). Ouabain-insensitive ATPase activity varied little (around 10 nmol Pi/min/mg) over the same concentration range, suggesting that only Na^+,K^+-ATPase activity is affected.

Effect of Spermine and Spermidine on ATP Hydrolysis by Na^+,K^+-ATPase

The modulation by ATP of Na^+,K^+-ATPase activity is also affected by polyamines (Fig. 2). At 10 mmol l^{-1}

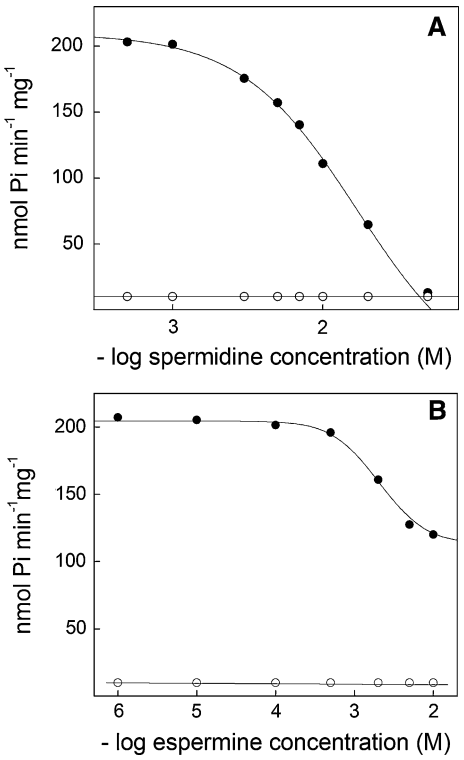

Fig. 1 Effect of spermidine and spermine on total ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus*. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-µg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction. *Filled circle* without ouabain; *open circle* with ouabain

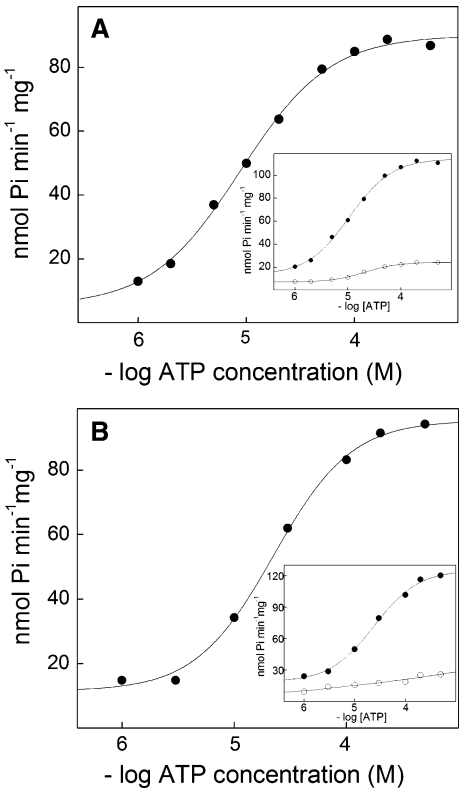

Fig. 2 Effect of ATP concentration on Na^+,K^+-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* in the presence of spermidine or spermine. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-µg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction: **a** 10 mM spermidine, **b** 10 mM spermine. *Insets filled circle* total ATPase activity; *open circle* ouabain-insensitive ATPase activity

spermidine, stimulation by ATP of Na^+,K^+-ATPase activity was considerably inhibited (Fig. 2a). As ATP concentration increased from 10^{-6} to 5×10^{-4} M, a maximum hydrolysis rate of 88.5 ± 3.0 nmol Pi/min/mg was estimated in contrast to 187.7 ± 7.1 nmol Pi/min/mg without spermidine (Table 1). At 10 mM spermine, a maximum rate of 94.3 ± 4.1 nmol Pi/min/mg was estimated over the same ATP concentration range (Fig. 2b). The figure insets show variation in total ATPase and ouabain-insensitive ATPase activities. Maximum rates for ouabain-insensitive ATPase activity of about 25 and 22 nmol Pi/min/mg were estimated in the presence and absence, respectively, of spermine and spermidine, again suggesting that these polyamines affect only the gill Na^+,K^+-ATPase activity.

Effect of Spermine and Spermidine on Modulation by Mg^{2+} of Na^+,K^+-ATPase Activity

The modulation by Mg^{2+} of gill tissue Na^+,K^+-ATPase activity was also affected by spermine and spermidine (Fig. 3). At 10 mM spermidine, the stimulation by Mg^{2+} of Na^+,K^+-ATPase activity (Fig. 3a) was only 46.5% (85.4 ± 2.8 nmol Pi/min/mg) compared to that without spermidine (183.5 ± 6.7 nmol Pi/min/mg) (Table 1). A maximum rate of 94.7 ± 4.4 nmol Pi/min/mg was estimated with 10 mM spermine (Fig. 3b). Insets show that total ATPase and ouabain-insensitive ATPase activities vary as a function of increased Mg^{2+} concentration in the presence of polyamines. The maximum rates of around 25 nmol Pi/min/mg suggest that spermine and spermidine do not affect the activity of Mg^{2+}-stimulated ATPases other than Na^+,K^+-ATPase.

Effect of Spermine and Spermidine on Modulation by K^+ of Na^+, K^+-ATPase Activity

Figure 4 shows stimulation of Na^+,K^+-ATPase by K^+ in the presence of the polyamines. As polyamine concentration

Table 1 Kinetic parameters for the stimulation by ATP, Mg^{2+}, Na^+, K^+ and NH_4^+ and inhibition by ouabain of Na^+,K^+-ATPase activity in posterior gill tissue of *C. ornatus* in the absence (control) or presence of spermine or spermidine

Effector	V_m (nmol/min/mg)			K_M or $K_{0.5}$ (μM)			n_H		
	Control	Spermine	Spermidine	Control	Spermine	Spermidine	Control	Spermine	Spermidine
ATP	187.7 ± 7.1	94.3 ± 4.1*	88.5 ± 3.0*	50.0 ± 2.5	23.0 ± 1.0*	10.5 ± 0.4*	1.0	1.4	1.3
Mg^{2+}	183.5 ± 6.7	94.7 ± 4.4*	85.4 ± 2.8*	266.0 ± 10.1	230.0 ± 8.7*	230.0 ± 8.5*	5.8	4.2	4.8
Na^+	175.9 ± 6.3	97.5 ± 4.6*	89.1 ± 3.1*	4180.0 ± 152.0	12300.0 ± 430.1*	13800.0 ± 523.3*	1.5	4.2	2.8
K^+	181.8 ± 6.9	97.5 ± 4.8*	91.0 ± 3.3*	890.4 ± 35.6	1670.0 ± 66.0*	1070.0 ± 42.9*	2.5	1.82	2.5
NH_4^+	240.7 ± 11.2	112.3 ± 5.2*	77.8 ± 2.3*	4440.0 ± 170.0	11000.0 ± 330.0*	7300.0 ± 219.0*	1.4	1.7	1.9
				K_I (μM)					
Ouabain				110.0 ± 2.6	190.8 ± 9.2*	115.0 ± 5.2			

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 in the absence (control) or presence of 10 mmol l^{-1} spermine or spermidine. Data are the mean ± SD from three different microsomal preparations

* $P \leq 0.05$ compared to control value

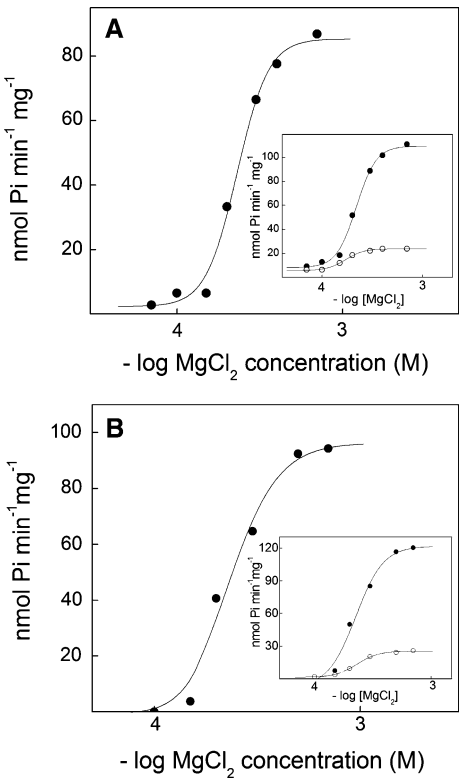

Fig. 3 Effect of magnesium concentration on Na^+,K^+-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* in the presence of spermidine or spermine. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-μg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction: **a** 10 mM spermidine, **b** 10 mM spermine. *Insets filled circle* total ATPase activity; *open circle* ouabain-insensitive ATPase activity

increased from 10^{-4} to 10^{-2} M, the maximum rates reached were 97.5 ± 4.8 (Fig. 4a) and 91.0 ± 3.3 nmol Pi/min/mg (Fig. 4b) for spermine and spermidine, respectively, corresponding to about 50–53% of the maximum rate estimated in their absence (181.8 ± 6.9 nmol Pi/min/mg, Table 1). Although increasing K^+ concentration resulted in a single saturation curve, effects were more evident in the case of spermine (Fig. 4a). A basal activity of around 12 nmol Pi/min/mg was estimated for spermine concentrations as low as 10^{-4} M. Figure insets show the variation in total ATPase and ouabain-insensitive ATPase activities as a function of increasing K^+ concentration. Maximum rates of around 23 nmol Pi/min/mg suggest that spermine and spermidine do not affect the activity of potassium-stimulated ATPases other than Na^+,K^+-ATPase.

Effect of Spermine and Spermidine on Modulation by Na^+ of Na^+,K^+-ATPase Activity

The stimulation by Na^+ of gill Na^+,K^+-ATPase in the presence of spermine and spermidine (Fig. 5) also resulted in maximum rates around 50–55% less (89.1 ± 3.1 and 97.5 ± 4.6 nmol Pi/min/mg for spermidine and spermine, respectively) than that in the absence of these polyamines (175.9 ± 6.3 nmol Pi/min/mg, Table 1). Na^+-stimulated, ouabain-insensitive ATPase activity was not observed (insets in Fig. 5).

Effect of Spermine and Spermidine on the Modulation by NH_4^+ of Na^+,K^+-ATPase Activity

The modulation by NH_4^+ of Na^+,K^+-ATPase was also affected by the presence of spermine and spermidine (Fig. 6). Maximum rates of 77.8 ± 2.3 (Fig. 6a) and 112.3 ± 5.2 nmol Pi/min/mg (Fig. 6b), estimated with spermidine and spermine, respectively, are 32 and 46% lower than for control conditions (240.7 ± 11.2 nmol Pi/min/mg) (Table 1). The figure insets show the variation

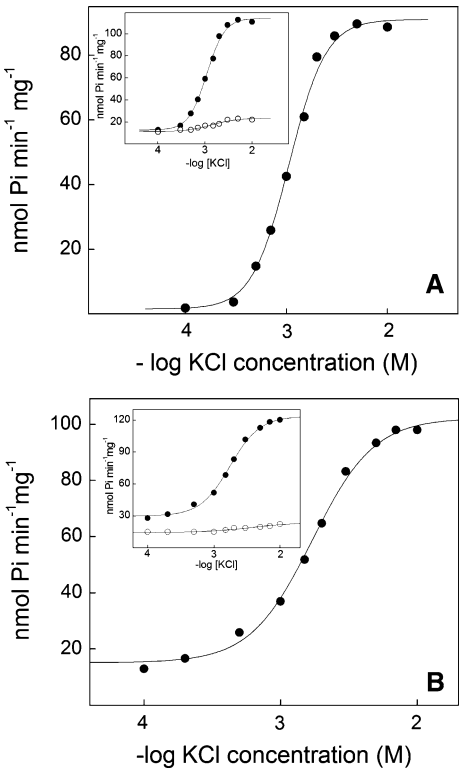

Fig. 4 Effect of potassium concentration on Na^+,K^+-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* in the presence of spermidine or spermine. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-μg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction: **a** 10 mM spermidine, **b** 10 mM spermine. *Insets filled circle* total ATPase activity; *open circle* ouabain-insensitive ATPase activity

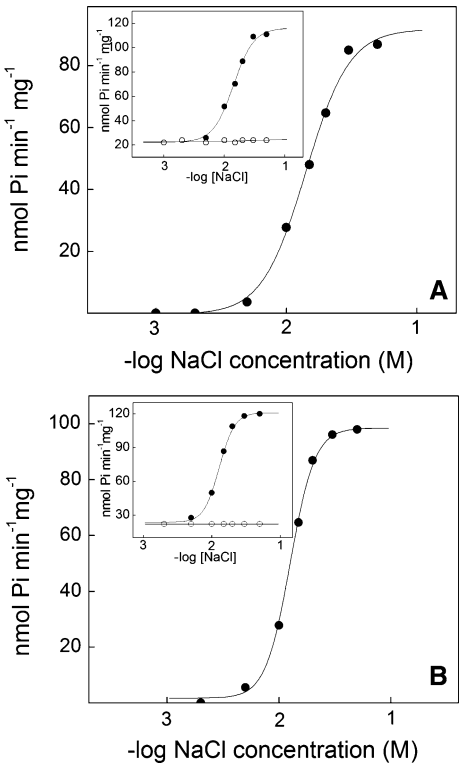

Fig. 5 Effect of sodium concentration on Na^+,K^+-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* in the presence of spermidine or spermine. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-μg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction: **a** 10 mM spermidine, **b** 10 mM spermine. *Insets filled circle* total ATPase activity; *open circle* ouabain-insensitive ATPase activity

in total ATPase and ouabain-insensitive ATPase activities as a function of increasing NH_4^+ concentration. Maximum rates of around 37 nmol Pi/min/mg were very similar to controls. However, the inhibition by excess NH_4^+ of the ouabain-insensitive ATPase activity was not seen in the presence of spermine or spermidine.

Effect of Spermine and Spermidine on Ouabain Inhibition of Na^+,K^+-ATPase Activity

Ouabain inhibition was also affected by the polyamines (Fig. 7). At 10 mM spermidine (Fig. 7a), total ATPase activity decreased from 110.9 ± 3.3 to 24.0 ± 0.7 nmol Pi/min/mg over the range 10^{-5}–5 × 10^{-3} M ouabain. For spermine, activity decreased from 120.1 ± 4.0 to 28.6 ± 1.0 nmol Pi/min/mg. The simple saturation kinetics suggests the involvement of a single ouabain-binding site, and the calculated K_I for ouabain in the presence of spermidine was 115.0 ± 5.2 μM, 66% lower (190.8 ± 9.2 μM) than that for spermine (Fig. 7b). The figure insets show the Dixon plots employed to estimate K_I.

Details of the kinetic parameters estimated for the modulation by ATP, Mg^{2+}, K^+, Na^+ and NH_4^+ and inhibition by ouabain are given in Table 1. In the absence of polyamines, ATP hydrolysis follows Michaelis–Menten kinetics, contrasting with the cooperative kinetics obeyed in the presence of spermine and spermidine. The calculated affinity for K^+ is higher in the presence of spermidine ($K_{0.5}$ = 1070.0 ± 42.9 μM) compared to that for spermine ($K_{0.5}$ = 1670.0 ± 66.0 μM), although both are higher than the control ($K_{0.5}$ = 890.4 ± 35.6 μM), suggesting that polyamines also affect $K_{0.5}$. Enzyme affinity for Na^+ was threefold lower ($K_{0.5}$ = 12300.0 ± 430.1 μM and $K_{0.5}$ = 13800.0 ± 523.3 μM for spermine and spermidine, respectively) when compared to that for the control ($K_{0.5}$ = 4180.0 ± 152.0 μmol l^{-1}). Na^+,K^+-ATPase affinity for NH_4^+ also decreased in the presence of spermidine

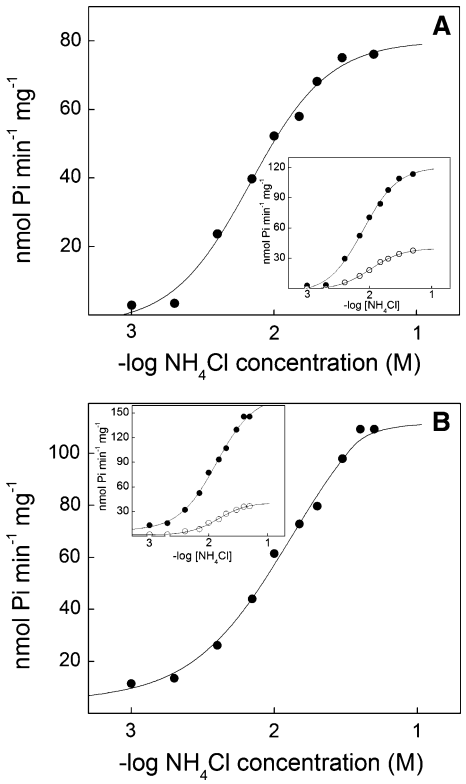

Fig. 6 Effect of ammonium concentration on Na^+,K^+-ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* in the presence of spermidine or spermine. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-µg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction: **a** 10 mM spermidine, **b** 10 mM spermine. *Insets filled circle*, total ATPase activity; *open circle* ouabain-insensitive ATPase activity

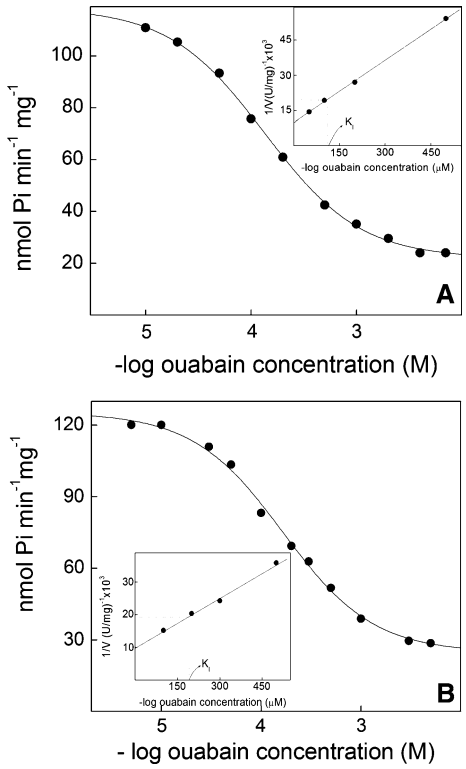

Fig. 7 Effect of ouabain on total ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus* in the presence of spermidine or spermine. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-µg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction: **a** 10 mM spermidine, **b** 10 mM spermine. *Insets* Dixon plot, showing only the ATP infinite saturation concentration (3 mM) curve, for the estimation of K_I, the enzyme–inhibitor complex dissociation constant. v_c represents the velocity corrected by subtraction of ouabain-insensitive ATPase activity at each ouabain concentration, where a plateau of inhibition has been reached

($K_{0.5}$ = 7300 ± 219.0 µM) and spermine ($K_{0.5}$ = 11000.0 ± 330.0 µM) when compared to the control ($K_{0.5}$ = 4440.0 ± 170.0 µM).

Effect of Spermine and Spermidine on the Modulation by Both K^+ and Na^+ of Na^+,K^+-ATPase Activity

Figure 8 shows the effect of the polyamines on total ATPase activity under suboptimal concentrations of K^+, Na^+ or both (Fig. 8). At 5 mmol l^{-1} Na^+ (suboptimal concentration) and 5 mmol l^{-1} K^+ (optimal concentration), maximum rates of 129.8 ± 5.2 and 122.8 ± 4.8 nmol Pi/min/mg were estimated for spermidine and spermine, respectively (Fig. 8a, d), in contrast to maximum rates of 207.0 ± 6.2 nmol Pi/min/mg in their absence (Fig. 1). At these concentrations, the apparent affinity of the enzyme for spermidine was almost unchanged at around 5 × 10^{-4} M, while that for spermine increased considerably from around 7 × 10^{-3} mol l^{-1} to around 5 × 10^{-4} mol l^{-1}. In the presence of 0.5 mM K^+ (suboptimal concentration) and 50 mM Na^+ (optimal concentration), maximum rates of 71.3 ± 2.8 and 60.0 ± 2.4 nmol Pi/min/mg were estimated for spermidine and spermine, respectively (Fig. 8b, e); the apparent affinity of the enzyme for the polyamines was around 5 × 10^{-4} M. In the presence of suboptimal concentrations of both Na^+ and K^+ (5 and 0.5 mM for Na^+ and K^+, respectively), maximum rates of 48.9 ± 1.9 and 48.0 ± 1.9 nmol Pi/min/mg were estimated for spermidine and spermine, respectively (Fig. 8c, f). There was a considerable increase in the apparent affinity of the enzyme to around 5 × 10^{-6} M for both polyamines. These findings suggest that spermine and spermidine compete for the cation binding sites on the enzyme molecule, affecting both V_m and $K_{0.5}$.

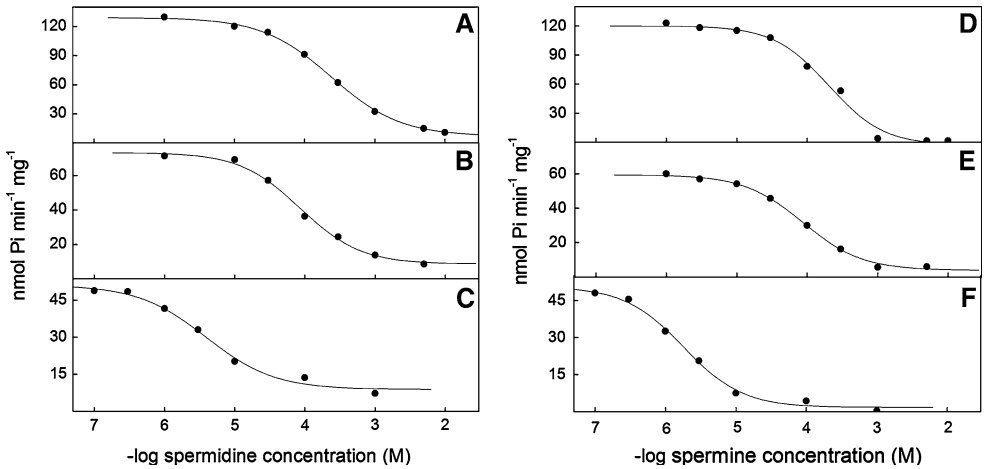

Fig. 8 Effect of spermidine and spermine, at suboptimal concentrations of sodium, potassium or both, on total ATPase activity in a microsomal fraction from the posterior gill tissue of *C. ornatus*. ATPase activity was estimated as described in "Materials and Methods" using duplicate aliquots of gill microsomal preparation containing 36.2-μg proteins. Three different microsomal fractions prepared using different pools of crabs were used to estimate the kinetic parameters. The figure shows a representative curve obtained from one gill microsomal fraction: **a**, **d** 5 mM NaCl, 5 mM KCl; **b**, **e** 50 mM NaCl, 0.5 mM KCl; **c**, **f** 5 mM NaCl, 0.5 mM KCl

Discussion

In this study we provide an extensive kinetic characterization of the effect of exogenous spermine and spermidine on the modulation by ATP, K^+, Na^+, Mg^{2+} and NH_4^+ and on the inhibition by ouabain of Na^+,K^+-ATPase activity in a posterior gill microsomal preparation from the euryhaline crab *C. ornatus* acclimated to 21‰ salinity. Our kinetic data reveal competition between spermine and spermidine and Na^+ and K^+ for the cation binding sites on the enzyme molecule, affecting both V_m and K_m for ATP hydrolysis. This is the first kinetic demonstration of competition between spermine and spermidine for the cation sites of a crustacean Na^+,K^+-ATPase.

Polyamines participate in the regulation of gene expression during acclimation processes (Waters et al. 1992) and play a role in amino acid metabolism and growth (Choo et al. 1991; Waters et al. 1992). By directly interacting with Na^+,K^+-ATPase from *C. sapidus* gill epithelium, polyamines may be involved in the regulation of osmotic and ionic homeostasis (Lovett and Watts 1995). While there is little information on their role in regulating crustacean gill Na^+,K^+-ATPase activity (Lovett and Watts 1995; Silva et al. 2008), spermine and spermidine are present at nanomolar titers in the gill tissue of *Eriocheir sinensis* (Péqueux et al. 2002).

Our data for *C. ornatus* disclose important differences compared to *C. danae*, also a euryhaline swimming crab (Silva et al. 2008). Inhibition of *C. ornatus* Na^+,K^+-ATPase activity is concentration-dependent regarding spermidine and spermine, in contrast to *C. danae* (Silva et al. 2008). $K_{0.5}$ values for Na^+, K^+ and NH_4^+ estimated for *C. ornatus* Na^+,K^+-ATPase activity are markedly affected by spermine and spermidine in contrast to the *C. danae* enzyme, where variation in $K_{0.5}$ in the presence of spermidine was found for Na^+ alone (Silva et al. 2008). Spermine and spermidine considerably affect V ($\approx$50%) Na^+, K^+ and NH_4^+ stimulation in contrast to minor variation in V for the *C. danae* enzyme in the presence of spermidine only (Silva et al. 2008). Putrescine had a negligible effect on *C. ornatus* Na^+,K^+-ATPase activity compared to the 20% inhibition seen for *C. danae* (Silva et al. 2008). However, it is difficult to envisage a physiologically important role for spermine in modulating the Na^+,K^+-ATPase present in the ionocyte plasma membranes of *C. ornatus* gills given that this polyamine is restricted to the cell nucleus (Tabor and Tabor 1984). However, spermine can function as a salvage compound and may serve as a reserve pool to be converted back to putrescine, as seen in vertebrates (Janne et al. 1991).

Cells exposed to high salt contents are subject to the deleterious effects of both osmotic pressure and increased ion concentration. Consequently, they trigger specific mechanisms to adjust intracellular osmolality. The accumulation of low molecular mass osmolytes, such as polyamines, provides an organism with a mechanism for salt stress tolerance (Bouchereau et al. 1999). Putrescine concentration increases during hyposmotic exposure of *Artemia* (Watts et al. 1994) and of *C. sapidus* (Lovett and Watts 1995) to hyperosmotic medium. While Na^+,K^+-ATPase activity decreases in *C. sapidus*, a direct correlation between increased biogenic amines and decreased enzyme activity has not been established (Lovett and Watts 1995).

The effect of spermine and spermidine on the Na^+ and K^+ binding sites on the *C. ornatus* enzyme differs

considerably from that for the *C. danae* enzyme, where spermidine inhibits pumping activity by competing with Na^+ at the Na^+-binding site, also inhibiting enzyme dephosphorylation (Silva et al. 2008). Apparently, the effect of the ionic concentration of spermine and spermidine on inhibition of Na^+,K^+-ATPase activity in *C. danae* is a consequence of both size and charge since, at physiological pH, spermine has a greater charge density (Silva et al. 2008). Based on the availability of positive charges within its structure, the polyamine showing the greatest charge density should exhibit greater interaction with the cation-binding domain of Na^+,K^+-ATPase. However, for the *C. ornatus* enzyme, the fact that spermidine was a stronger inhibitor than spermine suggests that polyamine inhibition of Na^+,K^+-ATPase activity is dependent on both the ionic charge radius of the molecule and aliphatic chain length. Thus, like the species-specific differences seen in ouabain affinity for Na^+,K^+-ATPase (Pressley 1992), the effect of polyamines on kinetic modulation of crustacean Na^+,K^+-ATPase activity also appears to be species-specific.

The modulation by Mg^{2+} of Na^+,K^+-ATPase activity obeyed cooperative kinetics and was independent of the presence of spermine and spermidine. Although V_m decreased considerably, Mg^{2+} affinity remained unchanged. Interpretation of the Mg^{2+} effect is complex since with ATP the activity measured is the sum of two effects: firstly, magnesium ions are essential for forming the ATP-Mg^{2+} substrate (Robinson and Pratap 1991); second, the metal ion binds to a specific site on the enzyme molecule at the cytoplasmic membrane face (Jorgensen et al. 1998). Further, since ATP and Na^+ induce rapid enzyme phosphorylation, Mg^{2+} binding cannot be separated from the phosphorylation reaction (Pilotelle-Bunner et al. 2009).

The kinetic analysis of the effect of spermine and spermidine on K^+ activation of ATP hydrolysis by the *C. ornatus* enzyme suggests mixed-type inhibition given the 50% decrease in V and slight change in K. These findings differ from those for the *C. danae* enzyme in which spermidine affected neither V nor K, obeying Michaelis–Menten kinetics (Silva et al. 2008).

With respect to Na^+, spermine and spermidine are apparently mixed-type inhibitors since they decrease V_m (twofold) and Na^+ affinity (threefold) of the *C. ornatus* enzyme, as also found for the *C. danae* enzyme (Silva et al. 2008). Canine kidney enzyme shows a similar effect for cation sites (Robinson et al. 1986). Spermine and spermidine may inhibit the *C. danae* gill Na^+,K^+-ATPase by two different mechanisms (Silva et al. 2008). At low Na^+ concentration (10 mM), spermine decreases the apparent affinity for Na^+ at the cytoplasmic sites, blocking E-P formation and inhibiting activity by 40–45%. At high Na^+ concentration (100 mM), the cation binding sites become saturated and both amines act as weak inhibitors, apparently stabilizing the phosphorylated intermediate and possibly reducing dephosphorylation rate (Silva et al. 2008). For *C. ornatus*, at suboptimal concentrations, competition between K^+ and Na^+ and spermine and spermidine was disclosed. For spermidine, in addition to a decrease in V_m, $K_{0.5}$ decreased markedly, from 5×10^{-4} M (for 5 mM NaCl and 5 mM KCl) to 8×10^{-6} M (for 5 mM NaCl and 0.5 mM KCl). Spermine showed similar behavior.

At physiological pH, up to 99% of hemolymph ammonia takes the NH_4^+ form. This ion can substitute for K^+ in basal Na^+,K^+-ATPase and for H^+ in the apical exchangers (Holliday 1985; Weihrauch et al. 1998, 1999; Morris 2001; Furriel et al. 2000; Masui et al. 2002). NH_4^+ may also substitute for K^+ as a counter-ion in Na^+ transport by the *C. sapidus* gill enzyme (Towle and Holleland 1987) and is actively transported by the vertebrate Na^+,K^+-ATPase (Mallery 1983; Wall 1996). In the absence of K^+, the apparent affinity of the *C. ornatus* gill enzyme for NH_4^+ is 5.4 ± 0.2 mM (Garçon et al. 2007), very similar to that of crab nerve (Skou 1960), *C. danae* gill (Masui et al. 2002) and vertebrate (Robinson 1970) enzymes. However, in the presence of spermine and spermidine, $K_{0.5}$ is twofold greater and associated with a 32–46% inhibition of Na^+,K^+-ATPase activity in *C. ornatus*, which contrasts with *C. danae* where spermine inhibits activity by around 26%, while NH_4^+ affinity is unchanged (Silva et al. 2008). Like the *C. danae* Na^+,K^+-ATPase (Masui et al. 2002), when fully saturated by NH_4^+, the *C. ornatus* enzyme is further activated by K^+ in a ouabain-sensitive manner, suggesting the existence of a second, previously unknown, independent ammonium binding site that affects both Na^+ and Mg^{2+} binding (Garçon et al. 2007, 2009). Such synergistic stimulation by K^+ and NH_4^+ has been reported in *C. danae* (Masui et al. 2002), *Clibanarius vitattus* (Gonçalves et al. 2006), *Macrobrachium olfersi* (Furriel et al. 2004) and *M. amazonicum* (Santos et al. 2007), suggesting a link between Na^+ hyperregulation, NH_4^+ excretion and Na^+,K^+-ATPase activity. Since spermine and spermidine interact with the cation-binding domain of Na^+,K^+-ATPase, we are currently investigating their role in fine-tuning gill Na^+,K^+-ATPase activity by K^+ and NH_4^+.

Acknowledgments This work constitutes part of a PhD thesis by D. P. G., financed by research grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Instituto Nacional de Ciência e Tecnologia (INCT) Adapta/Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM, grant 573976/2008-2). F. A. L., C. F. L. F. and J. C. M. received research scholarships from CNPq. D. P. G. received a postdoctoral scholarship from FAPESP, and M. N. L. and J. L. F. received research scholarships from CNPq. All experiments conducted in this study complied with currently applicable state and federal laws. We thank Nilton

Rosa Alves for technical assistance. This laboratory (F. A. L.) is integrated with the Amazon Shrimp Network (Rede de Camarão da Amazônia).

References

Axelsen KB, Palmgren MG (1998) Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol 46:84–101

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J (1999) Polyamines and environmental challenges: recent development. Plant Sci 140:103–125

Brues TC (1927) Occurrence of the marine crabs *Callinectes ornatus*, in brackish and freshwater. Am Nat 61:566–568

Choo PSTK, Smith TK, Cho CY, Ferguson HW (1991) Dietary excess of leucine influence growth and body composition of rainbow trout. J Nutr 121:1932–1939

Freire CA, Onken H, McNamara JC (2008) A structure–function analysis of ion transport in crustacean gills and excretory organs. Comp Biochem Physiol A Mol Integr Physiol 151:272–304

Furriel RPM, McNamara JC, Leone FA (2000) Characterization of Na^+,K^+-ATPase in gill microsomes of the freshwater shrimp *Macrobrachium olfersii*. Comp Biochem Physiol B Biochem Mol Biol 126:303–315

Furriel RPM, Masui DC, McNamara JC, Leone FA (2004) Modulation of gill Na^+,K^+-ATPase activity by ammonium ions: putative coupling of nitrogen excretion and ion uptake in the freshwater shrimp *Macrobrachium olfersii*. J Exp Zool A Comp Exp Biol 301:63–74

Garçon DP, Masui DC, Mantelatto FLM, McNamara JC, Furriel RPM, Leone FA (2007) K^+ and NH_4^+ modulate gill Na^+,K^+-ATPase activity in the blue crab, *Callinectes ornatus*: fine tuning of ammonia excretion. Comp Biochem Physiol A Mol Integr Physiol 147:145–155

Garçon DP, Masui DC, Mantelatto FLM, Furriel RPM, McNamara JC, Leone FA (2009) Hemolymph ionic regulation and adjustments in gill Na^+,K^+-ATPase activity during salinity acclimation in the swimming crab *Callinectes ornatus* (Decapoda, Brachyura). Comp Biochem Physiol A Mol Integr Physiol 154:44–55

Genovese G, Luchetti CG, Luquet CM (2004) Na^+,K^+-ATPase activity and gill ultrastructure in the hyper-hypo-regulating crab *Chasmagnathus granulatus* acclimated to dilute, normal, and concentrated seawater. Mar Biol 144:111–118

Glynn IM (1993) All hands to the sodium-pump. J Physiol 462:1–30

Gonçalves RR, Masui DC, McNamara JC, Mantelatto FLM, Garcon DP, Furriel RPM, Leone FA (2006) A kinetic study of the gill Na^+,K^+-ATPase and its role in ammonia excretion in the intertidal hermit crab, *Clibanarius vittatus*. Comp Biochem Physiol A Mol Integr Physiol 145:346–356

Haefner A Jr (1990) Natural diet of *Callinectes ornatus* (Brachyura: Portunidae) in Bermuda. J Crustac Biol 10:36–246

Holliday CW (1985) Na^+,K^+-ATPase activity and hypoosmoregulation in the brine shrimp, *Artemia salina*. Am Zool 25:A138–A138

Iarashi K, Kashiwagi K (2010) Modulation of cellular function by polyamines. Int J Biochem Cell Biol 42:39–51

Janne J, Alhone L, Leinonen P (1991) Polyamines: from molecular biology to clinical application. Ann Med 23:241–259

Jantaro S, Maenpaa P, Mulo P, Incharoensakdi A (2003) Content and biosynthesis of polyamines in salt and osmotically stressed cells of *Synechocystis* sp. PCC 6803. FEMS Microbiol Lett 228:129–135

Jayasundara N, Towle DW, Weihrauch D, Spanings-Pierrot C (2007) Gill-specific transcriptional regulation of Na^+/K^+-ATPase α-subunit in the euryhaline shore crab *Pachygrapsus marmoratus*: sequence variants and promoter structure. J Exp Biol 210:2070–2081

Jorgensen PL, Pedersen PA (2001) Structure–function relationships of Na^+, K^+, ATP, or Mg_2^+ binding and energy transduction in Na^+,K^+-ATPase. Biochim Biophys Acta 1505:57–74

Jorgensen PL, Nielsen JM, Rasmussen JH, Pedersen PA (1998) Structure–function relationships of E-1–E-2 transitions and cation binding in Na, K-pump protein. Biochim Biophys Acta 1365:65–70

Kalac P (2009) Recent advances in the research on biological roles of dietary polyamines in man. J Appl Biomed 7:65–74

Kaplan JH (2002) Biochemistry of Na^+,K^+-ATPase. Annu Rev Biochem 71:511–535

Kirschner LB (2004) The mechanism of sodium chloride uptake in hyperregulating aquatic animals. J Exp Biol 207:1439–1452

Krishnamurthy R, Bhagwat KA (1989) Polyamines as modulators of salt tolerance in rice cultivars. Plant Physiol 91:500–504

Lee KJ, Watts SA (1994) Specific activity of NA^+,K^+-ATPase is not altered in response to changing salinities during early development of the brine shrimp *Artemia franciscana*. Physiol Zool 67:910–924

Leone FA, Baranauskas JA, Furriel RPM, Borin IA (2005) SigrafW: an easy-to-use program for fitting enzyme kinetic data. Biochem Mol Biol Educ 33:399–403

Lin X, Fenn E, Veenstra RD (2006) An amino terminal lysine residue of rat connexin40 that is required for spermine block. J Physiol 570:251–269

Lovett DL, Watts SA (1995) Changes in polyamine levels in response to acclimation salinity in gills of the blue-crab *Callinectes-sapidus* rathbun. Comp Biochem Physiol B Biochem Mol Biol 115:115–119

Lucu C, Flik G (1999) Na^+-K^+-ATPase and Na^+/Ca^{2+} exchange activities in gills of hyperregulating *Carcinus maenas*. Am J Physiol Regul Integr Comp Physiol 276:R490–R499

Lucu C, Towle DW (2003) Na^+K^+-ATPase in gills of aquatic crustacea. Comp Biochem Physiol A Mol Integr Physiol 135:195–214

Luquet CM, Weihrauch D, Senek M, Towle DW (2005) Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*. J Exp Biol 208:3627–3636

Mallery CH (1983) A carrier enzyme basis for ammonium excretion in teleost gill-NH_4^+ stimulated Na^+-dependent ATPase activity in *Opsanus beta*. Comp Biochem Physiol A Mol Integr Physiol 74:880–897

Mañanes AAL, Meligeni CD, Goldemberg AL (2002) Response to environmental salinity of Na^+–K^+ATPase activity in individual gills of the euryhaline crab *Cyrtograpsus angulatus*. J Exp Mar Biol Ecol 274:75–85

Mantel LH, Farmer LL (1983) Osmotic and ion regulation. In: Mantel LH, Bliss DE (eds) The biology of crustacea. International anatomy and regulation, vol 5. Academic Press, New York, pp 53–161

Mantelatto FLM, Christofoletti RA (2001) Natural feeding activity of the crab *Callinectes ornatus* (Portunidae) in Ubatuba Bay (Sao Paulo, Brazil): influence of season, sex, size and molt stage. Mar Biol 138:585–594

Mantelatto FLM, Fransozo A (1999) Characterization of the physical and chemical parameters of Ubatuba Bay, northern coast of Sao Paulo State, Brazil. Rev Bras Biol 59:23–31

Mantelatto FLM, Fransozo A (2000) Brachyuran community in Ubatuba Bay, northern coast of Sao Paulo State, Brazil. J Shellfish Res 19:701–709

Marks MJ, Seeds NW (1978) A heterogeneous ouabain–ATPase interaction in mouse brain. Life Sci 23:2735–2744

Martin DW (2005) Structure–function relationships in the Na^+,K^+-pump. Semin Nephrol 25:282–291

Masui DC, Furriel RPM, McNamara JC, Mantelatto FLM, Leone FA (2002) Modulation by ammonium ions of gill microsomal (Na^+,K^+)-ATPase in the swimming crab *Callinectes danae*: a possible mechanism for regulation of ammonia excretion. Comp Biochem Physiol C Toxicol Pharmacol 132:471–482

Masui DC, Furriel RPM, Silva ECC, Mantelatto FLM, McNamara JC, Barrabin H, Scofano HM, Fontes CFL, Leone FA (2005) Gill microsomal (Na^+,K^+)-ATPase from the blue crab *Callinectes danae*: interactions at cationic sites. Int J Biochem Cell Biol 37:2521–2535

Morris S (2001) Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. J Exp Biol 204:979–989

Onken H, Riestenpatt S (1998) NaCl absorption across split gill lamellae of hyperregulating crabs, transport mechanisms and their regulation. Comp Biochem Physiol A Mol Integr Physiol 119:883–893

Pedersen PL (2007) Transport ATPases into the year 2008: a brief overview related to types, structures, functions and roles in health and disease. J Bioenerg Biomembr 39:349–355

Pegg AE (2009) Mammalian polyamine metabolism and function. IUBMB Life 61:880–894

Péqueux A (1995) Osmotic regulation in crustaceans. J Crustac Biol 15:1–60

Péqueux A, LaBras P, Cann-Moisan C, Coroff J, Sebert P (2002) Polyamines, indolamines, and catecholamines in gills and haemolymph of the euryhaline crab, *Eriocheir sinensis*. Effects of high pressure and salinity. Crustaceana 75:567–578

Pilotelle-Bunner A, Cornelius F, Sebban P, Kuchel PW, Clarke RJ (2009) Mechanism of Mg_2^+ binding in the Na^+,K^+-ATPase. Biophys J 96:3753–3761

Pressley TA (1992) Ionic regulation of Na^+-ATPase, K^+-ATPase expression. Semin Nephrol 12:67–71

Read SM, Northcote DH (1981) Minimization of variation in the response to different proteins of the Coomassie blue-g dye-binding assay for protein. Anal Biochem 116:53–64

Rebelo MF, Santos EA, Monserrat JM (1999) Ammonia exposure of *Chasmagnathus granulata* (Crustacea, Decapoda) Dana, 851. Accumulation in haemolymph and effects on osmoregulation. Comp Biochem Physiol A Mol Integr Physiol 122:429–435

Robinson JW (1970) Difference in sensitivity to cardiac steroids of (Na^+K^+)-stimulated ATPase and amino acid transport in intestinal mucosa of rat and other species. J Physiol 206:41–60

Robinson JD, Pratap PR (1991) (NA^+K^+)-ATPase—modes of inhibition by Mg^{2+}. Biochim Biophys Acta 1061:267–278

Robinson JD, Leach CA, Robinson LJ (1986) Cation sites, spermine, and the reaction sequence of the (NA^+K^+)-dependent ATPase. Biochim Biophys Acta 856:536–544

Rudolph FB, Baugher BW, Beissner RS (1979) Techniques in coupled enzyme assays. Methods Enzymol 63:22–42

Santos LCF, Belli NM, Augusto A, Masui DC, Leone FA, McNamara JC, Furriel RPM (2007) Gill Na^+, K^+-ATPase in diadromous, freshwater palaemonid shrimps: species-specific kinetic characteristics and alpha-subunit expression. Comp Biochem Physiol A Mol Integr Physiol 148:178–188

Schoner W, Scheiner-Bobis G (2007) Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth. Am J Physiol Cell Physiol 293:C509–C536

Silva ECC, Masui DC, Furriel RPM, Mantelatto FLM, McNamara JC, Barrabin H, Leone FA, Scofano HM, Fontes CFL (2008) Regulation by the exogenous polyamine spermidine of Na, K-ATPase activity from the gills of the euryhaline swimming crab *Callinectes danae* (Brachyura, Portunidae). Comp Biochem Physiol B Biochem Mol Biol 149:622–629

Skou JC (1960) Further investigations on a Mg^{2+} + Na^+-activated adenosinetriphosphatase, possibly related to the active, linked transport of Na^+ and K^+ across the nerve membrane. Biochim Biophys Acta 42:6–23

Tabor CW, Tabor H (1984) Polyamines. Annu Rev Biochem 53:749–790

Tassoni A, Antognoni F, Bagni N (1996) Polyamine binding to plasma membrane vesicle isolated from zucchini hypocotyls. Plant Physiol 110:817–824

Toner M, Vaio G, McLaughlin A, McLaughlin S (1988) Adsorption of cations to phosphatidylinositol 4,5-biphosphate. Biochemistry 27:7435–7443

Towle DW, Holleland T (1987) Ammonium ion substitutes for K^+ in ATP-dependent Na^+ transport by basolateral membrane-vesicles. Am J Physiol Regul Integr Comp Physiol 252:R479–R489

Towle DW, Weihrauch D (2001) Osmoregulation by gills of euryhaline crabs: molecular analysis of transporters 1. Am Zool 41:770–780

Wall SM (1996) NH_4^+ augments net acid secretion by a ouabain-sensitive mechanism in isolated perfused inner medullary collecting ducts. Am J Physiol Renal Physiol 39:F432–F439

Waters S, Khamis M, von der Decken A (1992) Polyamines in liver and their influence on chromatin condensation after 17-beta estradiol treatment of Atlantic salmon. Mol Cell Biochem 109:17–24

Watts SA, Lee KJ, Cline GB (1994) Elevated ornithine decarboxylase activity and polyamine levels during early development in the brine shrimp *Artemia franciscana*. J Exp Zool 270:426–431

Weihrauch D, Becker W, Postel U, Riestenpatt S, Siebers D (1998) Active excretion of ammonia across the gills of the shore crab *Carcinus maenas* and its relation to osmoregulatory ion uptake [B]. J Comp Physiol 168:364–376

Weihrauch D, Becker W, Postel U, Luck-Kopp S, Siebers D (1999) Potential of active excretion of ammonia in three different haline species of crabs [B]. J Comp Physiol 169:25–37

Weihrauch D, Morris S, Towle DW (2004) Ammonia excretion in aquatic and terrestrial crabs. J Exp Biol 207:4491–4504

Wu F, Grossenbacher D, Gehring H (2007) New transition state-based inhibitor for human ornithine decarboxylase inhibits growth of tumor cells. Mol Cancer Ther 6:1831–1839