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Original Communication

Gill (Na^+, K^+)-ATPase in the diadromous palaemonid shrimp *Macrobrachium amazonicum*: Kinetic characterization of K^+-phosphatase activity in juveniles and adults

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ABSTRACT

The kinetic properties of a microsomal gill (Na^+, K^+)-ATPase from juvenile and adult Amazon River shrimp *Macrobrachium amazonicum* were characterized using the synthetic substrate *p*-nitrophenylphosphate. The substrate was hydrolyzed revealing cooperative kinetics at maximum rates of 71.3 ± 1.1 U mg^{-1} and 82.7 ± 2.3 U mg^{-1} with $K_{0.5}$ = 1.45 ± 0.02 mmol L^{-1} and 1.52 ± 0.04 mmol L^{-1} for juveniles and adults, respectively. Stimulation of K^+-phosphatase activity by Mg^{2+} and K^+ also exhibited cooperative kinetics independently of ontogenetic stage. While the $K_{0.5}$ values estimated for Mg^{2+} and K^+ stimulation of K^+-phosphatase activity were very similar, maximum rates for adult shrimps were ≈1.8-fold greater than that for juveniles. NH_4^+ stimulation resulted in a ≈10-fold increase in $K_{0.5}$ for both stages compared to Mg^{2+} and K^+. Further, when stimulated by NH_4^+ plus K^+, *p*-nitrophenylphosphate hydrolysis was mostly unchanged, suggesting that NH_4^+ and K^+ bind to the same site. While $K_{0.5}$ values varied, stimulation by NH_4^+ plus K^+ was not synergistic. Increasing NH_4^+ or K^+ concentrations do not displace the corresponding ion from the cation-binding site. Ouabain partially inhibited *p*-nitrophenylphosphatase activity (K_I = 142.0 ± 5.6 µmol L^{-1} and 156.0 ± 6.2 µmol L^{-1} for juveniles and adults, respectively); however, inhibition was more effective when NH_4^+ was present (K_I = 117.1 ± 4.6 µmol L^{-1} and 47.2 ± 1.9 µmol L^{-1} for juveniles and adults, respectively). Although the activity of ATPases other than the (Na^+, K^+)-ATPase are considerably greater in adult shrimps, the kinetic properties of the gill K^+-phosphatase activity in the two life cycle stages, and the affinities for *p*-nitrophenylphosphate and ions are similar.

KEYWORDS: (Na^+, K^+)-ATPase, K^+-phosphatase activity, gill microsomes, *Macrobrachium amazonicum*, palaemonid shrimp, ontogenetic stage

INTRODUCTION

The (Na^+, K^+)-ATPase is an active, cation-transporting protein present in the plasma membranes of virtually all animal cells [1]. The enzyme is a member of the P_2-type ATPase family, forming a phosphorylated intermediate by the transfer of γ-phosphate from ATP to a conserved aspartate residue [2]. For each adenosine triphosphate molecule hydrolyzed, three Na^+ are transported out of and

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two K^+ into the cell by the pump [3] that alternates between two major conformational states, E_1 and E_2, according to a ping-pong alternate-access mechanism [4]. This membrane-bound enzyme consists of a catalytic α-subunit containing the conserved amino acid sequences characteristic of the P-type ATPase super family responsible for Na^+ and K^+ translocation and ATP hydrolysis [5, 6], and a β-subunit, required for folding, membrane insertion and delivery to the plasma membrane of the catalytically active α-subunits [7, 8]. In many cell types, the αβ-complex interacts with a small regulatory transmembrane protein, the γ subunit, belonging to the FXYD protein family [9, 10].

Besides ATP, the (Na^+, K^+)-ATPase also hydrolyzes other phosphate-donating substrates (K^+-phosphatase activity), like *p*-nitrophenylphosphate [11-15], O-methyl fluoresceinphosphate [16], acetylphosphate [17], 2,4 dinitrophenylphosphate and β-(2-furyl) acryloylphosphate [18]. While there is a strict correlation between the ouabain-inhibitable ATPase and K^+-phosphatase activities that correspond to different activities of the same enzyme, K^+-phosphatase activity is not yet well understood [11, 12, 15, 19-22]. The E_2 form may be the main conformational state involved in K^+-phosphatase activity, but K^+-stimulated enzyme phosphorylation by *p*-nitrophenylphosphate, not linked to cation transport, is still controversial [12, 23, 24].

While competitive inhibition by ATP of K^+-phosphatase activity is a strong evidence that *p*-nitrophenylphosphate and ATP are hydrolyzed at the same site on the (Na^+, K^+)-ATPase [20, 25], a separate binding site for *p*-nitrophenylphosphatase activity near the ATP binding site is recognized [26, 27]. Intracellular K^+ stimulates *p*-nitrophenylphosphate hydrolysis [14] while extracellular K^+ stimulates enzyme dephosphorylation [14, 28]; however, whether K^+-phosphatase activity involves a K^+-occluded conformation is uncertain [15]. A third Rb^+/K^+-binding site located on the α-subunit in the P-domain of the cytoplasmic moiety and not directly involved in the binding of the translocated ions has been suggested [29]. This site may be involved in the dephosphorylation of the E_2P state induced by K^+ binding to the transport sites when in their 'extracellular facing' configuration, and in the E_2 to E_1 transition of the dephosphorylated enzyme [29].

A great number of crustacean species occupy marine, fresh water, terrestrial or interface environments, and many show adaptations to the vast diversity of physical and chemical environments encountered [30, 31]. Marine species whose hemolymph osmolality accompanies that of the external medium, usually within strict limits, are essentially osmoconformers.

Hyperosmoregulating species inhabit dilute media or fresh water, and are constantly challenged by osmotic water influx and passive salt loss, for which they actively compensate; they are also less permeable to ion loss [32, 33]. Together with the excretory organs, the gills are responsible for ionic regulation in the Crustacea [34] and provide a selective interface across which salt is actively transported between the external environment and the internal *milieu*. The gills constitute a multi-functional effector organ system that contributes simultaneously to osmotic, excretory, acid-base and respiratory homeostasis [30, 34-38]. Many enzymes and transporters are involved in ion transport by crustacean gills, including the (Na^+, K^+)-ATPase, V-type H^+-ATPase ($V(H^+)$-ATPase), carbonic anhydrase, Cl^-/HCO_3^- exchanger and Na^+/H^+ exchanger [31, 34, 39]. The (Na^+, K^+)-ATPase is restricted to the basal membrane of the gill ionocytes and provides part of the driving force for the transepithelial movement of monovalent ions across the gill epithelia in brachyuran Crustacea [31, 37, 40-42]. In freshwater shrimp gills, a $V(H^+)$-ATPase located apically in the pillar cell flanges may complement the septal cell (Na^+, K^+)-ATPase in energizing osmoregulatory NaCl uptake from fresh water [31, 43, 44].

The palaemonid shrimps constitute one of the most diverse and widespread taxons that have successfully invaded freshwater from the ancestral marine habitat [45, 46], and their extant species exhibit varying degrees of adaptation to low salinity environments [31, 34, 47, 48]. The Amazon River prawn *Macrobrachium amazonicum* is endemic to South America [49, 50] and its presumptive natural distribution includes the Orinoco, Amazon, and Paraguay/Lower Paraná river basins [51]. This diadromous prawn has diversified into coastal populations that inhabit rivers close to estuaries, and continental populations living in rivers, lakes, and other inland water bodies [52, 53]. The two

groups apparently differ in external morphology and meristic characters [54]. Coastal populations of *M. amazonicum* exhibit a lengthy larval sequence dependent on brackish water for development to the post-larva. The juvenile stage then migrates back to fresh water to mature into the adult form [55]. Adult *M. amazonicum* are strong hyperosmotic and ionic regulators, an ability underpinned by gill (Na^+, K^+)-ATPase activity [44, 46]. While the kinetic characteristics of a gill Na^+, K^+-ATPase from various ontogenetic stages of *M. amazonicum* have been described [56, 57], little is known of the K^+-phosphatase kinetics of the gill (Na^+, K^+)-ATPase in different life cycle stages of *M. amazonicum*.

The present study furnishes an extensive kinetic characterization of K^+-phosphatase activity in a gill microsomal fraction from juvenile and adult *M. amazonicum* from fresh water. We show that K^+-phosphatase activity is not synergistically stimulated by K^+ plus NH_4^+, and that neither ion can displace the other from its respective binding site. Further, while K^+-phosphatase levels are fairly similar in juveniles and adults, the activity of ATPases other than the (Na^+, K^+)-ATPase are 2-fold greater in adults.

MATERIALS AND METHODS

Materials

All solutions were prepared using Millipore MilliQ ultrapure, apyrogenic water. (2-amino-2-hydroxymethyl)-propane-1-3-diol (Tris), N-(2-hydroxyethyl) piperazine-N'-ethane-sulfonic acid (HEPES), ouabain, alamethicin and *p*-nitrophenyl phosphate ditris salt (*p*NPP) were purchased from Sigma Chemical Co. (Saint Louis, USA). The protease inhibitor cocktail (1 mmol L^{-1} benzamidine, 5 µmol L^{-1} antipain, 5 µmol L^{-1} leupeptin, 1 µmol L^{-1} pepstatin A and 5 µmol L^{-1} phenyl-methane-sulfonyl-fluoride) was from Calbiochem (San Diego, USA). All other reagents were of the highest purity commercially available.

Shrimps

Amazon river shrimps, *Macrobrachium amazonicum*, were cultivated at the Aquaculture Center, UNESP, Jaboticabal, São Paulo, Brazil from broodstock collected at Furo das Marinhas near Santa Bárbara do Pará (1° 13.450" S; 48° 17.632" W), northeastern Pará State, Brazil, in 2001 [58]. The shrimps were transported to the laboratory in carboys containing aerated fresh water and were used immediately for microsomal preparation.

Gill dissection

Shrimps were anesthetized by chilling on crushed ice. The gills of juvenile and adult shrimps were dissected and transferred to 25 mL of homogenization buffer (20 mmol L^{-1} imidazole buffer, pH 6.8, 6 mmol L^{-1} EDTA, 250 mmol L^{-1} sucrose and the protease inhibitor cocktail), and maintained in crushed ice. For each homogenate, gills from 30-40 juvenile or 8-12 adult shrimps were used.

Preparation of the gill microsomal fraction

Gills were rapidly diced and homogenized in homogenization buffer (20 mL g^{-1} wet tissue) using a Potter homogenizer at 600 rpm. After centrifuging the crude homogenate at 20000 × g for 35 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 above, the two supernatants were pooled and centrifuged at 100000 × g for 90 min at 4 °C. The resulting pellet was re-suspended in homogenization buffer (10 mL g^{-1} wet tissue). Finally, 0.5-mL aliquots were rapidly frozen in liquid nitrogen and stored at -20 °C. Under these conditions, no appreciable changes in *p*NPP hydrolysis were seen after two-month's storage (juvenile and adult preparations V_M = 70.2 ± 2.5 U mg^{-1} and V_M = 83.5 ± 3.2 U mg^{-1} versus two month's freezing V_M = 67.9 ± 3.1 U mg^{-1} and V_M = 81.7 ± 2.5 U mg^{-1}, respectively). When required, the aliquots were thawed, placed on crushed ice and used immediately.

Measurement of *p*NPP hydrolysis

The hydrolysis of *p*NPP (*p*NPPase activity) by the gill microsomal fraction was assayed continuously, at 25 °C, monitoring the release of the *p*-nitrophenolate ion (ε_{410} nm pH 7.5 = 13160 mol^{-1} L cm^{-1}) in a Hitachi U-3000 spectrophotometer equipped with thermostatted cell holders. Standard conditions were 50 mmol L^{-1} HEPES buffer, pH 7.5, 10 mmol L^{-1} *p*NPP, 5 mmol L^{-1} $MgCl_2$, and 10 mmol L^{-1} KCl, in a final volume of 1.0 mL. Controls without enzyme were included in each experiment to quantify non-enzymatic hydrolysis of substrate. Initial velocities were constant for at least 15 min, provided that less than 5% of the substrate was hydrolyzed.

The reaction rate for each modulator was estimated in duplicate using aliquots from the same microsomal preparation. The mean values were used to fit each corresponding saturation curve, which was repeated three times utilizing different microsomal homogenates (N = 3). *p*NPPase activity was also assayed after 20 min incubation of the preparation with alamethicin (1 mg/mg protein) at 25 °C to demonstrate the presence or leaky and/or disrupted vesicles. Ouabain-insensitive *p*NPPase activity was estimated in the presence of 3 mmol L^{-1} ouabain. The difference in measured activity in the absence (total *p*NPPase activity) and presence of ouabain (ouabain-insensitive *p*NPPase activity) was considered to represent the K^+-phosphatase activity. Controls without added enzyme were included in each experiment to quantify non-enzymatic hydrolysis of substrate. One enzyme unit (U) is defined as the amount of enzyme that hydrolyzes 1.0 nmol of *p*NPP per minute at 25 °C. Specific activity is given as U per mg total protein.

Measurement of protein

Protein concentration was estimated according to [59], using bovine serum albumin as the standard.

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 *p*NPP hydrolysis were calculated using SigrafW software as described by [60]. The curves presented are those that best fit the experimental data. The kinetic parameters provided in the tables are calculated values and represent the mean ± SD derived from three different microsomal preparations (N = 3). The enzyme-inhibitor complex apparent dissociation constant, K_I, was estimated as described by [61]. SigrafW software can be freely obtained from http://portal.ffclrp.usp.br/sites/fdaleone/downloads.

RESULTS

The effect of increasing *p*NPP concentrations (10^{-5} mol L^{-1} to 2 × 10^{-2} mol L^{-1} *p*NPP) on K^+-phosphatase activity in gill microsomal preparations from adult and juvenile *M. amazonicum* is shown in Fig. 1. Under saturating K^+ (10 mmol L^{-1}) and Mg^{2+} (5 mmol L^{-1}) concentrations, the K^+-phosphatase activity of adult shrimp gills (Fig. 1A) obeyed cooperative kinetics (n_H = 1.5) with V_M = 82.7 ± 2.3 U mg^{-1} and $K_{0.5}$ = 1.52 ± 0.04 mmol L^{-1} (Table 1). There was no inhibition of K^+-phosphatase activity by excess *p*NPP above 2 × 10^{-2} mol L^{-1} (not shown). Ouabain-insensitive *p*NPPase activity was

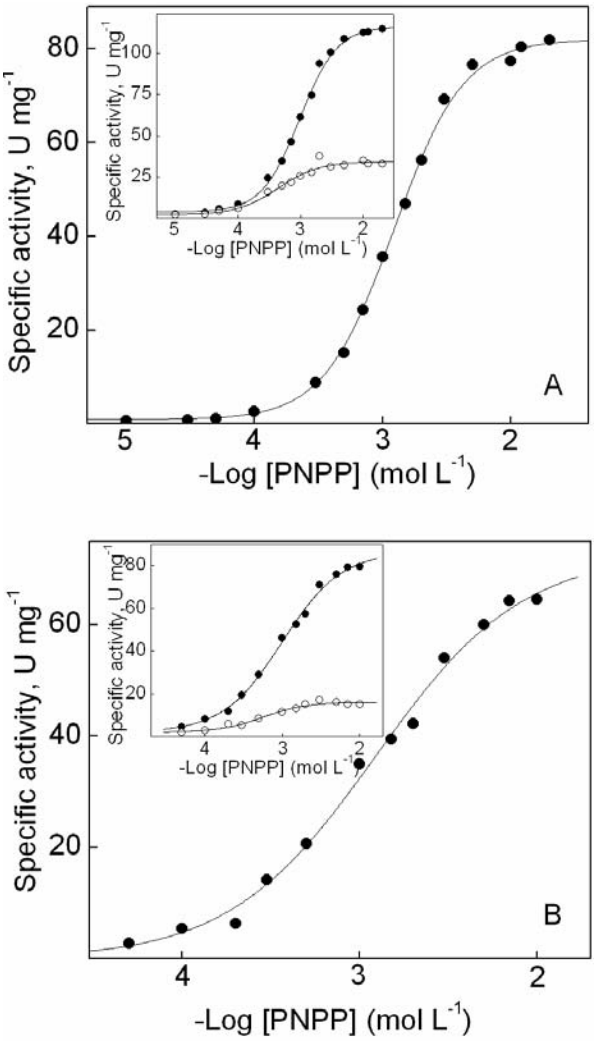

Fig. 1. Effect of *p*NPP concentration on K^+-phosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 5 mmol L^{-1} $MgCl_2$ and 10 mmol L^{-1} KCl in a final volume of 1.0 mL. Duplicate aliquots of three (N = 3) different gill microsomal preparations were used and the reaction was started by addition of 20.2 µg protein for adults or 9.4 µg for juveniles. Representative curves from one microsomal preparation are given. **A** - adult. **B** - juvenile. **Insets to the figures**: (●) total *p*NPPase activity. (○) ouabain-insensitive *p*NPPase activity.

Table 1. Kinetic parameters for the stimulation by *p*NPP, Mg^{2+}, K^+ and NH_4^+, and inhibition by Na^+ and ouabain of K^+-phosphatase activity in a microsomal fraction of gill tissue from adult and juvenile *Macrobrachium amazonicum*. Data are the mean ± SD (*N* = 3).

Effector	V (U mg^{-1})		$K_{0.5}$ (mmol L^{-1})		n_H	
	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile
*p*NPP	82.7 ± 2.3	71.3 ± 1.1	1.52 ± 0.04	1.45 ± 0.02	1.5	1.2
Mg^{2+}	84.5 ± 3.1	73.0 ± 0.8	1.09 ± 0.04	1.07 ± 0.01	2.0	1.4
K^+	85.8 ± 1.3	65.7 ± 1.2	1.91 ± 0.03	1.77 ± 0.03	1.9	2.0
NH_4^+	99.4 ± 1.4	65.7 ± 1.3	9.16 ± 0.12	9.40 ± 0.18	1.8	2.0

Inhibitor	K_I (mmol L^{-1})		K_I (µmol L^{-1})	
	Adult	Juvenile	Adult	Juvenile
Na^+	9.8 ± 0.2	6.7 ± 0.3	-	-
Ouabain	-	-	156.0 ± 6.2	142.0 ± 5.6
Ouabain + 50 mmol L^{-1} NH_4^+	-	-	47.2 ± 1.9	117.1 ± 4.6

stimulated by around 34 U mg^{-1} over the same *p*NPP concentration range, accounting for about 30% of total *p*NPPase activity, suggesting the presence of phosphohydrolases other than the (Na^+, K^+)-ATPase (inset to Fig. 1A). For juvenile gills (Fig. 1B), similar parameter values were found (V_M = 71.3 ± 1.1 U mg^{-1} and $K_{0.5}$ = 1.45 ± 0.02 mmol L^{-1}, n_H = 1.2) although ouabain insensitive *p*NPPase activity was about 15 U mg^{-1} (20% of total *p*NPPase activity). These findings suggest that while K^+-phosphatase levels are similar in adult and juvenile shrimp gills, the activities of ATPases other than (Na^+, K^+)-ATPase are considerably greater in adults.

Magnesium ions are essential for the K^+-phosphatase activity of the gill microsomal (Na^+, K^+)-ATPase from both juveniles and adults (Fig. 2); no K^+-phosphatase activity was detected in the absence of Mg^{2+}. Under saturating *p*NPP (10 mmol L^{-1}) and K^+ (10 mmol L^{-1}) concentrations, increasing Mg^{2+} concentrations (from 10^{-5} mol L^{-1} to 10^{-2} mol L^{-1}) stimulated K^+-phosphatase activity of adult gills to a maximum rate of V_M = 84.5 ± 3.1 U mg^{-1} with $K_{0.5}$ = 1.09 ± 0.04 mmol L^{-1} (Fig. 2A). Cooperative effects (n_H = 2.0) resulting from the metal ion-enzyme interaction suggest multiple binding sites for Mg^{2+}. The ouabain-insensitive *p*NPPase activity present was also stimulated up to 25 U mg^{-1} over the same Mg^{2+} concentration range (inset to Fig. 2A), corresponding to about 22% of Mg^{2+}-stimulated phosphohydrolases other than (Na^+, K^+)-ATPase. The enzyme from juvenile gills (Fig. 2B) showed a similar, cooperative effect (n_H = 1.4) with V_M = 73.0 ± 0.8 U mg^{-1} and $K_{0.5}$ = 1.07 ± 0.01 mmol L^{-1}. The ouabain-insensitive Mg^{2+}-stimulated *p*NPPase activity of juvenile gills (15 U mg^{-1}) is roughly 2-fold less than that in adult gills (insets to Figs. 2A and 2B).

The effect of increasing K^+ (from 10^{-5} mol L^{-1} to 2×10^{-2} mol L^{-1}) on K^+-phosphatase activity in the microsomal preparation from adult and juvenile *M. amazonicum* gills is shown in Fig. 3. Under saturating *p*NPP (10 mmol L^{-1}) and Mg^{2+} (5 mmol L^{-1}) concentrations, single saturation curves were found for adults (Fig. 3A) and juveniles (Fig. 3B). Maximal rates of V_M = 85.8 ± 1.3 U mg^{-1} with $K_{0.5}$ = 1.91 ± 0.03 mmol L^{-1} and V_M = 65.7 ± 1.2 U mg^{-1} with $K_{0.5}$ = 1.77 ± 0.03 mmol L^{-1} were estimated for adults and juveniles, respectively. Like Mg^{2+}, stimulation of ouabain-insensitive *p*NPPase activity of adult gills (≈28 U mg^{-1}) was almost 2-fold greater than that of juveniles (insets to Figs. 3A and 3B).

Under saturating *p*NPP (10 mmol L^{-1}) and Mg^{2+} (5 mmol L^{-1}) concentrations, and in the absence of K^+, the K^+-phosphatase activities were stimulated by increasing NH_4^+ (from 10^{-4} to 10^{-1} mol L^{-1}) (Fig. 4) to maximum rates of V_M = 99.4 ± 1.4 U mg^{-1} and V_M = 65.7 ± 1.3 U mg^{-1} for adults (Fig. 4A) and juveniles (Fig. 4B), respectively. The NH_4^+ affinities of the adult ($K_{0.5}$ = 9.16 ± 0.12 mmol L^{-1}) and

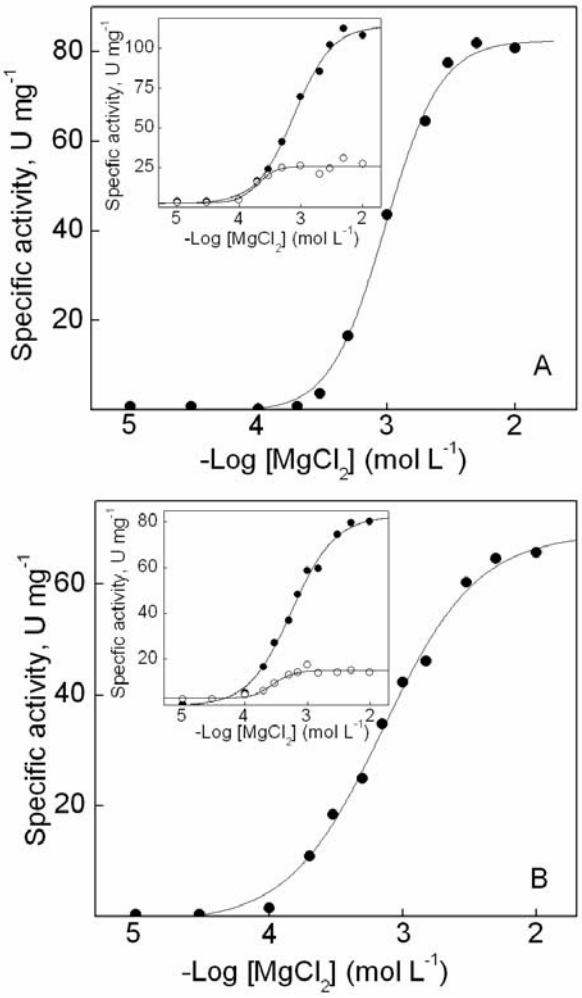

Fig. 2. Effect of $MgCl_2$ concentration on K^+-phosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 10 mmol L^{-1} *p*NPP and 10 mmol L^{-1} KCl in a final volume of 1.0 mL. Duplicate aliquots of three (*N* = 3) different gill microsomal preparations were used and the reaction was started by addition of 20.2 or 9.4 µg protein for adults and juveniles. Representative curves from one microsomal preparation are given. **A** - adult. **B** - juvenile. **Insets to the figures**: (●) total *p*NPPase activity. (○) ouabain-insensitive *p*NPPase activity.

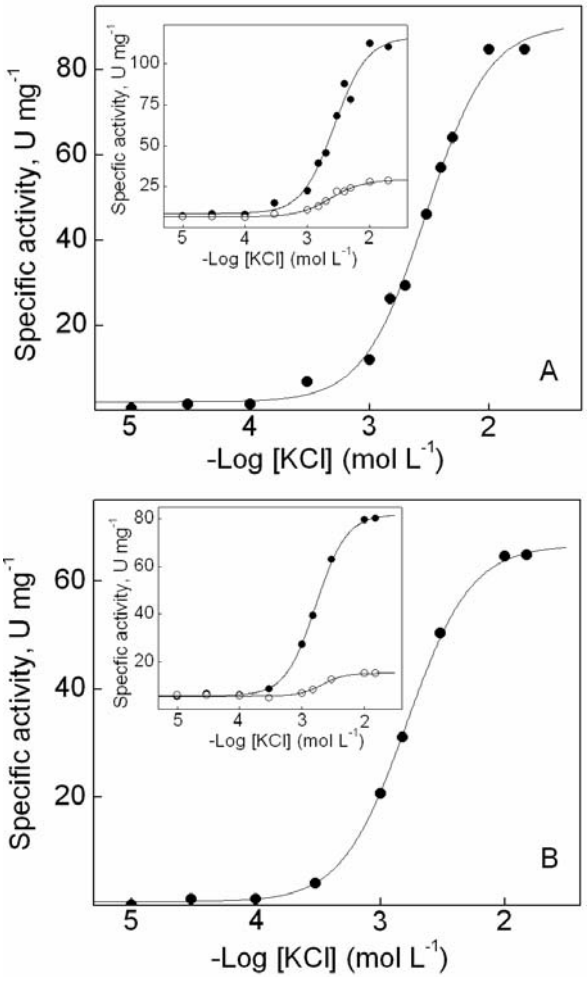

Fig. 3. Effect of KCl concentration on K^+-phosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 5 mmol L^{-1} $MgCl_2$ and 10 mmol L^{-1} *p*NPP in a final volume of 1.0 mL. Duplicate aliquots of three (*N* = 3) different gill microsomal preparations were used and the reaction was started by addition of 20.2 or 9.4 µg protein for adults and juveniles. Representative curves from one microsomal preparation are given. **A** - adult. **B** - juvenile. **Insets to the figures**: (●) total *p*NPPase activity. (○) ouabain-insensitive *p*NPPase activity.

juvenile ($K_{0.5}$ = 9.40 ± 0.18 mmol L^{-1}) enzymes were very similar. Stimulation of ouabain-insensitive *p*NPPase activity of adults (≈22 U mg^{-1}) was almost 2-fold greater than that for juveniles (insets to Figs. 4A and 4B**).**

Sodium ions below 10^{-3} mol L^{-1} had no effect on gill *p*NPPase activity in adult or juvenile gill preparations (Fig. 5). However, as Na^+ concentration increased up to 5 × 10^{-1} mol L^{-1}, *p*NPPase activity was considerably inhibited (≈7 U mg^{-1}), and calculated K_I values for Na^+ inhibition of *p*NPPase activity were 9.8 ± 0.2 mmol L^{-1} and 6.7 ± 0.3 mmol L^{-1} for adults and juveniles, respectively (insets to Figs. 5A and 5B).

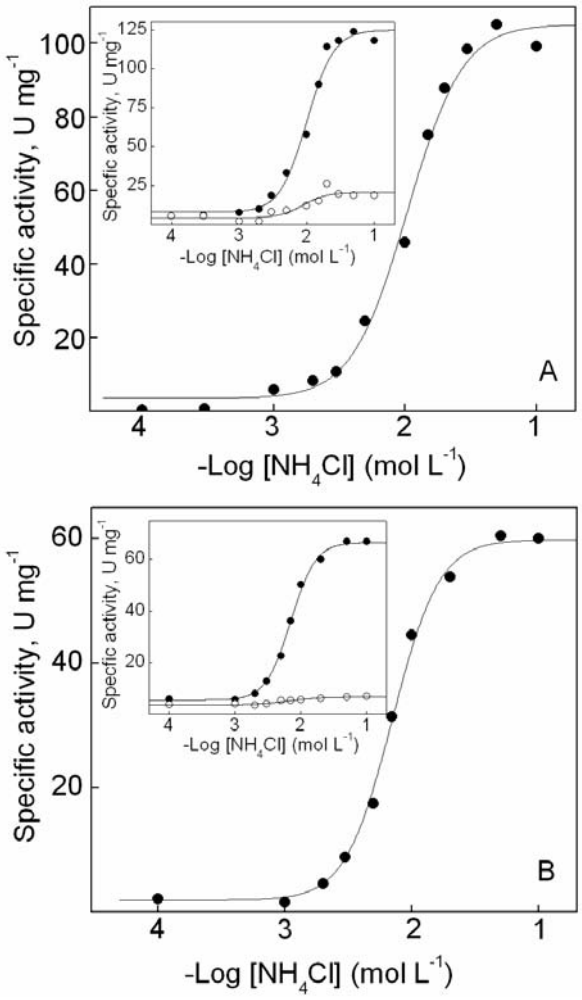

Fig. 4. Effect of NH_4Cl concentration on K^+-phosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 5 mmol L^{-1} $MgCl_2$ and 10 mmol L^{-1} *p*NPP in a final volume of 1.0 mL. Duplicate aliquots of three (N = 3) different gill microsomal preparations were used and the reaction was started by addition of 20.2 or 9.4 µg protein for adults and juveniles. Representative curves from one microsomal preparation are given. **A** - adult. **B** - juvenile. **Insets to the figures**: (●) total *p*NPPase activity. (○) ouabain-insensitive *p*NPPase activity.

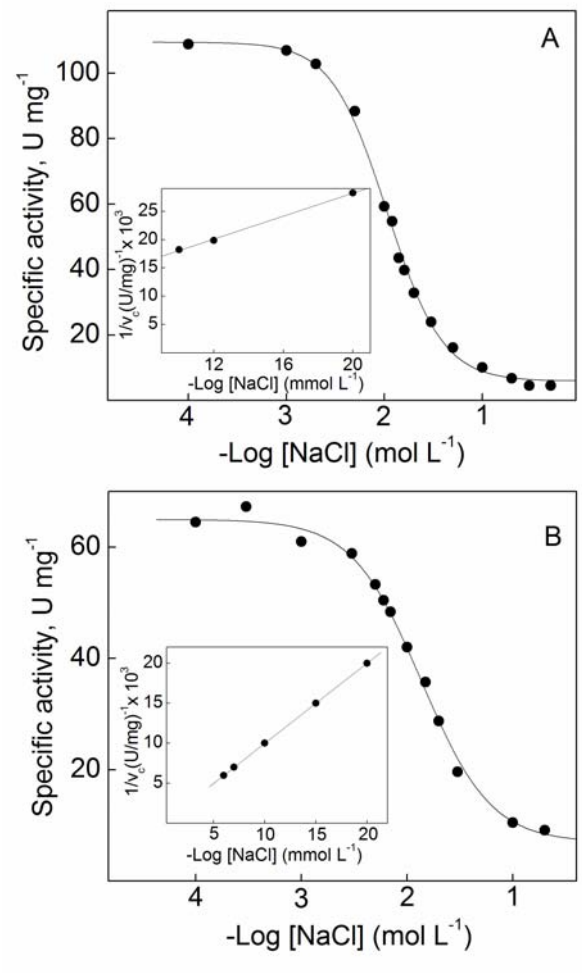

Fig. 5. Effect of NaCl concentration on *p*-nitrophenylphosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} KCl and 10 mmol L^{-1} *p*NPP in a final volume of 1.0 mL. Duplicate aliquots of three (N = 3) different gill microsomal preparations were used and the reaction was started by addition of 20.2 or 9.4 µg protein for adults and juveniles. Representative curves from one microsomal preparation are given. **A** - adult. **B** - juvenile. **Insets to the figures**: Dixon plots for K_I in which v_c is the reaction rate corresponding to the K^+-phosphatase activity.

The effect of a wide ouabain concentration range on the *p*NPPase activity of adult and juvenile gill microsomal fractions is shown in Fig. 6. Under optimal concentrations of *p*NPP (10 mmol L^{-1}), Mg^{2+} (5 mmol L^{-1}), and K^+ (10 mmol L^{-1}) or NH_4^+ (50 mmol L^{-1}), increasing ouabain concentrations up to 7 mmol L^{-1} inhibited almost 70% of adult *p*NPPase activity (Fig. 6A). The inhibition pattern corresponds to that of a single binding site model with a calculated apparent K_I value of 156.0 ± 6.2 µmol L^{-1} (inset to Fig. 6A). When *p*NPPase activity was assayed with 50 mmol L^{-1} NH_4^+, the profile was similar, although K_I decreased to 47.2 ± 1.9 µmol L^{-1} (inset to Fig. 6A). For the juvenile enzyme, a

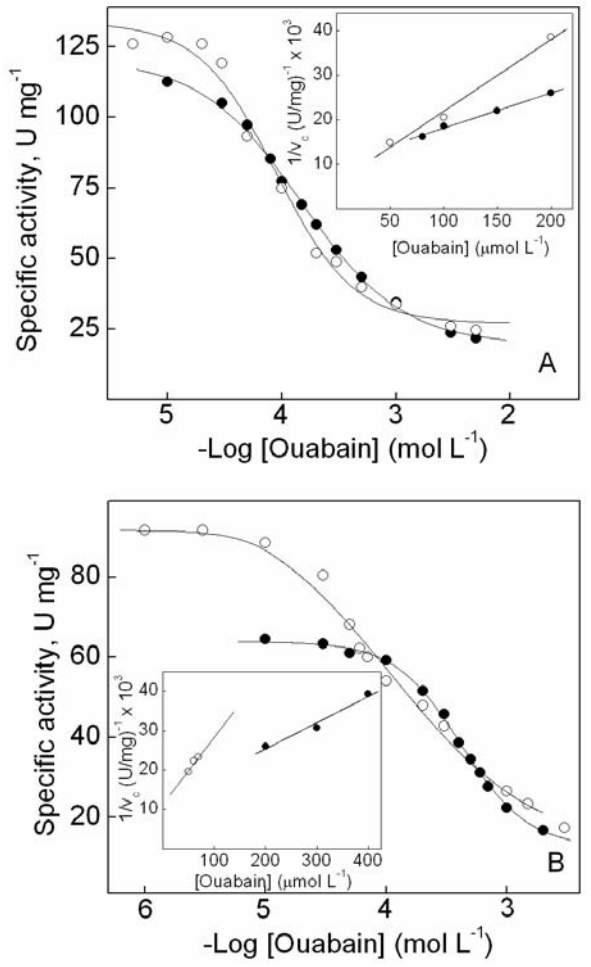

Fig. 6. Effect of ouabain concentration on *p*-nitrophenylphosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} KCl and 10 mmol L^{-1} *p*NPP in a final volume of 1.0 mL, in the absence (●) and presence of 50 mmol L^{-1} NH_4Cl (○). Duplicate aliquots of three (N = 3) different gill microsomal preparations were used and the reaction was started by addition of 20.2 or 9.4 μg protein for adults and juveniles, respectively. Representative curves from one microsomal preparation are given. **A** - adult. **B** - juvenile. **Insets to the figures**: Dixon plots for K_I in which v_c is the reaction rate corresponding to the K^+-phosphatase activity.

single binding site pattern also was obtained, with maximum inhibition of around 80%. Dixon plots for K_I gave values of 142.0 ± 5.6 μmol L^{-1} (inset to Fig. 6B) and 117.1 ± 4.6 μmol L^{-1} (inset to Fig. 6B) in the absence and presence of NH_4^+, respectively.

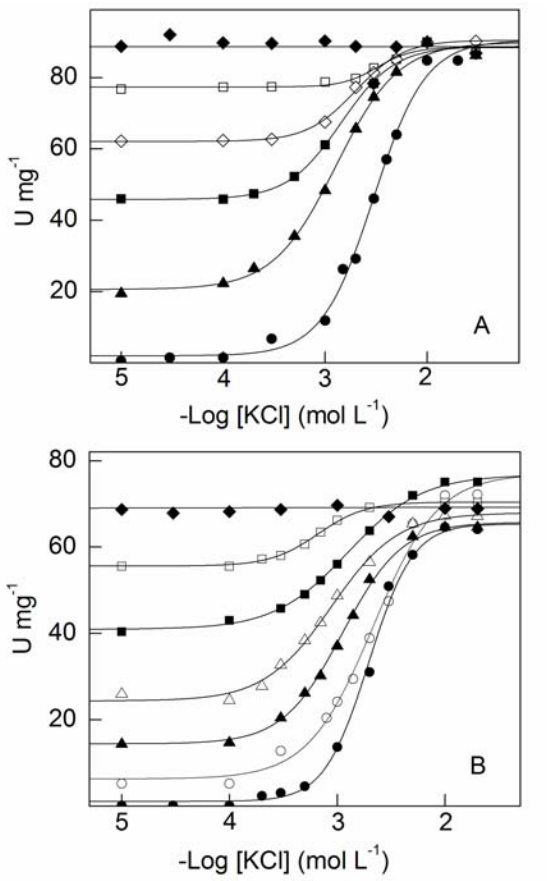

Fig. 7. Effect of KCl concentration on K^+-phosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*, in the presence of NH_4Cl. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 5 mmol L^{-1} $MgCl_2$ and 10 mmol L^{-1} *p*NPP in a final volume of 1.0 mL. Duplicate aliquots of three (*N* = 3) different gill microsomal preparations were used and the reaction was started by addition of 20.2 or 9.4 μg protein for adults and juveniles, respectively. Representative curves from one microsomal preparation are given. NH_4^+ concentrations: (●) none; (○) 1 mmol L^{-1}; (▲) 5 mmol L^{-1}; (Δ) 7 mmol L^{-1}; (■) 10 mmol L^{-1}; (◊) 14 mmol L^{-1}; (□) 20 mmol L^{-1}; (♦) 50 mmol L^{-1}. **A** - adult. **B** - juvenile.

Table 1 summarizes the calculated values of the kinetic parameters for these different modulators and inhibitors of K^+-phosphatase activity in gill preparations from adult and juvenile *M. amazonicum*.

The effect of NH_4^+ on K^+ stimulation of K^+-phosphatase activity in the gill microsomal fraction is shown in Fig. 7. In the absence of NH_4^+, stimulation of K^+-phosphatase activity in adults

by K^+ from 10^{-5} mol L^{-1} to 5×10^{-2} mol L^{-1} exhibited cooperative kinetics (n = 1.9) and gave a single saturation curve with $V_M = 85.8 \pm 1.3$ U mg^{-1} and $K_{0.5} = 1.91 \pm 0.03$ mmol L^{-1} (Fig. 7A). At fixed NH_4^+ concentrations (1 mmol L^{-1} to 50 mmol L^{-1}), K^+-phosphatase activity was slightly stimulated by K^+ over the above concentration range to a maximum rate of 95 U mg^{-1}, obeying cooperative kinetics and showing a 2-fold increase in $K_{0.5}$ values. There was no evidence of synergistic stimulation for the combined modulation of enzyme activity by both NH_4^+ and K^+. For juveniles, findings were very similar (Fig. 7B) except for a 5-fold increase in V/K at increased fixed NH_4^+ concentrations (Table 2).

The effect of K^+ on NH_4^+ stimulation of gill microsomal K^+-phosphatase activity in juveniles and adults is shown in Fig. 8. Under saturating *p*NPP (10 mmol L^{-1}) and Mg^{2+} (5 mmol L^{-1}) concentrations and in the absence of K^+, stimulation of K^+-phosphatase activity by NH_4^+ from 10^{-5} mol L^{-1} to 5×10^{-2} mol L^{-1} exhibited cooperative kinetics for both adults (Fig. 8A) and juveniles (Fig. 8B). However, NH_4^+ stimulation was ≈65% greater in adults than in juveniles (Table 2). At fixed K^+ concentrations (1 mmol L^{-1} to 10 mmol L^{-1}) maximum rates varied little for the adult enzyme (Fig. 8A); rates in juveniles (Fig. 8B) were unchanged. Additional K^+ (1 mmol L^{-1} to 10 mmol L^{-1}) resulted in a 4-fold decrease in adult $K_{0.5}$ compared to a 2-fold decrease in juvenile $K_{0.5}$ (Table 2). No synergistic effects were seen with both K^+ and NH_4^+.

The inhibition of total *p*NPPase activity by ouabain suggests that the (Na^+, K^+)-ATPase constitutes 70 to 80% of the total K^+-phosphatase activity present in the microsomal preparation from adult and juvenile gills (Table 3). The lack of additional inhibitory effects by thapsigargin and ethacrynic acid seems to exclude Ca^{2+}- and Na^+-ATPases, respectively. Inhibition by ouabain plus orthovanadate, and ouabain plus theophylline, suggest a minor contribution of neutral phosphatases to ouabain-insensitive enzyme activity in adult shrimp, but not in juveniles. The substantial inhibition by oligomycin, aurovertin B and bafilomycin A_1 is a strong evidence for the presence of F_0-F_1- and V(H^+)-ATPases, respectively, in both adult and juvenile gill microsomal fractions.

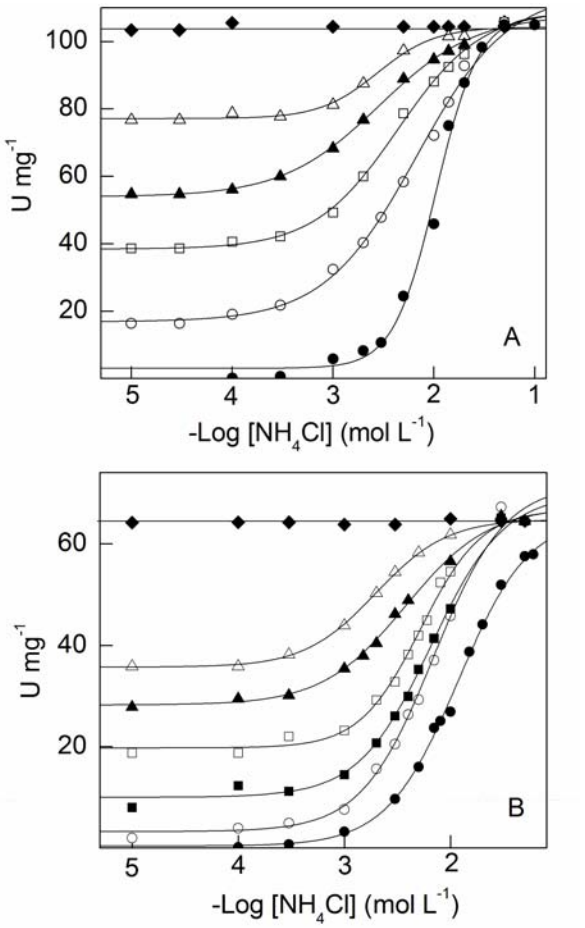

Fig. 8. Effect of NH_4Cl concentration on K^+-phosphatase activity in a gill microsomal fraction from *Macrobrachium amazonicum*, in the presence of KCl. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes, pH 7.5, containing 5 mmol L^{-1} $MgCl_2$ and 10 mmol L^{-1} *p*NPP in a final volume of 1.0 mL. Duplicate aliquots of three ($N = 3$) different gill microsomal preparations were used and the reaction was started by addition of 20.2 or 9.4 µg protein for adults and juveniles, respectively. Representative curves from one microsomal preparation are given. K^+ concentrations: (●) none; (○) 1 mmol L^{-1}; (■) 1.5 mmol L^{-1}; (□) 2 mmol L^{-1}; (▲) 3 mmol L^{-1}. (Δ) 5 mmol L^{-1}; (♦) 10 mmol L^{-1}. **A** - adult. **B** - juvenile.

Both F_0-F_1- and V(H^+)-ATPase activities are higher in adults compared to juveniles (6.8 U mg^{-1}).

DISCUSSION

We provide an extensive kinetic characterization of the modulation by *p*NPP, K^+, Mg^{2+} and NH_4^+, and inhibition by ouabain and Na^+, of gill microsomal

Table 2. Kinetic parameters for the stimulation by both K^+ and NH_4^+ of K^+-phosphatase activity in a microsomal fraction of gill tissue from adult and juvenile *Macrobrachium amazonicum*. Data are the mean ± SD (*N* = 3).

$[K^+]$ (mmol L^{-1})	$[NH_4^+]$ (mmol L^{-1})	V (U mg^{-1})		$K_{0.5}$ (mmol L^{-1})		n_H		(V/K)×10^6	
		Adult	Juvenile	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile
Variable	0	85.8 ± 1.3	65.7 ± 1.3	1.91 ± 0.05	1.77 ± 0.03	1.9	2.0	45	37
Variable	1	-	88.3 ± 2.6	-	1.92 ± 0.05	-	1.8	-	46
Variable	5	95.6 ± 3.5	75.1 ± 2.2	1.37 ± 0.05	1.22 ± 0.04	1.3	1.4	70	61
Variable	7	-	74.7 ± 2.3	-	1.20 ± 0.06	-	1.3	-	62
Variable	10	94.0 ± 3.4	84.6 ± 2.5	1.64 ± 0.06	1.70 ± 0.05	1.6	1.2	57	49
Variable	14	92.5 ± 3.4	-	2.06 ± 0.08	-	1.9	-	45	-
Variable	20	93.2 ± 3.4	77.3 ± 3.1	3.88 ± 0.14	0.50 ± 0.01	1.8	1.9	24	154
Variable	50	86.1 ± 3.1	66.7 ± 1.7	-	-	-	-	-	-
0	Variable	99.4 ± 4.8	65.7 ± 2.6	9.16 ± 0.44	9.40 ± 0.28	1.7	2.0	11	7
1	Variable	111.6 ± 4.6	66.7 ± 1.7	6.47 ± 0.26	5.66 ± 0.14	1.7	1.5	17	12
1.5	Variable	-	65.9 ± 2.6	-	5.30 ± 0.21		1.2	-	13
2	Variable	105.1 ± 4.3	63.2 ± 2.5	4.13 ± 0.17	5.12 ± 0.20	1.0	2.0	25	12
3	Variable	104.4 ± 4.3	62.9 ± 2.6	2.97 ± 0.12	3.50 ± 0.14	1.0	2.0	35	18
5	Variable	100.7 ± 4.1	65.7 ± 2.7	2.50 ± 0.10	4.57 ± 0.18	1.5	3.5	40	14
10	Variable	105.4 ± 4.3	62.5 ± 2.5	-	-	-	-	-	-

Table 3. Effect of various inhibitors on *p*-nitrophenylphosphatase activity in a microsomal fraction of gill tissue from adult and juvenile *Macrobrachium amazonicum*. Data are the mean ± SD (*N* = 3).

Inhibitor	V (U mg^{-1})		Specific ATPase activity (%)		P-type ATPase likely present
	Adult	Juvenile	Adult	Juvenile	
Control	119.0 ± 3.8	88.4 ± 3.5	-	-	-
Ouabain (3 mmol L^{-1})	34.0 ± 1.7	17.4 ± 0.9	71.4	80.3	Na^+, K^+
Orthovanadate (50 µmol L^{-1})	29.7 ± 1.1	18.0 ± 0.5	75.0	79.6	-
Ouabain (3 mmol L^{-1}) + Orthovanadate (50 µmol L^{-1})	28.6 ± 0.5	16.9 ± 0.2	4.5	-	Neutral phosphatase
Ouabain (3 mmol L^{-1}) + Ethanol (20 µL mL^{-1})	32.6 ± 0.8	17.6 ± 0.2	-	-	-
Ouabain (3 mmol L^{-1}) + DMSO (20 µg mL^{-1})	34.7 ± 0.9	17.3 ± 0.5	-	-	-
Ouabain (3 mmol L^{-1}) + Aurovertin B (10 µmol L^{-1})	20.4 ± 1.2	10.3 ± 0.1	11.4	8.0	F_0F_1
Ouabain (3 mmol L^{-1}) + Oligomycin (1.0 µg mL^{-1})	19.4 ± 0.9	9.8 ± 0.4	12.2	8.6	F_0F_1
Ouabain (3 mmol L^{-1}) + Bafilomycin A_1 (0.4 µmol L^{-1})	15.0 ± 0.7	10.6 ± 0.4	16.0	7.7	$V(H^+)$
Ouabain (3 mmol L^{-1}) + Ethacrynic acid (2 mmol L^{-1})	34.0 ± 1.5	18.3 ± 0.7	-	-	Na^+
Ouabain (3 mmol L^{-1}) + Theophylline (5 mmol L^{-1})	28.5 ± 0.6	18.3 ± 0.7	4.5	-	Neutral phosphatase
Ouabain (3 mmol L^{-1}) + Thapsigargin (0.5 µmol L^{-1})	34.7 ± 2.1	17.6 ± 0.5	-	-	Ca^{2+}

(Na^+, K^+)-ATPase activity in adult and juvenile *M. amazonicum*. There are no remarkable differences in the enzyme kinetic parameters regarding gill K^+-phosphatase activity in the two life cycle stages, and the enzyme affinities for *p*NPP and ions are similar. The lack of synergistic stimulation of *p*NPP hydrolysis by NH_4^+ plus K^+ suggests that the NH_4^+ binding sites are unavailable for cation binding in the E_2 enzyme form of both ontogenetic stages.

While ATP has been used to estimate (Na^+, K^+)-ATPase specific activity in crustacean gill homogenates from different ontogenetic stages [57] or when acclimated to different salinities [62-65], studies using *p*NPP reveal the convenience of this synthetic substrate for comparative studies [20-22]. The gill K^+-phosphatase activity of adult and juvenile *M. amazonicum* is about 2-fold less than the respective ATPase activity of (Na^+, K^+)-ATPase [57]. There is a similar ratio between these two activities for the gill enzyme from *Callinectes danae* [21], *Clibanarius vittatus* [66], *Macrobrachium olfersi* [20, 67], wild type *M. amazonicum* [68], and *Cancer pagurus* axon membranes [25]. Like *M. olfersi* [20], *C. danae* [21] and *C. vittatus* [69], a single family of binding sites for *p*NPP hydrolysis is also present in *M. amazonicum*. However, this single binding site contrasts with the high- and low-affinity binding sites, revealed by a biphasic curve for ATP hydrolysis in *C. danae* [70], *C. vittatus* [66], *Dilocarcinus pagei* [71] and wild type *M. amazonicum* [68]. The absence of high-affinity sites for *p*NPP hydrolysis by the juvenile and adult enzymes is consistent with the negligible hydrolytic activity detected below 10^{-5} mol L^{-1} *p*NPP. The gill enzyme affinity for *p*NPP of adult ($K_{0.5}$ = 1.52 mmol L^{-1}) and juvenile (1.45 mmol L^{-1}) *M. amazonicum* is similar to that for many crustaceans (1.0 to 2.5 mmol L^{-1}) [18, 20, 21, 56, 67, 72].

$K_{0.5}$ values of around 1 mmol L^{-1}, calculated for stimulation by Mg^{2+} of the K^+-phosphatase activity of juvenile and adult *M. amazonicum*, were similar to those for *C. vitattus* [69], *C. pagurus* [25], *C. danae* [21], *M. olfersi* [20, 67] and for wild type *M. amazonicum* [56].

Except for *C. pagurus* [18] site-site interactions have been reported for Mg^{2+} stimulation of K^+-phosphatase activity in several crustaceans [20, 21, 56, 67-69]. Magnesium ions are essential for the ATPase and K^+-phosphatase activities of (Na^+, K^+)-ATPases from various sources [12, 20, 21, 24, 56, 67, 73, 74], and in the presence of both ATP and Na^+, Mg^{2+} induces immediate enzyme phosphorylation [75, 76]. Consequently, Mg^{2+} binding cannot be separated from the phosphorylation reaction, and it is easier to study Mg^{2+} stimulation using *p*NPP rather than ATP. At least one separate Mg^{2+}-binding site is important for both ATPase and *p*NPPase activities [77]. However, in certain species, excess Mg^{2+} may inhibit K^+-phosphatase activity reflecting competition between Mg^{2+} and K^+ for the K^+-activating sites of the phosphatase reaction [28]. In mammals, Mg^{2+} inhibition has been attributed to binding to the E_2K enzyme form, decreasing affinity for ATP at the rate limiting step of the reaction cycle [78, 79].

The gill enzyme from adult and juvenile *M. amazonicum* does not hydrolyze *p*NPP in the presence of Na^+ alone, a characteristic noted by others [12]. However, at saturating K^+ concentrations, *p*NPPase activity is strongly inhibited (≈90%) by high Na^+ concentrations [20-22, 67, 69, 72].

Stimulation by both K^+ and NH_4^+ of *M. amazonicum* gill K^+-phosphatase activity disclosed kinetic properties comparable to those of *C. danae* [21, 72] and *M. olfersi* [80], which contrasts with the unique synergistic stimulation of K^+-phosphatase activity seen in *C. ornatus* acclimated to low salinity [22]. $K_{0.5}$ values for K^+-stimulated K^+ -phosphatase activity in juveniles (1.77 ± 0.03 mmol L^{-1}) and adults (1.91 ± 0.03 mmol L^{-1}) are similar to those for ATP hydrolysis [57] and for gill microsomal K^+-phosphatase activity in *M. olfersi* [20], *C. danae* [21] and *C. ornatus* [22]. The high $K_{0.5}$ values for NH_4^+ stimulation of adult (9.16 ± 0.12 mmol L^{-1}) and juvenile (9.4 ± 0.18 mmol L^{-1}) *M. amazonicum* are similar to those for *C. danae* [21, 72], *M. olfersi* [80] and *C. ornatus* [22]. Stimulation of *p*NPP hydrolysis by NH_4^+ may reflect substitution of K^+ by NH_4^+ at the same binding site [81].

Synergistic stimulation of ATP hydrolysis by K^+ and NH_4^+ is a well-known feature of the crustacean gill (Na^+, K^+)-ATPase [64-66, 68]. K^+ and NH_4^+ can bind at different sites on the enzyme molecule [70] or NH_4^+ can bind to two sites while K^+ binds to only one site [82]. However, except for 21‰ salinity-acclimated *C. ornatus* [22], *p*NPP hydrolysis

by the crustacean enzyme is not synergistically stimulated, apparently owing to the fact that the sites responsible for stimulation of (Na^+, K^+)-ATPase activity by NH_4^+ (or K^+) under saturating K^+ (or NH_4^+) concentrations are unavailable for cation binding to the E_2 enzyme form.

The single ouabain titration curves obtained for the gill microsomal enzymes from juvenile and adult *M. amazonicum* exclude the presence of different (Na^+, K^+)-ATPase isoforms. Ouabain inhibits K^+-phosphatase activity with apparent K_I's of 156.0 ± 6.2 and 142.0 ± 4.6, for adult and juveniles, respectively. Apparently, life cycle stage does not affect ouabain inhibition of K^+-phosphatase activity. Estimated K_I values are similar to those for *Xiphopenaeus kroyeri* [83] and 21‰ salinity-acclimated *C. ornatus* [22], but are discretely higher compared to fresh caught *C. danae* [21] and *M. olfersi* [20]. K_I values for ouabain inhibition of K^+-phosphatase activity of the (Na^+, K^+)-ATPase from diverse sources range from 3 μmol L^{-1} to 200 μmol L^{-1} [11, 25]. Interestingly, ouabain-insensitive *p*NPPase activity increased 2-fold in the adults, suggesting that during development from the juvenile stage onwards phosphohydrolytic enzymes other than the (Na^+, K^+)-ATPase may be required for osmoregulation.

The partial inhibition of total *p*NPPase activity by ouabain suggests the presence of phosphohydrolytic enzymes other than the (Na^+, K^+)-ATPase that can hydrolyze *p*NPP under the experimental conditions employed. The additional inhibition by oligomycin and aurovertin B of this ouabain-insensitive K^+-phosphatase activity strongly suggests the presence of an F-type ATPase in the microsomal fraction. Mitochondrial membrane fragments may contaminate the preparation, given that the gill intralamellar septal cells of *M. amazonicum* contain abundant mitochondria [44, 56]. The inhibition by bafilomycin A_1 of ouabain-insensitive K^+-phosphatase activity suggests the presence of a gill $V(H^+)$-ATPase, corroborating the $V(H^+)$-ATPase mRNA expression seen in *M. amazonicum* gill tissue [44].

The crustacean gill epithelium continues to provide an insightful model for investigating kinetic regulatory capabilities and responses. The (Na^+, K^+)-ATPases examined to date in the different crustacean groups clearly exhibit different kinetic behaviors and site-site interactions, and their comparative investigation should help to elucidate the nature of the biochemical processes underlying osmoregulatory ability.

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CONFLICT OF INTEREST STATEMENT

We state that there are no conflicts of interest with respect to the present article.

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