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Polyamines regulate phosphorylation–dephosphorylation kinetics in a crustacean gill (Na^+, K^+)-ATPase

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Abstract Aiming to clarify the mechanism of inhibition of (Na^+, K^+)-ATPase activity by polyamines, we examined the effects of exogenous putrescine, spermidine, and spermine on the kinetic behavior of phosphoenzyme-linked partial reactions using a microsomal gill (Na^+, K^+)-ATPase from juvenile and adult *M. amazonicum*, a freshwater palaemonid shrimp. The time course of phosphointermediate formation is greater (0.089 ± 0.006 s^{-1}) in adults than in juveniles (0.053 ± 0.003 s^{-1}) for spermidine, but similar to juveniles (0.059 ± 0.004 s^{-1}) for putrescine. Maximum phosphointermediate formation for the (Na^+, K^+)-ATPase from juveniles decreased by 46% and 32% with spermidine and putrescine, respectively. In adults, maximum phosphointermediate levels decreased by 50% and 8%, respectively. For both spermidine and putrescine, dephosphorylation rates were higher for adults than for juveniles, and were higher than in controls without polyamines. Spermine had a negligible effect (<10%) on phosphorylation/dephosphorylation rates of both juvenile and adult enzymes. This is the first report on the effects of polyamines on phosphoenzyme-linked partial reactions in juvenile and adult *M. amazonicum* gill (Na^+, K^+)-ATPases. Our findings suggest that the phosphorylation/dephosphorylation steps of this gill enzyme may be regulated by polyamines during ontogenetic development.

Keywords (Na^+, K^+)-ATPase · Phosphorylation · Dephosphorylation · Polyamines · Freshwater shrimp, *Macrobrachium amazonicum* · Ontogenetic stage

Introduction

The sodium–potassium ATPase is an ATP-powered ion pump that establishes elevated Na^+ and K^+ concentration gradients across the plasma membranes of all animal cells by exchanging cytoplasmic Na^+ for extracellular K^+, converting the chemical energy derived from ATP hydrolysis into asymmetrical ion distributions [1, 2]. The (Na^+, K^+)-ATPase is an oligomeric, tissue-specific protein [3], consisting of a catalytic α-subunit and a β-subunit in equimolar ratios, together with an FXYD2 peptide, the γ-subunit [4–6]. The α-subunit, of ≈110 kDa, consists of 10 transmembrane segments and contains the protein kinase, and the nucleotide-, ouabain- and cation-binding sites [7]. The β-subunit, of ≈31 kDa, is a highly glycosylated, single span, type II membrane protein associated with transmembrane helices αM7 to αM10 [8]. This subunit is required for the correct folding, stabilization, and expression of the active α-subunit protomer in the plasma membrane, and for occlusion of the K^+ binding sites [9]. In many cell types, the αβ-complex interacts with a small regulatory transmembrane protein belonging to the FXYD

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family, a group of small amphiphilic peptides that exhibit the FXYD motif. This ≈7-kDa γ-subunit is a single-span membrane protein associated with transmembrane helix αM9, and regulates pump activity [5, 10, 11]. This γ-subunit (FXYD2) represents a functional constituent of the crustacean (Na^+, K^+)-ATPase [12] that responds to pig FXYD2 by increasing NH_4^+ affinities and the maximum rate of ATP hydrolysis, without causing major changes in ATP or Na^+ and K^+ affinities [12].

Like other P-type ATPases, the (Na^+,K^+)-ATPase forms a phosphorylated enzyme intermediate (EP) owing to the transfer of the γ-phosphate from ATP to a conserved aspartate residue, D_{369}, in the P-domain [7, 13]. During its catalytic cycle, the (Na^+, K^+)-ATPase takes on either the E_1 or the E_2 conformation, depending on the binding, debinding and occlusion of Na^+ and K^+ at their respective ion-binding sites, and the phosphorylation or dephosphorylation of the D_{369} residue [13–16]. ATP and ADP bind to the (Na^+, K^+)-ATPase in the E_1 conformation with similar affinities, although the properties of the enzyme when in ATP and ADP-complexes are very different due to rearrangement of the N and A domains relative to the P domain [17]. Thus, ATP binding induces changes strikingly different from ADP binding, resulting in a structural transition from an'open' to a'closed' conformation that facilitates phosphorylation [18].

The same aspartic acid residue is phosphorylated both by ATP and inorganic phosphate and is mutually exclusive for these substrates [19]. Phosphorylation of the (Na^+, K^+)-ATPase by Mg^{2+}-ATP at low physiological Na^+ concentrations or by inorganic phosphate and Mg^{2+}, without K^+, results in the formation of phosphorylated intermediates, mainly E_2P. Dephosphorylation of E_2P formed from ATP is accelerated by K^+, but that from inorganic phosphate is retarded [20]. E_2P is the main component of the phosphorylated enzyme while E_1P occurs only at high Na^+ concentrations or when the enzyme is partially inhibited by N-ethyl maleimide or oligomycin [21, 22]. E_1P may not bind to ouabain while E_2P binds to ouabain in the absence of free Mg^{2+} [23, 24].

Enzyme phosphorylation is not adequately explained by the Albers-Post model, since the sum of ADP-sensitive and K^+-sensitive pools of the measured phosphoenzyme excede total EP by 150% [25]. At least one more phosphorylated intermediate is known from electric eel and pig kidney preparations [25–27]. Thus, EP is sensitive to both ADP and K^+ and is likely an intermediate form that appears after the formation of E_1P and before E_2P, carrying at least one Na^+ still bound at the cation sites [25]. Several signaling pathways are thought to regulate (Na^+, K^+)-ATPase function; however, most of the mechanisms underlying phosphorylation remain elusive. Indeed, whether phosphorylation is important for auto-regulation of (Na^+, K^+)-ATPase activity should be investigated to clarify the role of phosphorylation in modulating enzyme activity [28].

In osmoregulating crustaceans, various organs such as the gills, antennal glands, and intestine are involved in ion transport [29–31]. Various enzymes and ion transporters participate in translocating ions across the gill epithelia, including the (Na^+,K^+)-ATPase, V(H^+)-ATPase and carbonic anhydrase, and the Cl^-/HCO_3^- and Na^+/H^+ exchangers [32, 33]. Although its role in osmoregulation varies depending on the organism and cell type, the (Na^+, K^+)-ATPase is the main enzyme that underpins osmoregulatory ability [33–35]. The (Na^+, K^+)-ATPase is particularly abundant in the basal invaginations of the gill epithelial ionocytes [36–38].

The Amazon River shrimp *Macrobrachium amazonicum* is endemic to South America [39, 40] and its presumptive natural distribution includes the Orinoco, Amazon, and Paraguay/Lower Paraná river basins [41]. This diadromous shrimp has diversified into coastal populations that inhabit rivers close to estuaries, and continental populations living in rivers, lakes, and other inland water bodies [42, 43]. Based on significant morphological differences between geographically separated populations from the Amazon delta and the Pantanal region of Brazil, the latter population has been designated as a new species, *Macrobrachium pantanalensis* [44]. Although these two groups differ in external morphology and meristic characters [45], recent findings suggest, however, that these populations belong to the same species [46]. 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 [47]. Adult *M. amazonicum* are strong hyperosmotic and ionic regulators [48], an ability underpinned by gill (Na^+, K^+)-ATPase activity that has been kinetically characterized in several ontogenetic stages [49–51].

The polyamines putrescine, spermidine, and spermine are ubiquitous, polycationic metabolites present in both prokaryotic and eukaryotic cells that play various roles in cell growth and differentiation. They are positively charged, basic nitrogen compounds (Fig. 1) of low molecular weight, synthesized by microorganisms, plants, and animals. Their regulated biosynthetic pathways are very intricate and have attracted much attention in recent decades [52–56]. Two general mechanisms regulate intracellular polyamine titers: ATP concentration and Na^+ gradient-dependent transport across the cell membrane, together with *de novo* biosynthesis [57–60]. Polyamines are presumed to have multiple effects on a large number of cellular events such as stabilization of acidic cellular components [52–54]; modulating V(H^+)-ATPase pump activity [56]; interacting with membrane components [61–63]; formation of ternary compounds with Mg^{2+}-ATP affecting the catalytic activity

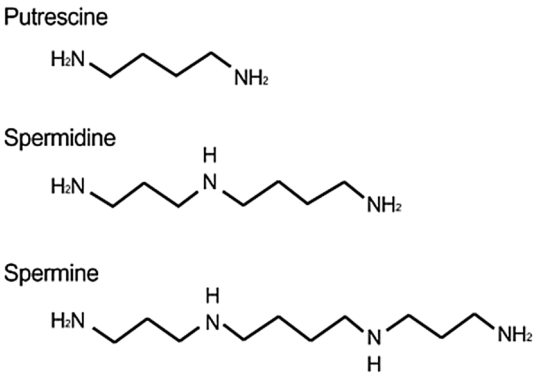

Fig. 1 Molecular structure of putrescine, spermidine and spermine

of protein kinases [64]; acting as scavengers of reactive oxygen species and free radicals and as stimulators of the cellular antioxidant system [65]; binding to phospholipids [66]; inhibiting (Na^+, K^+)-ATPase activity in various vertebrate tissues by binding to amino acid residues [67–71]; affecting mitochondrial calcium homeostasis; modulating RNA-, DNA- and ATP-related functions; and modulating ion channel function [53].

In euryhaline decapods, polyamines present in the hemolymph and gill tissues [72, 73] may be involved in osmoregulatory mechanisms [72, 74, 75]. Putrescine, spermidine, and spermine titers of between 16 and 150 nmol g^{-1} wet tissue have been measured in the gills of the crabs *Eriocheir sinensis* and *Callinectes sapidus* [72, 73]; the anterior and posterior gills of *E. sinensis* show differences in polyamine concentrations [73]. Exposure of *C. sapidus* to seawater increases putrescine and spermidine titers in the posterior gills and decreases (Na^+, K^+)-ATPase activity [72]. Putrescine, spermine, and spermidine used at 1 to 5 mmol L^{-1} in vitro inhibit *C. danae* gill (Na^+, K^+)-ATPase by 40% [74]. Competition between spermine and spermidine and Na^+ and K^+ for the cation binding sites on the enzyme affect both V_M and K_M for ATP hydrolysis by the gill (Na^+,K^+)-ATPase of low salinity-acclimated *C. ornatus* [75]. The effects of exogenous polyamines on gill microsomal (Na^+, K^+)-ATPase activity in the freshwater shrimp *M. amazonicum* at varying ATP, Mg^{2+}, Na^+, and K^+ concentrations [76] reveal that over the range of 10^{-5} to 2.10^{-1} mol L^{-1}, putrescine and spermidine, respectively, inhibited activity in juveniles by 43% and 97%, and in adults by 35% and 72%. Spermine had no effect in either stage [76]. K_I values for inhibition by spermidine and putrescine of (Na^+, K^+)-ATPase activity in juveniles were, respectively, 3.2 ± 0.2 mmol L^{-1} and 55.8 ± 1.7 mmol L^{-1}, and 14.3 ± 1.1 mmol L^{-1} and 23.7 ± 1.6 mmol L^{-1} in adults (M.N. Lucena, unpublished data). These findings reveal ontogenetic stage-specific effects, although the role of polyamines in regulating (Na^+, K^+)-ATPase activity remains to be clarified. Some organisms respond to saline stress by increasing polyamine titers [77]. However, little information is available on the effects of polyamines either on activity or phosphorylation of the crustacean (Na^+, K^+)-ATPase *in vitro* [74, 75]. This plethora of sometimes conflicting results does not explain adequately the role of polyamines in either osmoregulation or the ontogeny of crustaceans.

To better understand the mechanism by which polyamines inhibit ATP hydrolysis by the gill (Na^+, K^+)-ATPase in the present study, we examine the effects of spermidine, putrescine, and spermine on the formation, stability, and dephosphorylation of the (Na^+, K^+)-ATPase phosphoenzyme.

Materials and Methods

Material

All solutions were prepared using Millipore MilliQ ultrapure, apyrogenic water. Tris (hydroxymethyl) amino methane (Tris), ATP di-Tris salt, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), imidazole, pyruvate kinase (PK), lactic dehydrogenase (LDH), NADH, EDTA, perchloric acid, phosphoric acid, sucrose, sodium pyrophosphate, putrescine, spermidine, and spermine were purchased from the 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). [γ-^{32}P]P_i was from the Brazilian Institute for Atomic Energy (IPEN). All enzymes employed in [γ-^{32}P]ATP synthesis were purchased from Boehringer Mannheim (Germany).

Shrimps

Amazon river shrimps, *Macrobrachium amazonicum*, were produced at the Aquaculture Center, UNESP, Jaboticabal, São Paulo, Brazil from brood stock collected in fresh water at Furo das Marinhas near Santa Bárbara do Pará (1° 13′ 25″ S; 48° 17′ 40″ W), northeastern Pará State, Brazil, in 2001 [78]. Juveniles were collected from freshwater rearing tanks and held in carboys containing 32 L aerated fresh water from the rearing tank. Adult male and non-ovigerous female shrimps were collected from freshwater ponds and maintained in carboys containing 32 L aerated pond water. Juveniles and adults were used in stage C of the intermolt cycle, confirmed by stereoscopic microscopy [79]. The juvenile is an early benthonic freshwater stage while adult shrimps are well established in fresh water.

Gill dissection

For each homogenate prepared, shrimps were anesthetized by chilling on crushed ice immediately before dissection and gill homogenization. After removal of the branchiostegites, all the gills from juvenile (20 individuals/preparation, ≈700 µg wet gill mass) and adult (20 individuals/preparation, ≈6 g wet gill mass) shrimps were rapidly dissected, diced, and homogenized in a Potter homogenizer (600 rpm) in 20 mmol L^{-1} imidazole buffer, pH 6.8, containing 6 mmol L^{-1} EDTA, 250 mmol L^{-1} sucrose, and a protease inhibitor cocktail (20 mL buffer/g wet tissue). A high-yield, gill microsomal fraction was prepared by differential centrifugation as follows.

Preparation of gill microsomes

After centrifuging the crude extract at 20,000×g for 35 min at 4 °C, the supernatant was placed on crushed ice and the pellet was re-suspended in an equal volume of the imidazole homogenization buffer. After further centrifugation as above, the two supernatants were gently pooled and centrifuged at 100,000×g for 90 min at 4 °C. The resulting pellet, containing the microsomal fraction, was homogenized in 20 mmol L^{-1} imidazole buffer, pH 6.8, containing 6 mmol L^{-1} EDTA and 250 mmol L^{-1} sucrose (15 mL buffer/g wet tissue). Finally, 0.5-mL aliquots were rapidly frozen in liquid nitrogen and stored at −20 °C. No appreciable loss of (Na^+, K^+)-ATPase activity was seen after two-month's storage at −20 °C. Enzyme activity, measured immediately after microsome preparation, was considered to represent 100% (Na^+, K^+)-ATPase activity (activity at time = 0 h). When required, the stored aliquots were thawed, placed on crushed ice and used within an 8-h period. Prior to all experiments, enzyme activity was measured. When activity was <80% of that measured at time = 0 h, the preparation was discarded.

Measurement of ATP hydrolysis

When necessary, ATPase activity was assayed at 25 °C using a pyruvate kinase/lactate dehydrogenase coupling system in which ATP hydrolysis was coupled to NADH oxidation according to [80]. The kinetic parameters V_M (maximum velocity), K_M (Michaelis–Menten constant), and the n_H (Hill coefficient) values were calculated according to [81].

Effect of exogenous polyamines on enzyme phosphorylation and dephosphorylation

The effect of exogenous spermidine and putrescine on phosphorylation and dephosphorylation of the gill (Na^+, K^+)-ATPase of juveniles was evaluated by preincubating the enzyme with 10 mmol L^{-1} spermidine or 25 mmol L^{-1} putrescine for 10 min, at 25 °C, before the phosphorylation/dephosphorylation assays. The adult gill preparation was preincubated with 20 mmol L^{-1} spermidine or 50 mmol L^{-1} putrescine. These concentrations correspond to those providing 50% inhibition of the respective (Na^+, K^+)-ATPase activity (for details see [76]). Controls were performed using choline chloride to evaluate the influence of ionic strength on inhibitory effects.

Synthesis of [γ-^{32}P]ATP

Synthesis of [γ-^{32}P]ATP was performed as described by [82] modified according to [83].

Gill (Na^+, K^+)-ATPase phosphorylation

The enzyme (30 µg) was preincubated at 4 °C for 10 min in an assay medium containing 50 mmol L^{-1} HEPES buffer, pH 7.5, 5 mmol L^{-1} $MgCl_2$, 100 mmol L^{-1} NaCl, and 0.5 mmol L^{-1} EGTA with or without spermine, spermidine, or putrescine. The phosphorylation reaction was initiated by adding 0.02 mL of 1.25 mmol L^{-1}[γ-^{32}P]ATP in a final volume of 0.5 mL. After 5 min at 4 °C, the phosphorylation reaction was stopped by adding 200 µL of 125 mmol L^{-1} perchloric acid containing 5 mmol L^{-1} phosphoric acid and 5 mmol L^{-1} sodium pyrophosphate. The resulting mixture containing the ^{32}P-phosphorylated enzyme (EP) was filtered on 0.45 µm HAWP 29,325 Millipore filters. The filters were washed three times with 2 mL of 125 mmol L^{-1} perchloric acid then four times with 50 mmol L^{-1} perchloric acid [84] and were dried over an air flow. Radioactivity was counted using a Packard Tri-Carb 2100 LSC Liquid Scintillator spectrometer. Controls were performed using the enzyme previously denatured with perchloric acid before the addition of [γ-^{32}P]-ATP. Each phosphorylation curve was repeated three times using a different microsomal preparation ($N = 3$).

Dephosphorylation of the [γ-^{32}P]ATP-phosphorylated gill (Na^+, K^+)-ATPase

After 5-min phosphorylation of the gill (Na^+,K^+)-ATPase by 0.02 mL of 1.25 mmol L^{-1} [γ-^{32}P]ATP at 4 °C, as above, EP dephosphorylation was initiated using an eightfold dilution (3.8 mL final volume of non-radioactive assay reaction) with 12.5 mmol L^{-1} ATP, prepared in the same assay medium, and performed for up to 60 s [85, 86]. The radioactivity remaining on the filters was estimated as above. Each dephosphorylation curve was repeated three times using a different microsomal preparation ($N = 3$).

Effect of K^+ on [γ-^{32}P]ATP-phosphorylated gill (Na^+, K^+)-ATPase dephosphorylation

After 5-min phosphorylation by 0.02 mL of 1.25 mmol L^{-1} [γ-^{32}P]ATP of the gill (Na^+,K^+)-ATPase, at 4 °C, as above, EP dephosphorylation was performed for 60 s at 0 °C, by adding K^+ over a wide concentration range (5.10^{-5} to 5.10^{-2} mol L^{-1}). The radioactivity remaining on the filters was estimated as above. Each dephosphorylation curve was repeated three times using a different microsomal preparation ($N=3$).

Measurement of protein

Protein concentration was estimated according to [87], using bovine serum albumin as the standard.

Estimation of kinetic constants for enzyme phosphorylation/dephosphorylation

The time course of (Na^+, K^+)-ATPase phosphoenzyme formation (phosphorylation) was characterized as a first-order rate constant, k_{obs}, calculated by the ratio Ln $2/t_{0.5}$, where $t_{0.5}$ is the time for phosphorylation to reach half $[EP]_{max}$ [85]. The first-order phosphorylation rate constant, k_{phos}, was calculated from the ratio $r_0/[EP]_{max}$ [85], where r_0 represents EP formation (nmol.mg protein^{-1}) at time $=t_{0.5}$ (s). The dephosphorylation curves were characterized by first-order dephosphorylation rate constants for the disappearance of EP, k_{dephos}, estimated from the ratio Ln $2/t_{0.5}$ for the decay of EP.

Statistical analyses

Data were analyzed using a one-way analysis of variance (polyamine) followed by Student–Newman–Keuls (SNK) multiple means testing. Effects and differences were considered significant at $P \leq 0.05$. The kinetic parameters furnished in the tables are calculated values and represent the mean (±SEM) derived from the three ($N=3$) different microsomal preparations.

Results

Demonstration of the absence of sealed membrane vesicles

The (Na^+, K^+)-ATPase activity of gill microsomal preparations from juvenile and adult shrimps, assayed without alamethicin showed maximum activities of 194.4 ± 9.6 and 133.3 ± 6.4 nmol Pi min^{-1} mg^{-1} protein, respectively. (Na^+, K^+)-ATPase activity assayed with increasing alamethicin concentrations (1 to 20 µg/µg microsomal protein) showed activities of 190.8 ± 10.6 and 139.3 ± 7.9 nmol Pi min^{-1} mg^{-1} protein for juveniles and adults, respectively. These findings indicate that sealed vesicles were not present in the assay reaction and that the substrate is fully accessible to the enzyme.

Effect of spermidine and putrescine on [γ-^{32}P]ATP phosphorylation of gill (Na^+, K^+)-ATPase

Spermidine and putrescine inhibited (Na^+, K^+)-ATPase phosphorylation in juveniles and adult shrimp gill preparations. In the absence of spermidine or putrescine, maximum EP formation in juveniles was 1.57 ± 0.14 nmol.mg^{-1} (Fig. 2), decreasing to 0.84 ± 0.05 nmol.mg protein^{-1} and 1.06 ± 0.09 nmol.mg protein^{-1} with spermidine or putrescine, respectively. These values represent decreases of 46% and 32% with respect to the maximum capacity of EP formation. Compared to the control first-order rate constant (k_{obs} = 0.087 ± 0.005 s^{-1}), those for spermidine and putrescine were 0.053 ± 0.003 s^{-1} and 0.049 ± 0.003 s^{-1}, respectively. Using the $t_{0.5}$ from each phosphorylation curve, first-order rate constants (k_{phos}) of 0.063 ± 0.003 s^{-1}, 0.025 ± 0.001 s^{-1}, and 0.041 ± 0.002 s^{-1} were estimated for control, spermidine, and putrescine, respectively.

In adults, maximum EP formation without polyamines was 0.96 ± 0.08 nmol mg^{-1} (Fig. 2). With spermidine or putrescine, maximum rates of EP formation were 0.48 ± 0.04 nmol.mg protein^{-1} and 0.89 ± 0.07 nmol.mg protein^{-1}, respectively, representing 50% and 92% of the maximum capacity for EP formation. Rate constants (k_{obs}) for adult EP formation were 0.089 ± 0.006 s^{-1} and 0.059 ± 0.004 s^{-1} for spermidine and putrescine, respectively, similar to the control (0.079 ± 0.005 s^{-1}). Similar phosphorylation rate constants (k_{phos}) were estimated for spermidine (0.030 ± 0.001 s^{-1}) and putrescine (0.039 ± 0.002 s^{-1}), about ≈35% lower than the control (0.054 ± 0.003 s^{-1}) (Table 1).

Negligible inhibition (< 10%) was found for gill (Na^+, K^+)-ATPase phosphorylation by [γ-^{32}P] in the presence of spermine for both juveniles and adults (data not shown).

Effect of spermidine and putrescine on K^+-mediated dephosphorylation of [γ-^{32}P]ATP-phosphorylated gill (Na^+, K^+)-ATPase

In the absence of polyamines and K^+, the maximum EP titer for juvenile (Na^+, K^+)-ATPase was 1.57 ± 0.14 nmol.mg protein^{-1} (Fig. 3). However, with K^+ from 10 to 4 mol L^{-1} to 3.10^{-2} mol L^{-1}, EP levels decreased to 0.11 ± 0.008 nmol.mg protein^{-1}. With putrescine and without K^+, the maximum EP titer was 1.06 ± 0.09 nmol.mg protein^{-1} decreasing to 0.02 ± 0.001 nmol.mg protein^{-1}

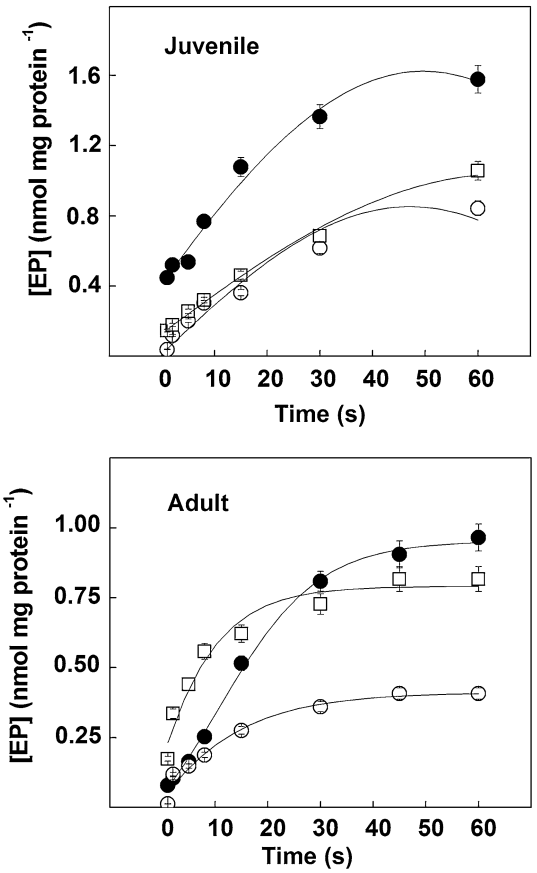

Fig. 2 Effect of spermidine and putrescine on [γ-^{32}P]ATP phosphorylation of gill (Na^+, K^+)-ATPase from juvenile and adult *M. amazonicum*. The gill enzyme from juvenile or adult (≈30 µg) shrimps was preincubated for 10 min at 4 °C in the assay medium; phosphorylation was initiated by adding 1.25 mmol L^{-1} [γ-^{32}P]ATP, as described in the "Material and methods" section. Mean values from duplicate reactions were used to fit each corresponding curve, which was repeated utilizing three different microsomal preparations (±SEM, $N=3$). Juvenile: (*filled circle*) Control; (*open circle*) 10 mmol L^{-1} spermidine; (*open square*) 25 mmol L^{-1} putrescine. Adult: (*filled circle*) Control; (*open circle*) 20 mmol L^{-1} spermidine; (*open square*) 50 mmol L^{-1} putrescine

with increasing K^+ over the same concentration range. Similarly, with spermidine and without K^+, maximum EP formation was 0.84 ± 0.06 nmol.mg $protein^{-1}$. As K^+ increased from 10 to 4 mol L^{-1} to 3.10^{-2} mol L^{-1}, EP level decreased to 0.01 ± 0.001 nmol.mg $protein^{-1}$.

In adults, maximum EP formation was 1.07 ± 0.08 nmol.mg $protein^{-1}$ without polyamines and K^+ (Fig. 3). K^+-stimulated dephosphorylation over the range $2.5.10^{-4}$ mol L^{-1} K^+ to 3.10^{-2} mol L^{-1} K^+ reduced EP levels to 0.11 ± 0.008 nmol.mg $protein^{-1}$. As seen in juveniles, with putrescine and spermidine and without K^+, maximum EP levels were 0.89 ± 0.06 nmol.mg $protein^{-1}$ and 0.48 ± 0.04 nmol.mg $protein^{-1}$, respectively. As K^+ increased over the range of $2.5.10^{-4}$ mol L^{-1} to 3.10^{-2} mol L^{-1}, EP levels decreased to 0.08 ± 0.004 nmol.mg $protein^{-1}$ and 0.009 ± 0.0001 nmol.mg $protein^{-1}$, for putrescine and spermidine, respectively.

Negligible EP title was obtained in the presence of spermine for both juveniles and adults (not shown).

Effect of spermidine and putrescine on dephosphorylation of γ-32[P]ATP-phosphorylated gill (Na^+, K^+)-ATPase

In the absence of polyamines and with 20 mmol L^{-1} KCl, dephosphorylation of juvenile EP decreased from 1.60 ± 0.13 nmol.mg $protein^{-1}$ to 0.58 ± 0.09 nmol.mg $protein^{-1}$ with a rate constant of 0.063 ± 0.003 s^{-1} after eightfold dilution (Fig. 4), a surprisingly high dephosphorylation offset (0.58 ± 0.09 nmol.mg $protein^{-1}$) not seen in adults. However, with putrescine the initial EP concentration decreased from 0.85 ± 0.08 nmol.mg $protein^{-1}$ to 0.08 ± 0.004 nmol.mg $protein^{-1}$ after a 60 s reaction (Fig. 4). For spermidine, the initial EP concentration decreased from 0.42 ± 0.04 nmol.mg $protein^{-1}$ to 0.009 ± 0.001 nmol.mg^{-1} (Fig. 4). Single dephosphorylation first-order rate constants (k_{dephos}) of 0.252 ± 0.015 s^{-1} and 0.207 ± 0.012 s^{-1} were estimated for spermidine and

Table 1 Effect of spermidine and putrescine on the time course ($t_{0.5}$) of phosphoenzyme formation (k_{obs}, k_{phos}), and on dephosphorylation (k_{dephos}) of [γ-^{32}P]ATP-phosphorylated gill (Na^+, K^+)-ATPase from juvenile and adult *M. amazonicum*

Parameter (s^{-1})	Juvenile			Adult		
	Control	Spermidine (10 mmol L^{-1})	Putrescine (25 mmol L^{-1})	Control	Spermidine (20 mmol L^{-1})	Putrescine (50 mmol L^{-1})
$t_{0.5}$	8.95	12.85	14.15	8.78	7.81	11.35
k_{obs}	0.087 ± 0.005	0.053 ± 0.003	0.049 ± 0.003	0.079 ± 0.005	0.089 ± 0.006	0.059 ± 0.004
k_{phos}	0.063 ± 0.003	0.025 ± 0.001	0.041 ± 0.002	0.054 ± 0.003	0.030 ± 0.001	0.039 ± 0.002
k_{dephos}	0.127 ± 0.007	0.252 ± 0.015	0.207 ± 0.012	0.022 ± 0.001[a] 0.376 ± 0.022[b]	0.115 ± 0.007[a] 0.888 ± 0.053[b]	0.017 ± 0.001[a] 0.410 ± 0.025[b]

[a]Phosphorylation fast step

[b]Phosphorylation slow step, estimated from respective components of the biphasic kinetic response

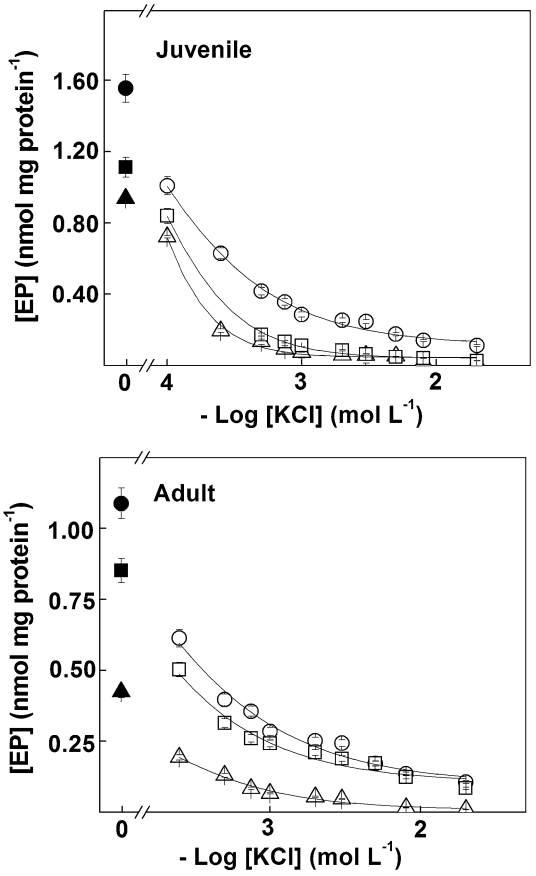

Fig. 3 Effect of spermidine and putrescine on K^+-mediated dephosphorylation of [γ-^{32}P]ATP-phosphorylated gill (Na^+,K^+)-ATPase from juvenile and adult *M. amazonicum*. The gill enzyme from juvenile or adult (≈30 µg) shrimps was preincubated for 10 min at 4 °C in the assay medium; phosphorylation was initiated by adding 1.25 mmol L^{-1} [γ-^{32}P]ATP, as described in the "Material and Methods" section. Dephosphorylation was initiated by adding increasing KCl concentrations to the reaction medium. Mean values from duplicate reactions were used to fit each corresponding curve, which was repeated using three different microsomal preparations (±SEM, $N=3$). Juvenile: (*open circle*) Control; (*open triangle*) 10 mmol L^{-1} spermidine; (*open square*) 25 mmol L^{-1} putrescine. Adult: (*open circle*) Control; (*open triangle*) 20 mmol L^{-1} spermidine; (*open square*) 50 mmol L^{-1} putrescine. Filled symbols (*filled circle, filled triangle, filled square*) represent the EP concentrations prior to the addition of K^+ for the control, spermidine, and putrescine assays, respectively, at the same polyamine concentrations

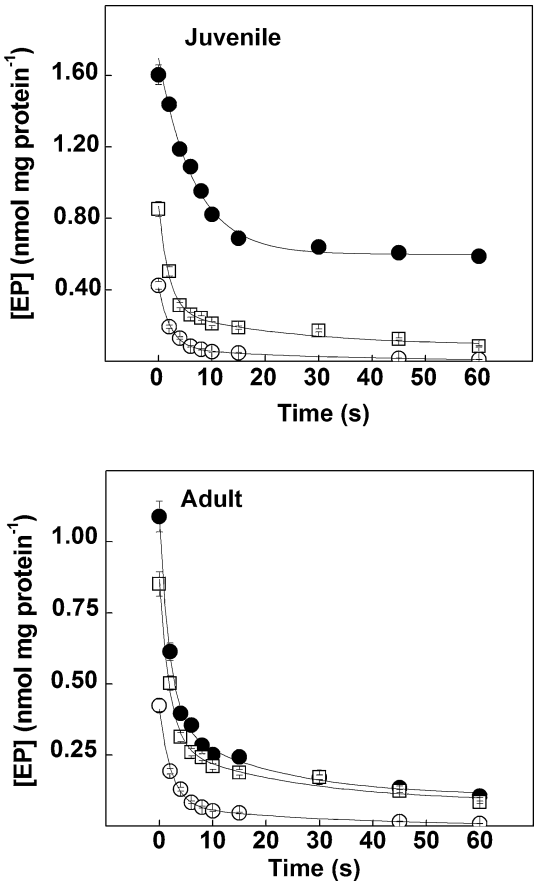

Fig. 4 Effect of spermidine and putrescine on dephosphorylation of [γ-^{32}P]ATP-phosphorylated gill (Na^+,K^+)-ATPase from juvenile and adult *M. amazonicum*. The gill enzyme from juvenile or adult (≈30 µg) shrimps was preincubated for 10 min at 4 °C in the assay medium; phosphorylation was initiated by adding 1.25 mmol L^{-1} [γ-^{32}P]ATP, as described in the "Material and methods" section. Dephosphorylation was initiated after 5-min reaction time by diluting the reaction medium eightfold with 12.5 mmol L^{-1} ATP. Mean values for duplicate reactions were used to fit each corresponding curve, each of which was repeated utilizing three different microsomal preparations (±SEM, $N=3$). Juvenile: (*filled circle*) Control; (*open circle*) 10 mmol L^{-1} spermidine; (*open square*) 25 mmol L^{-1} putrescine. Adult: (*filled circle*) Control; (*open circle*) 20 mmol L^{-1} spermidine; (*open square*) 50 mmol L^{-1} putrescine

putrescine, respectively, almost twofold greater than the control (k_{dephos}= 0.127 ± 0.007 s^{-1}) (Table 1).

Interestingly, for the adult enzyme, initial EP values of 1.08 ± 0.09 nmol.mg $protein^{-1}$ decreased to 0.13 ± 0.009 nmol.mg $protein^{-1}$, after 60 s, following a biphasic dephosphorylation kinetic process with rate constants of 0.022 ± 0.001 s^{-1} and 0.376 ± 0.022 s^{-1}, for the fast and slow dephosphorylation steps, respectively (Fig. 4; Table 1). Dephosphorylation assayed with putrescine resulted in EP concentrations varying from 0.80 ± 0.07 nmol.mg $protein^{-1}$ to 0.12 ± 0.008 nmol.mg $protein^{-1}$ after 60 s (Fig. 4). For spermidine, EP concentration decreased from 0.45 ± 0.03 nmol.mg $protein^{-1}$ to 0.009 ± 0.001 nmol.mg $protein^{-1}$ (Fig. 4). For both polyamines, dephosphorylation also followed a biphasic kinetic process. For the fast step, first-order rate constants of 0.115 ± 0.007 s^{-1} and 0.017 ± 0.001 s^{-1} were estimated for spermidine and putrescine, respectively (Table 1). For the slow step, first-order rate constants were 0.888 ± 0.053 s^{-1} and 0.410 ± 0.025 s^{-1} for spermidine and putrescine, respectively.

There was a negligible effect of spermine on gill (Na^+, K^+)-ATPase dephosphorylation by [γ-^{32}P] in both juveniles and adults (data not shown).

Discussion

We have examined the effects of the polyamines spermine, putrescine, and spermidine on the phosphorylation/dephosphorylation rates of a gill (Na^+, K^+)-ATPase from juvenile and adult *M. amazonicum*. Putrescine and spermidine impair either phosphoenzyme formation, and stimulate EP decomposition, which reduces the net hydrolysis velocity. The time course of phosphointermediate (EP) formation is greater in adult shrimps than in juveniles for spermidine, but similar for putrescine. Dephosphorylation rates were higher in adults than juveniles for spermidine and putrescine and were always higher than in controls without polyamines. Spermine had negligible effects on phosphorylation/dephosphorylation rates in both juvenile and adult shrimps.

Both spermidine and putrescine partially inhibited formation of the phosphorylated intermediate form of the gill (Na^+, K^+)-ATPase in adult and juvenile *M. amazonicum*. The inhibitory effect of spermidine is similar to that seen in the (Na^+, K^+)-ATPases from blue crab gill [74] and mammalian brain microsomal membrane [70]. However, although EP levels in adult shrimps estimated at a high Na^+ concentration are similar to those of the blue crab *C. danae* [74], putrescine had little effect on EP levels in adult shrimps, even at a high Na^+ concentration, suggesting that this polyamine stabilizes the steady-state phosphorylated intermediate in adult *M. amazonicum*.

The E_2P dephosphorylation is K^+-dependent and is enhanced by polyamines. This suggests that the binding of these organic cations to the (Na^+, K^+)-ATPase increases water entropy, destabilizing the acyl bond at the substrate binding site, increasing the rate of dephosphorylation [88]. Our findings suggest that polyamines inhibit *M. amazonicum* gill (Na^+, K^+)-ATPase activity during at least two steps of the catalytic cycle. Since putrescine and spermidine increased the EP dephosphorylation rate, polyamines may replace potassium ions at these cation sites, increasing dephosphorylation rates. However, these polyamines decreased EP formation, inhibiting ATP hydrolysis, with a possible increase in the E_1-Na^+ form, which may act as a rate-limiting step in the hydrolysis cycle of the *M. amazonicum* gill (Na^+, K^+)-ATPase.

In addition to charge density, data from [76] suggest that the effectiveness of polyamines as inhibitors may be species-specific. Spermidine, possessing three positive charges, inhibits (Na^+, K^+)-ATPase activity more effectively than putrescine with two positive charges; spermine, with four positive charges, has no inhibitory effect. In contrast to the *C. danae*, enzyme in which the greater size and charge density of spermine induced stronger inhibitory effects [74], in *M. amazonicum*, putrescine, that has a smaller size and charge density, was a more effective inhibitor of (Na^+, K^+)-ATPase activity than spermine. Apparently, the presence of four positive charges in spermine hinders its interaction with the inhibitory site on the *M. amazonicum* (Na^+, K^+)-ATPase, suggesting species-specific differences at the cation sites. The negligible inhibition by spermine of gill (Na^+, K^+)-ATPase activity in both juvenile and adult *M. amazonicum* contrasts with the considerable inhibition seen in *C. danae* (≈20%) and *C. ornatus* (≈58%) and *Clibanarius vittatus* (≈48%) [74, 75]. In vertebrate tissue, spermine stimulates (Na^+, K^+)-ATPase activity under specific low ionic and substrate concentrations [71]. However, it is difficult to envisage a role for spermine in modulating (Na^+, K^+)-ATPase activity in the ionocyte plasma membrane of *M. amazonicum* gills since this polyamine is restricted to the cell nucleus [55]. The high spermine concentration found in the gills of *C. sapidus* suggests that spermine may function as a salvage compound or reserve pool to be converted back to putrescine, as seen in vertebrates [72, 89].

Putrescine exerted a greater inhibitory effect on gill (Na^+, K^+)-ATPase activity in adult (≈37%) and juvenile (≈40%) *M. amazonicum* [76] compared to *C. danae* (≈20%) [74] and *C. ornatus* [75], but only slightly affected ATP binding in both juvenile and adult *M. amazonicum*. In the brine shrimp *Artemia franciscana* and in *C. sapidus*, the decrease in gill (Na^+, K^+)-ATPase activity is accompanied by an increase in putrescine concentration in the gill tissue [72, 90]. Putrescine increases the apparent $K_{0.5}$ for Na^+, allosterically affecting *C. sapidus* (Na^+, K^+)-ATPase activity [72]. Spermidine also affected the maximum rate of ATP hydrolysis in juveniles (≈60%) and adults (≈50%) [76] similarly to *C. ornatus* [75] and to *C. danae* [74]. The slight alterations in K_M for ATP in the *M. amazonicum* enzyme incubated with polyamines contrasts with the 5-fold greater affinity for the *C. ornatus* enzyme [75]. Apparently, the effects of polyamines on ATP hydrolysis by the *M. amazonicum* gill (Na^+, K^+)-ATPase reflect a mixed-type inhibition [76].

The greater the availability of positive charges in polyamine structure, the greater the ease of interaction with the cation-binding domain of the (Na^+, K^+)-ATPase, a motif classically described as rich in acidic amino acid residues in mammals [69, 91]. Owing to structure and charge differences, competition between Mg^{2+}-ATP and spermidine or putrescine for the same binding site on the (Na^+, K^+)-ATPase seems unlikely [67]. However, under suboptimal Na^+ and K^+ concentrations in the assay conditions, putrescine, spermine, and spermidine significantly

inhibit the ATPase reaction [70, 74, 75], suggesting that the polyamines probably act at multiple sites during the (Na^+, K^+)-ATPase reaction cycle, inducing conformational changes that prevent substrate binding [70]. However, spermidine did not affect (Na^+, K^+)-ATPase affinity for ATP [74]. These different inhibitory effects of polyamines on (Na^+, K^+)-ATPase are not uncommon. At physiological pH, their amino groups are protonated and the fact that these positive charges are distributed along the different lengths of the carbon chain may allow specific interactions of each polyamine, leading to different effects on different targets.

Polyamines inhibit pumping activity by competing with Na^+ at the Na^+-binding sites, and by inhibiting enzyme dephosphorylation [74]. Our data suggest that phosphorylation rates alone are reduced in the presence of polyamines, reinforcing their inhibitory profile in this partial reaction of the ATPase cycle. Since the phosphorylation- and cation-binding sites communicate through a helix extending from Ala_{749} to Phe_{786} (sheet α1), and since this helix begins about 0.5 nm from the phosphorylation site, enzyme phosphorylation may induce H5 helix movement, causing a local conformational change at the cation-binding site that modifies cation affinity [92]. Whether this finding reflects structural differences between the juvenile and adult enzymes remains to be clarified.

(Na^+, K^+)-ATPase activity is altered during the ontogeny of *M. amazonicum* [51]. However, whether these changes are due to regulation of pre-existing enzyme or to increased gene transcription and mRNA translation, or to post-translational modifications remains unclear. (Na^+, K^+)-ATPase from mammalian sources is regulated by protein kinase A, C and tyrosine kinase-related receptors like the insulin and EGF receptors [11, 93–96]. The effects of polyamines on the gill (Na^+, K^+)-ATPase behavior of *M. amazonicum* [76, 80] may reflect ontogenetic changes that correlate with the regulation of endogenous enzyme activity such as protein kinase phosphorylation or protein–protein interactions. Another important source of regulation comes from interaction with members of the FXYD family of proteins in which cation affinity and V_M are the parameters usually critically regulated by FXYDs [97, 98]. However, despite some findings on protein kinase and FXYD-linked regulation of the *C. danae* (Na^+, K^+)-ATPase [12], whether crustacean ATPases respond to these modulators similarly to the mammalian ATPase is largely unknown.

Conclusions

Polyamines may be involved in osmotic and ionic regulation by interacting directly with the (Na^+, K^+)-ATPase. They may be carried by the hemolymph to target tissues such as the gills when transport or metabolic adjustments are required [73]. Since polyamines can alter membrane permeability and ion transport [99], they may participate in adjustments to fluctuations in environmental salinity such as alterations in membrane permeability to water and ions. Our findings for juvenile and adult *M. amazonicum* suggest that the inhibitory effects of putrescine and spermidine on the kinetic behavior of the gill (Na^+, K^+)-ATPase may be both stage- and species-specific, and are apparently due to differences in phosphoenzyme formation/decomposition. However, whether the changes in (Na^+, K^+)-ATPase activity in the gills of *M. amazonicum* might be regulated *in situ* by polyamine levels remain to be investigated. Further elucidation of the biochemical and physiological functions of polyamines should contribute to a better understanding of their putative role in regulating cell activities.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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