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Na^+-dependent and Na^+-independent mechanisms for inorganic phosphate uptake in *Trypanosoma rangeli*

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ABSTRACT

Background: Trypanosoma rangeli is dependent on the presence of exogenous orthophosphate (P_i) for maximal growth and ecto-phosphatase activity is responsible for P_i supply under low P_i. Here we investigated the mechanisms of P_i uptake.
Methods: We investigated the kinetics of $^{32}P_i$ transport, its Na^+ and H^+ dependence, its correlation with the Na^+-ATPase and H^+-ATPase, and gene expression of the Na^+:P_i cotransporter and Na^+-ATPase.
Results: T. rangeli grown under limiting P_i transports this anion to the cytosol in the absence and presence of Na^+, suggesting that influx is mediated by both Na^+-independent and Na^+-dependent transporters. Cloning studies demonstrated that this parasite expresses a P_i transporter not previously studied in trypanosomatids. The H^+ ionophore, carbonylcyanide-p-trifluoromethoxyphenylhydrazone, decreased both components of $^{32}P_i$ influx by 80–95%. The H^+-ATPase inhibitor, bafilomycin A_1, inhibited the Na^+-independent mechanism. Furosemide, an inhibitor of ouabain-insensitive Na^+-ATPase, decreased both uptake mechanisms of $^{32}P_i$ to the same extent, whereas ouabain had no effect, indicating that the former is the pump responsible for inwardly directed Na^+ and the electric gradients required by the transporters. Parasite growth in high P_i had a lower P_i influx than that found in those grown in low P_i, without alteration in TrPho89 expression, showing that turnover of the transporters is stimulated by P_i starvation.
Conclusions: Two modes of P_i transport, one coupled to Na^+-ATPase and other coupled to H^+-ATPase seem to be responsible for P_i acquisition during development of *T. rangeli*.
General significance: This study provides the first description of the mechanism of P_i transport across the plasma membrane of trypanosomatids.

1. Introduction

Trypanosomatids are a group of protozoa that parasitize a large number of eukaryotic organisms [1], and in the Trypanosomatidae family, the genus *Trypanosoma* comprises digenetic flagellates that usually have insects as vectors which infect human beings and other animal hosts [1,2]. *Trypanosoma rangeli* is a hemoflagellate protozoan with special behavior because it infects humans, a great number of other mammals, and also its triatomine vectors [3]. Since human infection with *T. rangeli* has been described in several countries, the occurrence of single or infections mixed with *T. cruzi* must be considered in areas where these parasites are sympatric [4].

Little is known about energetic metabolism and transport of nutrients across the plasma membrane of *T. rangeli*, which is strongly dependent on the presence of inorganic phosphate (P_i) in the culture medium to achieve maximal growth [5]. We have recently demonstrated that P_i depletion increases ecto-phosphatase activity of the parasite [6], which was considered to be an adaptative mechanism to make P_i available for cell functioning. P_i is an essential nutrient for cell functions in all forms of life since it is required in most

Abbreviations: FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; LIT, liver infusion tryptose medium; TcENA, *T. cruzi* ouabain-insensitive Na^+-ATPase; TrENA, *T. rangeli* ouabain-insensitive Na^+-ATPase

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metabolic processes, including the biosynthesis of nucleic acids and phospholipids, energy metabolism, and signal transduction [7–9], with transport across the plasma membrane being the first step for its intracellular the utilization [8]. In *Saccharomyces cerevisiae*, two P_i transport systems have been detected, one has a low affinity for external P_i ($K_m \approx 800$ μM), and the other a higher affinity $K_m \approx 0.5$ μM) [10]. The high-affinity system consists of two P_i transporters, Pho84p and Pho89p; the first is a H^+:P_i cotransporter with an optimum acidic pH for P_i uptake [11], while Pho89p is a Na^+:P_i cotransporter that is more active at alkaline pH [12]. Expression of both PHO84 and PHO89 genes occurs in P_i starvation and is regulated by P_i through the kinase/cycline-mediated PHO pathway [13] in a process that ultimately leads to the scavenging and specific uptake of P_i from extracellular sources [14].

The aim of the present work has been to investigate: (i) whether *T. rangeli* has a P_i transport system and to measure the uphill P_i uptake capacity across the plasma membranes of parasites grown under P_i depletion, and (ii) any possible association with the transport of other species of ions. We have identified a Na^+-dependent and a Na^+-independent phosphate transporter in *T. rangeli* that seem to be functionally coupled to an ouabain-insensitive Na^+-ATPase and a bafilomycin A_1-sensitive H^+ pump, respectively.

2. Materials and methods

2.1. Materials

Reagents were purchased from Merck or Sigma Chemical Co., except where specifically stated. Radioactive orthophosphate ($^{32}P_i$) was purchased from Instituto de Pesquisas Energéticas e Nucleares (IPEN). [γ-^{32}]ATP was obtained as per Maia et al. [15]. Distilled water deionized with a MilliQ system of resins (Millipore Corp.) was used in the preparation of all solutions.

2.2. Parasites and growth conditions

Macias strain of *T. rangeli* (supplied by Dr Maria Auxiliadora Sousa, Fiocruz, Rio de Janeiro, Brazil) was maintained in liver infusion tryptose medium (LIT) supplemented with 20% fetal calf serum (Cripion) at 28 ± 2 °C, sub-cultivated in the same fresh medium for 5 days – when the parasites had reached stationary phase of growth – and used to obtain plasma membrane preparations. For PCR analysis, the cells were cultivated for 2 days when in exponential phase and at 5 days. The LIT medium used in the 5-day culture contained low P_i (2 mM), as previously described [6]; high P_i (50 mM) was used in some controls. Before the transport experiments, the parasites were collected from the culture medium by centrifugation (1500 ×*g* at 4 °C for 10 min) and washed 3 times with a cold buffer solution containing 100 mM sucrose, 20 mM KCl and 50 mM Tris–HCl (pH 7.2) to remove remaining traces of P_i. Unlabeled P_i was undetectable in the third supernatant of washing, using the Fiske and Subbarow method [16]. Parasite viability was assessed before and after incubation by observing their motility under a light microscopy, by Trypan blue dye exclusion [17], or by measuring the levels of lactate dehydrogenase activity in the medium [6]. For staining, the cells were incubated in the presence of 0.01% Trypan blue for 10 min in assay buffer. Viability was unaffected by the conditions employed in the experiments.

2.3. P_i transport assays

Intact cells (1.0×10^7 cells/ml) of *T. rangeli* were incubated at 25 °C in a reaction mixture (0.2 ml) containing (unless otherwise stated in the figure legends) 140 mM NaCl (or 140 mM choline chloride to study the Na^+-independent component of P_i transport), 5 mM KCl, 25 mM HEPES–Tris (pH 7.4), 1 mM $MgCl_2$ and 100 μM $^{32}P_i$ (0.5 mCi/μmol). Uptake was initiated by addition of $^{32}P_i$ and, except for Fig. 1, was arrested 60 min later by adding 0.2 ml of an ice-cold solution containing 140 mM choline chloride, 5 mM KCl, 25 mM HEPES–Tris (pH 7.4) and 1 mM $MgCl_2$. Following 3 washes with the same ice-cold buffer, the cells were disrupted by addition of 0.1% SDS and the suspension counted in a scintillation counter (Packard). Blank uptake values were obtained by exposing the cells to a cold $^{32}P_i$-reaction mixture kept on ice for 60 min [18]. For determination of the transport affinity ($K_{0.5}$) and maximal rate (V_{max}), $^{32}P_i$ uptake was measured at P_i concentrations ranging 5–500 μM in the absence or presence of Na^+. When the effect of Na^+ concentration on P_i uptake was analyzed, the P_i concentration was kept constant (100 μM) and NaCl varied from 0 (nominal) to 50 mM. Routine controls for Na^+ contamination in the assay solutions carried out by flame spectrometry indicated values of 0.1 mM or less. In this experiment, choline chloride was also used to keep medium osmolarity constant at ≈300 mOsM. When indicated, the Na^+ ionophore monensin (100 μM) was used to collapse the Na^+ gradient. To assess the effect of perturbation of the parasite plasma membrane potential gradient on P_i uptake (and possibly the H^+ distribution across the membrane), the H^+ ionophore, carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 10 μM), or the H^+-pump inhibitor, bafilomycin A_1 (100 nM), were used. The Na^+-dependent component of phosphate uptake in each condition was measured, in experiments run in parallel, as the difference between total uptake in the presence of Na^+ and that in the presence of choline chloride (Na^+-independent component).

2.4. TrPho89 cloning

A total of 10^8 parasites was homogenized in TRIzol (Invitrogen) and RNA extracted from the samples following the manufacturer's instructions. Total RNA concentrations were determined spectrophotometrically using Nanodrop ND-1000 (Thermo Fisher Scientific). Five micrograms of total RNA was used to synthesize cDNA samples using high-capacity cDNA reverse transcription kit (Applied Biosystems). TrPho89 RNA was amplified in a PCR reaction using Taq DNA Polymerase (Fermentas) under the following conditions: one cycle

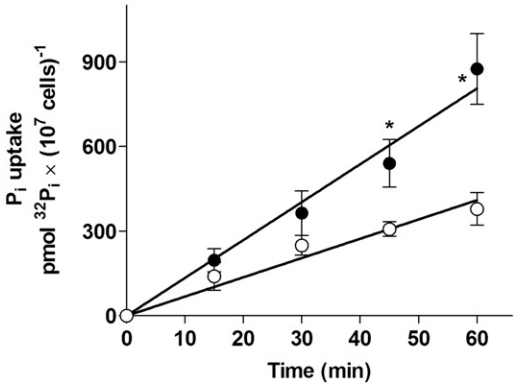

Fig. 1. Time-course of P_i uptake by epimastigote cells of *T. rangeli* by Na^+-independent and Na^+-dependent mechanisms. $^{32}P_i$ uptake was assayed in intact cells grown in P_i-depleted medium (with 100 μM $^{32}P_i$, at pH 7.4); see Materials and methods (subsection 2.3). Empty circles: Na^+-independent uptake measured in the presence of 140 mM choline chloride. Filled circles: Na^+-dependent component calculated, at each time, as the difference between the total uptake in the presence of 140 mM NaCl and the Na^+-independent uptake. At the times on the abscissa, the suspensions were filtered, washed and counted. Data are means ± S.E.M. of 6 determinations carried out with cell suspensions from different cultures. *Statistical difference ($P<0.05$) between the time-matched groups assayed by unpaired *t*-test.

for 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C, and finally one cycle for 10 min at 72 °C. PCR amplification was performed using primers designed for *T. cruzi* Pho89 (Genbank ID: XP813912) as follows:

TcPho89f, 5′-GGCTCCCTTAATATGCGTCA-3′ (forward);
TcPho89r, 5′-CCGACTCCCAGACAAATGAT-3′ (reverse).

The PCR product was run on agarose gel electrophoresis and the amplicon band was cut out. DNA was purified from the agarose using Wizard SV Gel and PCR Clean-Up System (Promega). TrPho89 was inserted on pGEM-T vector (Promega) and used to transform *E. coli* DH10B strain (Invitrogen). Positive clones were sequenced at Laboratório Sonda (Rio de Janeiro, Brazil). The sequence obtained for partial TrPho89 mRNA was deposited in GenBank (ID: GU989248). This sequence was compared to GenBank database using Blastx algorithm [19].

2.5. Quantitative PCR (qPCR)

Total RNA was extracted as before. One microgram of total RNA treated with RNase-free DNase I (Fermentas) was used to synthesize cDNA samples using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TrPho89 and TrENA expression were determined by qPCR analysis, using an Applied Biosystems StepOnePlus™ Real-Time PCR System. SYBR Green PCR Master Mix (Applied Biosystems) was used under the following conditions: one cycle for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C, and a melt curve stage. TrPho89 and TrENA PCR amplification involved the following primers:

TrPho89F, 5′-AAGTTTTGCGGCAGCTGTAT-3′;
TrPho89R, 5′-GAGAAGACGGCAAATCCAAG-3′;
TcENAF, 5′-GCTCCTTTG CCGTCGGGG TC-3′;
TcENAR, 5′-GGCGAGAACACCCCAGTGCC-3′.

TcENA primers were designed using the sequence given in the Genbank database (ID: AB107891). All assays were done in triplicate and TrGAPDH (GenBank ID: AF053742) expression was used for normalization. Primers for TrGAPDH PCR amplification were:

TrGAPDHF, 5′-GCAGCTCCATCTACGACTCC-3′ (forward);
TrGAPDHR, 5′-AGTATCCCCACTCGTTGTCG-3′ (reverse).

2.6. Preparation of plasma membranes

An enriched plasma membrane fraction from *T. rangeli* was obtained as described elsewhere [20]. Briefly, parasites were disrupted by abrasion using glass beads on an ice-bath. The resulting homogenate was mixed with 25 ml of a solution containing 10 mM HEPES–Tris (pH 7.4), 400 mM mannitol, 10 mM KCl, 1 mM $Mg(CH_3COO)_2$, 1 mM phenylmethylsulfonyl fluoride (trypsin, chymotrypsin, thrombin and papain inhibitor), 10 μM E-64 (cysteine proteases inhibitor) and 1 μM pepstatin (an aspartyl protease inhibitor). After centrifugation at 1000 ×*g* to remove the beads, unbroken cells and large cell debris, the resulting supernatant was centrifuged at 5000 ×*g* (20 min), 16,000 ×*g* (40 min) and 105,000 ×*g* (60 min). The final pellet was resuspended in 150 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol and 75 mM HEPES–Tris (pH 7.4), and immediately applied to a continuous density gradient of 18% Percoll supplemented with 250 mM sucrose and 12 mM Tris–HCl (pH 7.4), which was centrifuged at 40,000 ×*g* for 60 min. Four regions were seen, clearly separated by thin interfaces at the end of centrifugation, and the second from the top of the tubes with the highest ouabain-insensitive Na^+-ATPase (TrENA) activity was carefully recovered by aspiration and mixed with an equal volume of 250 mM sucrose to give a suspension containing 10–20 mg protein/ml, assayed by the method of Lowry et al. [21] with bovine serum albumin as the standard. This fraction also had the highest activity of 3′-nucleotidase, considered the plasma membrane marker in trypanosomatids [20].

2.7. Determination of Na^+-ATPase and H^+-ATPase activities

Furosemide-sensitive (ouabain-insensitive) Na^+-ATPase [22,23] activity was measured via $^{32}P_i$ released from [γ-^{32}P]ATP as previously described [24]. The membranes (0.5 mg/ml) were incubated for 60 min in an assay medium containing 20 mM HEPES–Tris (pH 7.0), 120 mM NaCl, 5 mM [γ-^{32}P]ATP (0.2 μCi/μmol), 10 mM $MgCl_2$ and 1 mM ouabain in the absence or presence of 1 mM furosemide. The reaction was stopped by adding 1 vol of activated charcoal in 0.1 M HCl, the suspension centrifuged at 1500 ×*g*, and an aliquot of the clear supernatant counted in the liquid scintillation counter. Na^+-ATPase activity was calculated as the difference between the $^{32}P_i$ released in the absence and presence of furosemide.

The bafilomycin-sensitive H^+-ATPase was assayed as follows. Membranes (0.5 mg/ml) were suspended in a reaction mixture containing 130 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES–Tris (pH 7.2) and 5 mM [γ-^{32}P]ATP (~0.2 μCi/μmol) in the absence or presence of 1 μM bafilomycin A_1. The reaction was stopped after 60 min as described earlier for Na^+-ATPase activity. H^+-ATPase activity was calculated as the difference between the $^{32}P_i$ released in the absence and presence of bafilomycin A_1. These experimental conditions are optimal for examining H^+ transport in *T. cruzi* [25].

2.8. Determination of P_i in the intestine and haemolymph of *Rhodnius prolixus*

The P_i concentration in the haemolymph of *R. prolixus*, the main vector of *T. rangeli*, was measured by a colorimetric phosphate method [16], previously used to assay P_i concentration in the intestine of the insect [6].

2.9. Statistical analysis

All experiments were performed in triplicate, with similar results obtained from at least 3 different cell suspensions or membrane fractions. Differences between groups were analyzed using Student's *t*-test, whereas one-way ANOVA followed by Tukey's post-test was used to verify differences within the experimental groups involving inhibitors. Statistical significance was set at $P<0.05$. The program Sigma Plot/11.0 (Systat Software Inc.) was used to measure transport kinetic parameters and the functions were adjusted to the experimental points by non-linear regression (see later the corresponding equations).

3. Results and discussion

There are no literature reports describing the transport of P_i across the plasma membrane of trypanosomatids. *T. rangeli* cells grown in low P_i conditions incorporate the anion at a rate of 7 pmol per min^{-1} per 10^7 cells in the presence of choline chloride (the Na^+-independent component, open circles in Fig. 1) and 22 pmol per min^{-1} per 10^7 cells in the presence of Na^+ (the total rate). The Na^+-dependent component, 15 pmol per min^{-1} (filled circles in Fig. 1), calculated as the difference between the total P_i transport measured in Na^+-containing media minus the Na^+-independent component) *T. rangeli* therefore expresses at least two P_i transport systems. We have previously demonstrated that proliferation of *T. rangeli* depends of the presence P_i in the culture medium [5], and that the ecto-phosphatase activity of the parasite is highest when grown in a P_i-starved medium [6]. Therefore, it is reasonable to propose a functional coupling between ecto-phosphatase and the P_i transporter ensures effective uptake, particularly when the level of P_i decreases in the culture medium. During its evolution (~2 weeks) inside the intestine of its vector, *Rhodnius prolixus,* and towards the haemolymph, the parasite faces different P_i levels

(16 mM in the intestine [6]; 0.6 mM in the haemolymph). Therefore, the existence of a transporter that senses external P_i is of the utmost relevance for the parasite life cycle. P_i uptake capacity coupled with Na^+ appears to be essential for growth and survival of other parasites, in an extra- or intra-cellular milieu, as in *Plasmodium falciparum* [26]. This suggests that an electrochemical gradient of Na^+ is one of the driving forces for P_i uptake in *T. rangeli*, as described in many other cells [27], thus preserving the capacity to adjust velocity to cellular P_i demands.

The Na^+ concentration dependence of the rate (v) of the total P_i uptake (Fig. 2A) showed Michaelis–Menten characteristics with an additional component:

$$v\,P_i\ \text{uptake} = v_{chol} + \left\{V_{max} \times \left[Na^+\right] / \left(K_{0.5,Na} + \left[Na^+\right]\right)\right\} \quad (1)$$

where v_{chol} is the velocity in the absence of Na^+ (140 mM choline chloride; 7.5 ± 0.4 pmol × min^{-1} × (10^7 cells)$^{-1}$) and, with their usual meanings, the values of apparent $K_{0.5,\ Na}$ and V_{max} were 1.2 ± 0.3 mM and 22.0 ± 1.2 pmol × min^{-1} × (10^7 cells)$^{-1}$, respectively. The very high affinity for Na^+ should allow P_i uptake by parasites in a very low Na^+ environment, such as the interior of the cells of salivary glands where metacyclogenesis occurs [28], thereby allowing the transporter to be saturated and reach V_{max}. The experiment in Fig. 2A, however, is insufficient to suggest the stoichiometry of the transport.

The significant uptake activity of the cells in the presence of choline alone seen in Fig. 1 clearly indicates the existence of another P_i transport system independent of Na^+. This view is supported by the addition of monensin reducing P_i accumulation inside cells in the presence of NaCl to the level observed in the presence of choline alone (v_{chol}, Eq. (1); Fig. 2B). Therefore, approximately one third of the total uphill P_i uptake in *T. rangeli* is not driven by an inwardly oriented Na^+ gradient. In view of the findings shown in Figs. 1 and 2, the following experiments were carried out to characterize both components (the Na^+-independent and the Na^+-dependent) of P_i uptake.

In contrast to the Na^+ curve, the dependence on P_i concentration of P_i uptake showed sigmoidal behavior for both components (Fig. 3):

$$v\,P_i\ \text{uptake} = V_{max} \times [P_i]^n / \left\{\left(K_{0.5,Pi}\right)^n + [P_i]^n\right\} \quad (2)$$

For the Na^+-independent component, the Hill coefficient (n), $K_{0.5,\ Pi}$ and V_{max} values were 2.6 ± 0.1, 45 ± 7 μM and 6.6 ± 0.7

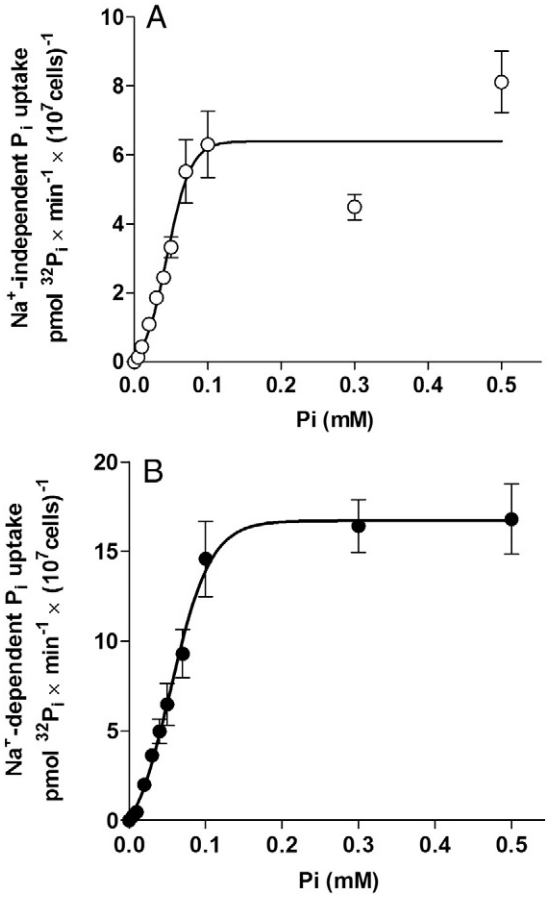

Fig. 3. P_i concentration dependence of P_i influx. $^{32}P_i$ uptake was measured in living parasites in media containing 140 mM NaCl or 140 mM choline chloride at pH 7.4, supplemented with the given concentrations of potassium orthophosphate (P_i, 5–500 μM) and traced with $^{32}P_i$ (25 μCi/ml assay). The Na^+-independent component (A, empty circles) was that measured in the presence of choline chloride; the Na^+-dependent component (B, filled circles) was calculated as described in the legend to Fig. 1. Data are means ± S.E.M. (n = 5). The function described by Eq. (2) (see text) was adjusted to the data point by non-linear regression ($r^2 = 0.90$ in A; $r^2 = 0.99$ in B). The kinetic parameters are given in the text.

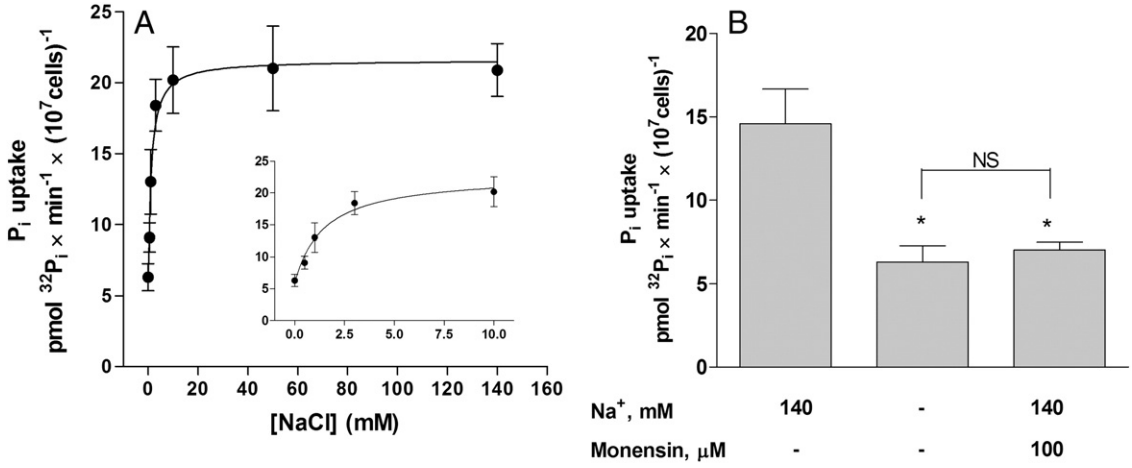

Fig. 2. Dependence of P_i influx on Na^+ concentration and Na^+ gradient. (A) $^{32}P_i$ uptake was assayed in intact cells grown in P_i-depleted medium. The assays (subsection 2.3) contained 100 μM $^{32}P_i$, at pH 7.4, with increasing NaCl concentrations (nominal 0 − 140 mM). Medium osmolarity was maintained at 300 mOsM by choline chloride supplementation. The inset allows better visualization of the concentration dependence at low Na^+. Data are means ± S.E.M. of at least 3 determinations carried out with different cultures. The smooth line in A was adjusted to the filled data point by non-linear regression using Eq. (1) (see text; $r^2 = 0.98$). (B) $^{32}P_i$ uptake in the combinations of NaCl and monensin shown on the abscissa. Choline chloride (140 mM) replaces for NaCl in Na^+-free medium. Data are means ± S.E.M. (n = 6). *Statistically different ($P < 0.05$) from uptake in the presence of Na^+ in the absence of monensin; NS, not significantly different.

pmol × min^{-1} × (10^7 cells)$^{-1}$, respectively. For the Na^+-dependent component, the respective values were 2.3 ± 0.2, 58 ± 3 μM and 17.1 ± 0.6 pmol × min^{-1} × (10^7 cells)$^{-1}$. The data clearly show that at least two different mechanisms for P_i uptake with similar affinities, but different transport capacities and energized by different ionic gradients, are present in *T. rangeli*. It is important to take into account that both the affinity and the transport capacity values could not represent solely the kinetic properties of the P_i transporters themselves, since they can be influenced by the sequestration of the anion by metabolism, as proposed for nucleobase uptake [29]. Moreover, the sigmoidicity of the curves may reflect the superimposed influence of a rapid metabolization of internalized P_i rather than an intrinsic kinetic property of the transporters.

The apparent micromolar affinities for P_i described here could also reflect various affinities in distinct transporters in the plasma membrane of *T. rangeli*, as is the case for *S. cerevisiae*. This yeast has evolved two high-affinity phosphate transport systems to adjust the uptake capacity for different P_i concentrations in various environments [30]. In addition to several low-affinity constitutive transporters [30], *S. cerevisiae* expresses high-affinity Pho84p H^+-coupled and very high-affinity Pho89p cation-coupled symporters [10]. These symporters are upregulated by the PHO pathway in response to low external P_i [31].

A partial mRNA sequence from a putative *T. rangeli* P_i transporter has been cloned and sequenced. Blastx analysis indicated a great similarity to other putative P_i transporters encoded by trypanosomatids and with the well-characterized *P. falciparum* transporter (Table 1). Moreover, the *T. rangeli* P_i transporter has similarity with the well-characterized *S. cerevisiae* transporter PHO89 [12]. The amino acid sequence deduced from its mRNA shows it has a conserved PHO-4 domain, also present in the yeast Na^+:P_i symporter [32]. These results confirm that the mRNA cloned sequence probably encodes a P_i transporter.

The rate of P_i uptake at pH 6.4 was approximately two-fold higher than at pH 8.4 for both the Na^+-independent and the Na^+-dependent components (Fig. 4). The increased transport rates at the lower pH may reflect a preference for $H_2PO_4^-$ over HPO_4^{2-}. As P_i has a pK_a of 7.1 for the second H^+ under physiological conditions, the ratio of monovalent ($H_2PO_4^-$) to divalent (HPO_4^{2-}) P_i decreases as the extracellular pH increases from 6.4 to 8.4. Fig. 4 shows that the influence of pH is different for the Na^+-independent and the Na^+-dependent components. P_i uptake by the Na^+-independent mechanism slowly decreases (>50%) from pH 6.4 to 8.4, without statistical difference between pH 6.4 and 6.8. In contrast, P_i uptake by the Na^+-dependent component decreases by approximately one third at the same pH interval and transport then remains nearly constant over a pH interval in which the ratio $H_2PO_4^-$/ HPO_4^{2-}gradually changes from 2:1 to 1:20, suggesting that HPO_4^{2-} can also be transported. In *P. falciparum*, the carrier-mediated influx of P_i takes place through a transporter displaying a strong preference for $H_2PO_4^-$ over HPO_4^{2-} [26]. The observed difference in pH dependence of the two mechanisms may reflect different affinities of the carriers for the acidic and alkaline forms of the anion. However, the inherent (and unknown) pH-dependence of the P_i transporters in *T. rangeli* could also favor a higher velocity of uptake at lower pH values, as well as producing

Table 1
Comparison of aligned amino acid sequences of partial phosphate transporter from *Trypanosoma rangeli* and other organisms.

Accession number	Species	Identity (%)	Similarity (%)
XP818641	*Trypanosoma cruzi*	98	99
XP001466587	*Leishmania infantum*	63	81
XP847723	*Leishmania major*	61	79
XP001566413	*Leishmania braziliensis*	60	78
NP009855	*Saccharomyces cerevisiae*	38	60
XP001614191	*Plasmodium vivax*	36	64
XP001350133	*Plasmodium falciparum*	34	62

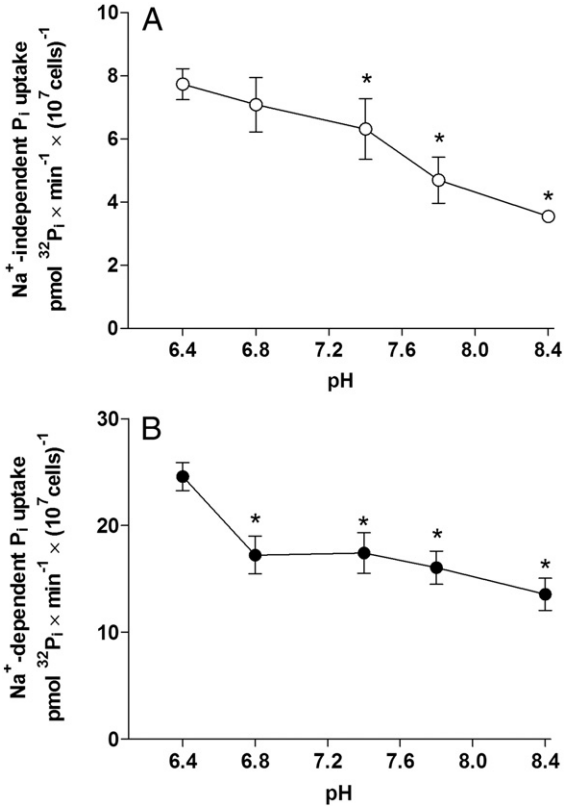

Fig. 4. pH dependence of P_i uptake. $^{32}P_i$ uptake was measured in intact cells using 100 μM $^{32}P_i$. The Na^+-independent (A, empty circles) and the Na^+-dependent (B, filled circles) components were measured as described in the legend to Fig. 1. Medium pH was adjusted to the values shown on the abscissa by adding concentrated HCl (6.4 and 6.8) or KOH (7.4, 7.8 and 8.4). The ratio $H_2PO_4^-$/HPO_4^{2-} varied from 5:1 at pH 6.4 to 1:20 at pH 8.4. Controls showed cells were viable during the experiments. Data are means ± S.E.M of 4 determinations with different cell suspensions. *Significantly different from the values obtained at pH 6.4 by the unpaired *t*-test.

different shapes in the curves. Given the observation that there is an important contribution of a Na^+-independent component of P_i uptake (Figs. 1 and 2), the data in Fig. 4 raise the possibility that a supply of H^+ to the cytosol does occur upon dissociation of $H_2PO_4^-$, thus the need for an efficient mechanism for H^+ extrusion may be functionally coupled to P_i entry. To explore the possibility that preservation of an electrogenic H^+ pumping activity is required for P_i uptake, the effects of FCCP (the H^+ ionophore) and bafilomycin A_1 (the inhibitor of the plasma membrane H^+ pump) were investigated.

Depolarization of the parasite plasma membrane using FCCP (Fig. 5) strongly reduced P_i uptake by both the Na^+-independent and Na^+-dependent mechanisms (the latter being barely detectable in the presence of the ionophore), supporting the view that an electric potential gradient Δψ is necessary for an electrogenic (influx of a positive net charge) or electroneutral transport of P_i coupled with Na^+ or not. In *S. cerevisiae*, the highest-affinity Pho89 plasma membrane Na^+-coupled P_i transporter demonstrates a Michaelian dependence on Na^+ concentration [31], and the P_i uptake activity was reduced in the presence of carbonyl cyanide m-chlorophenylhydrazone, showing that disruption of Δψ (and of a H^+ gradient) limits Na^+-coupled P_i influx mediated by the Pho89 transporter [32]. In *T. cruzi*, the H^+-ATPase plays a significant role in the regulation of Δψ at all developmental stages, as shown by the suppression of Δψ by the H^+-ATPase inhibitors, dicyclohexylcarbodiimide diethylstilbestrol, N-ethylmaleimide and bafilomycin A_1 [33]. The H^+-dependent transmembrane electric field could aid in reorientation of the symporter after P_i intracellular release to facilitate the beginning of a new transport cycle, as proposed for mammalian Na^+:P_i transporters [34].

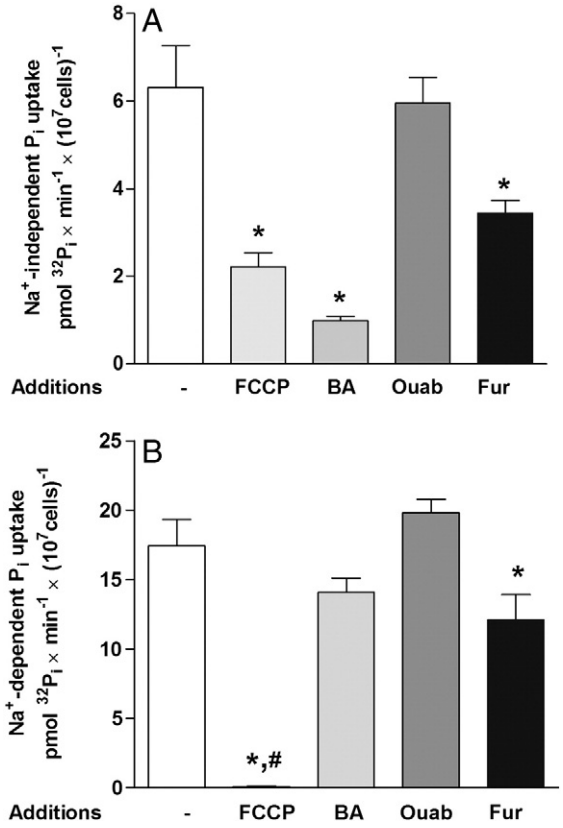

Fig. 5. Effects of H^+ ionophore and inhibitors of H^+-ATPase, $(Na^+ + K^+)$ATPase and Na^+-ATPase on P_i influx. P_i uptake by intact cells was assayed in the presence of 100 μM $^{32}P_i$ (pH 7.4), without or with addition of 10 μM FCCP, 100 nM bafilomycin A_1 (BA), 2 mM ouabain (Ouab) or 2 mM furosemide (Fur), as shown on the abscissa. The Na^+-independent (A) and the Na^+-dependent (B) components were measured as described in the legend to Fig. 1. Data are means ± S.E.M. (n = 6). Differences were estimated by one-way ANOVA followed by Tukey's post-test. *Significantly different from the control without additions; # in B indicates difference between the FCCP and furosemide groups.

Mammalian Na^+:P_i transporters use the inwardly created Na^+ electrochemical gradient created by the $(Na^+ + K^+)$ATPase to drive P_i import. This is clearly not the case for *T. rangeli* for two reasons: (i) $(Na^+ + K^+)$ ATPase activity is very low in its plasma membrane (not shown), and (ii) addition of ouabain does not affect $^{32}P_i$ uptake (Fig. 5). The inhibition of $^{32}P_i$ uptake by furosemide points to an ouabain-resistant, furosemide-sensitive Na^+-pumping activity as being responsible for generating an inwardly directed Na^+ gradient in *T. rangeli* cells to energize uphill Na^+:P_i entry. The ouabain-insensitive Na^+-ATPase activity has previously been described in *T. cruzi* [35]. It was subsequently cloned and purified from this parasite and called TcENA [22]; this characterization was very recently achieved in mammalian cells [23]. Even though the trypanosomatid and mammal ouabain-resistant and furosemide-sensitive Na^+-ATPases are structurally and kinetically different, Fig. 5 gives support for the idea that an alternative mechanism can be responsible for the generation of the Na^+ gradient required for the secondary active transport of other chemical species such as P_i.

Two other observations from Fig. 5 deserve special mention: (i) whereas the Na^+-independent component of P_i uptake is sensitive to bafilomycin A_1, the Na^+-dependent is not; (ii) furosemide, the inhibitor of the Na^+-ATPase, inhibits both components to approximately the same extent. Inhibition of the Na^+-independent component by bafilomycin A_1 suggests a coupling between P_i and H^+ transport, which depends of a H^+ sustained gradient. Conversely, the Na^+-dependent uptake being insensitive to inhibition of the bafilomycin-sensitive H^+-ATPase indicates that the Na^+:P_i coupled influx may be independent of a H^+ gradient, independently of a possible partial influence of the latter on $\Delta\psi$. The ~50%-inhibition of the Na^+-independent P_i by furosemide, is compatible with the view that the Na^+-ATPase participates in the generation of the membrane potential in *T. rangeli*. When the potential is partially collapsed, P_i uptake decreases, as in the case of addition of FCCP. There is no information about the electrogenicity of the Na^+-ATPase in trypanosomatids [22,35], but the fact that the turnover of a Na^+-ATPase from a marine microalga is associated with the generation of a transmembrane $\Delta\psi$ [36] opens up this possibility in our case.

The experiments presented in Fig. 6A demonstrated a high Na^+-ATPase activity in membranes isolated from *T. rangeli* grown in a medium with low (2 mM) P_i, whereas this activity was >10-fold lower in membranes from cells grown in high (50 mM) P_i. Up-regulation of the Na^+-ATPase has been implicated in the response of yeast cells to stress, represented by an alkaline environment that allows more efficient acquisition of P_i via the PHO89p Na^+:P_i symporter [37]. The fact the H^+-ATPase activity remained unchanged under the same conditions (Fig. 6B) reveals that, despite its influence in the Na^+-independent P_i transport mechanism (Fig. 5A), modulation of this pump does not participate in the adaptative response to varying extracellular P_i in *T. rangeli*.

The importance of the high-affinity Na^+-dependent and Na^+-independent (possibly H^+-dependent) P_i transport systems for *T. rangeli* cells grown under low P_i starvation conditions has been

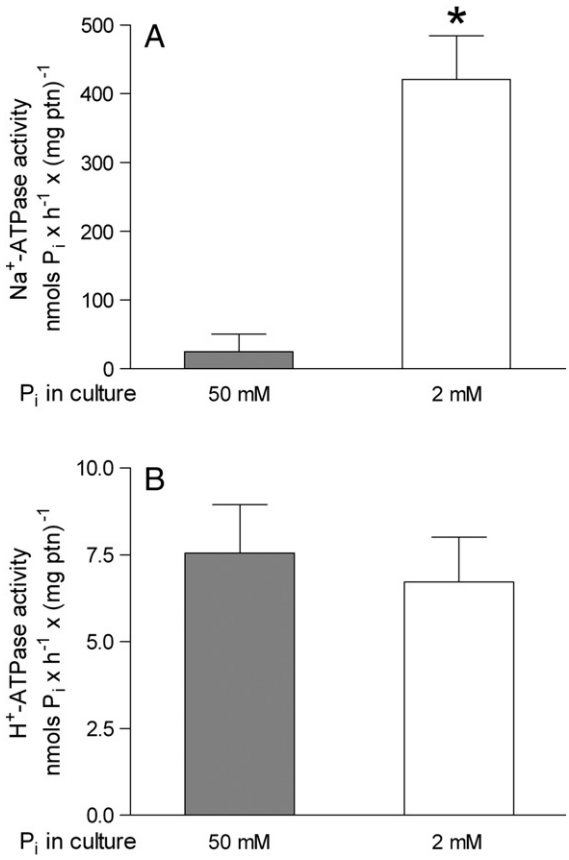

Fig. 6. Ouabain-resistant, furosemide-sensitive Na^+-ATPase activity (A) and bafilomycin A_1-sensitive H^+-ATPase (B) in membranes isolated from *T. rangeli* epimastigotes grown in high (50 mM, gray bars) or low (2 mM, empty bars) P_i during culture, as shown on the abscissae. The enriched plasma membrane fraction obtained from *T. rangeli* cells was assayed for Na^+-ATPase and H^+-ATPase as described in Materials and methods (subsection 2.7). Data are means ± S.E.M. of 4 determinations carried out with membranes obtained from different parasite cultures. Asterisk denotes a significant difference in Na^+-ATPase when compared to that in cells grown at low P_i (unpaired *t*-test).

confirmed by the data shown in Fig. 7. In parasites grown in high (50 mM) P_i culture medium, both components of Pi uptake were reduced compared with parasites grown at low (2 mM) P_i. The fact that the Na^+-independent and the Na^+-dependent mechanisms increase 40 and 140%, respectively, when parasites are in low P_i during their growth is in line with the observation that the bafilomycin-sensitive H^+-ATPase, the important adjuvant of the Na^+-independent transport (Fig. 5A), is not affected by changes in external P_i (Fig. 6B). The fact that Na^+-ATPase activity found in parasites grown in high P_i (Fig. 6A) decreased by >90% whereas P_i transport is only 30% (the Na^+-independent component, Fig. 7A) or 50% lower (the Na^+-dependent component, Fig. 7B), may indicate that the small Na^+-ATPase activity suffices to generate an electric and a Na^+ gradients that can sustain a significant P_i uptake.

The different profiles of inhibition of the two components (notably the inhibition by bafilomycin A_1 of the Na^+-independent component, without any influence on the Na^+-dependent component) (Fig. 5) support the interpretation that there are at least 2 mechanisms, although the possibility that a single transporter is responsible for P_i uptake in *T. rangeli* could not be discarded. The similarities in the kinetic constants in the presence and absence of Na^+ could favor this alternative.

Interestingly, parasites grown in low or high P_i have with the same TrPho89 and TrENA mRNA levels during the exponential and the stationary phase of growth (data not shown). Despite the marked influence of external P_i during growth on Na^+-ATPase (Fig. 6A) and on P_i influx (Fig. 7), expression of these genes may not be related to the adaptative events induced by low and high P_i in culture during growth. Thus, these two results support the idea that increase in P_i uptake capacity under starvation conditions is related to translational and/or post-translational modifications

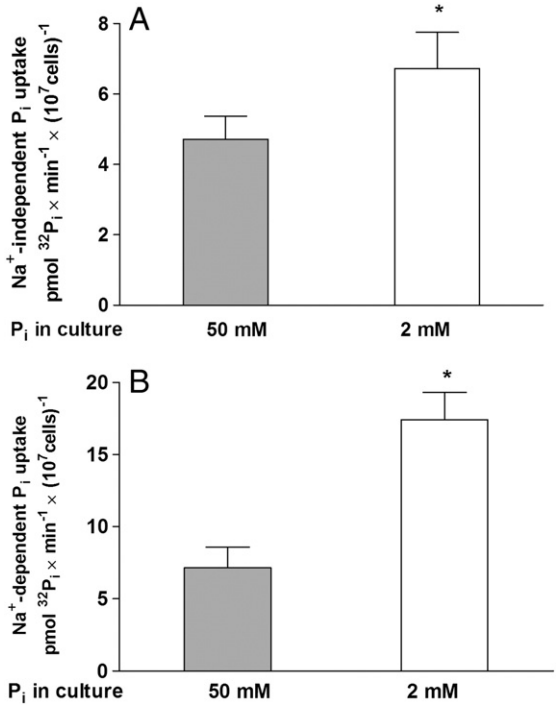

Fig. 7. P_i uptake activity of living *T. rangeli* grown in high (50 mM, gray bars) or low (2 mM) P_i concentrations, as shown on the abscissae. $^{32}P_i$ was assayed in intact cells as described in subsection 2.3 using 100 μM $^{32}P_i$ (pH 7.4). The Na^+-independent (A) and the Na^+-dependent (B) components were measured as described in the legend to Fig. 1. Data are means ± S.E.M. (n = 6) of determinations of cell suspensions from different cultures. Differences were estimated by unpaired *t*-test. For both components, an asterisk denotes a significant difference compared with cells grown at low P_i in culture medium.

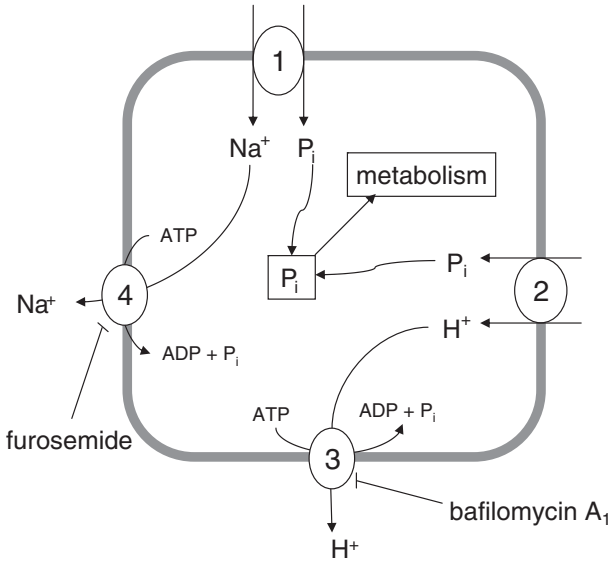

Fig. 8. Proposed P_i uptake mechanisms in *T. rangeli* functionally coupled to plasma membrane bafilomycin A_1-sensitive H^+-ATPase and ouabain-resistant, furosemide-sensitive Na^+-ATPase. Arrows indicate the direction of ion fluxes. For further description, see text.

affecting TrPho89 and TrENA, the more widely adaptative strategies in trypanosomatids [38,39], although on/off short-term mechanisms of activation for these transporters need to be considered. The ouabain-insensitive Na^+-ATPase is a particular target for a delicate ensemble of kinase-mediated regulatory mechanisms [40]. In this regard, it is noteworthy that, in mammals, P_i was recently postulated to be a specific signaling molecule that can activate several signaling pathways to modulate P_i transporters downstream [41]. An appropriate P_i-sensing mechanism has also been proposed [41]. Therefore, we might speculate that extracellular P_i levels are the primary signals for cascades that modulate TrPho89 and TrENA activity, although this requires confirmation.

In summary, the results presented here demonstrate that mechanisms of P_i transport in *T. rangeli*, energized by inwardly directed Na^+ and H^+ gradients and depressed under normal P_i supply, are activated when the parasite is grown in a medium with restricted P_i availability (Fig. 7). Fig. 8 graphically summarizes the ensemble of coupled transport events that may be essential for P_i acquisition during epimastigotes development. The Na^+:P_i (1 at the top) and H^+:P_i (2, right) allow incorporation of the anion into the cytosol and its delivery to P_i-requiring metabolic pathways of the parasite. Coupled H^+ extrusion by a bafilomycin A_1 sensitive ATP-driven pump (3, bottom) and Na^+ extrusion mediated by the ouabain-resistant and furosemide-sensitive Na^+-ATPase (4, left) allow a continuous steady influx of P_i in Na^+-independent and Na^+-dependent manners, according to metabolic requirements. It is also postulated that modulation of high-affinity primed P_i transporters and Na^+-ATPase, which are lower in an abundant P_i supply when low-affinity carriers probably suffice [42], plays a central role in fine-tuning the rates of the transporters to the intracellular P_i demands.

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References

[1] D'Alessandro, Biology of *Trypanosoma* (*Herpetosoma*) *rangeli* Tejera, 1920, in: W.H. Lumsden, D.A. Evans (Eds.), Biology of the Kinetoplastida, Academic Press, London, 1976, pp. 327–404.

[2] E.S. Garcia, P. Azambuja, Development and interactions of *Trypanosoma cruzi* within the insect vector, Parasitol. Today 7 (1991) 240–244.

[3] M.B. Figueiredo, F.A. Genta, E.S. Garcia, P. Azambuja, Lipid mediators and vector infection: *Trypanosoma rangeli* inhibits *Rhodnius prolixus* hemocyte phagocytosis by modulation of phospholipase A2 and PAF-acetylhydrolase activities, J. Insect Physiol. 54 (2008) 1528–1537.

[4] E.C. Grisard, Salivaria or Stercoraria? The *Trypanosoma rangeli* dilemma, Kinetoplastid Biol. Dis. 1 (2002) 5.

[5] A.L. Fonseca-de-Souza, C.F. Dick, A.L. Dos Santos, F.V. Fonseca, J.R. Meyer-Fernandes, *Trypanosoma rangeli*: a possible role for ecto-phosphatase activity on cell proliferation, Exp. Parasitol. 122 (2009) 242–246.

[6] C.F. Dick, A.L. Dos-Santos, A.L. Fonseca-de-Souza, J. Rocha-Ferreira, J.R. Meyer-Fernandes, *Trypanosoma rangeli*: differential expression of ecto-phosphatase activities in response to inorganic phosphate starvation, Exp. Parasitol. 124 (2010) 386–393.

[7] A. Torriani, From cell membrane to nucleotides: the phosphate regulon in *Escherichia coli*, Bioessays 12 (1990) 371–376.

[8] C. Auesukaree, T. Homma, H. Tochio, M. Shirakawa, Y. Kaneko, S. Harashima, Intracellular phosphate serves as a signal for the regulation of the PHO pathway in *Saccharomyces cerevisiae*, J. Biol. Chem. 279 (2004) 17289–17294.

[9] M.G. Lamarche, B.L. Wanner, S. Crépin, J. Harel, The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis, FEMS Microbiol. Rev. 32 (2008) 461–473.

[10] B.L. Persson, A. Berhe, U. Fristedt, P. Martinez, J. Pattison, J. Petersson, R. Weinander, Phosphate permeases of *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1365 (1998) 23–30.

[11] M. Bun-ya, M. Nishimura, S. Harashima, Y. Oshima, The PHO84 gene of *Saccharomyces cerevisiae* encodes an inorganic phosphate transporter, Mol. Cell. Biol. 11 (1991) 3229–3238.

[12] P. Martinez, B.L. Persson, Identification, cloning and characterization of a derepressible Na^+-coupled phosphate transporter in *Saccharomyces cerevisiae*, Mol. Gen. Genet. 258 (1998) 628–638.

[13] C. Auesukaree, T. Homma, Y. Kaneko, S. Harashima, Transcriptional regulation of phosphate-responsive genes in low-affinity phosphate-transporter-defective mutants in *Saccharomyces cerevisiae*, Biochem. Biophys. Res. Commun. 306 (2003) 843–850.

[14] N. Ogawa, J. DeRisi, P.O. Brown, New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis, Mol. Biol. Cell 11 (2000) 4309–4321.

[15] J.C.C. Maia, S.L. Gomes, M.H. Juliani, Preparation of (gamma-^{32}P)- and (alpha-^{32}P)-nucleoside triphosphates with high specific activity, in: C. Morel (Ed.), Genes and antigens of parasites, a laboratory manual, Editora Fundação Oswaldo Cruz, Rio de Janeiro, 1983, pp. 146–167.

[16] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.

[17] M.S. Leite, R. Thomaz, F.V. Fonseca, R. Panizzutti, A.E. Vercesi, J.R. Meyer-Fernandes, *Trypanosoma brucei brucei*: biochemical characterization of ecto-nucleoside triphosphate diphosphohydrolase activities, Exp. Parasitol. 115 (2007) 315–323.

[18] H.P. De Koning, C.J. Watson, L. Sutcliffe, S.M. Jarvis, Differential regulation of nucleoside and nucleobase transporters in *Crithidia fasciculata* and *Trypanosoma brucei brucei*, Mol. Biochem. Parasitol. 106 (2000) 93–107.

[19] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[20] C.M. Pinheiro, E.S. Martins-Duarte, R.B. Ferraro, A.L. Fonseca-de-Souza, M.T. Gomes, A.H.C.S. Lopes, M.A. Vannier-Santos, A.L.S. Santos, J.R. Meyer-Fernandes, *Leishmania amazonensis*: biological and biochemical characterization of ecto-nucleoside triphosphate diphosphohydrolase activity, Exp. Parasitol. 114 (2006) 16–25.

[21] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[22] K. Iizumi, Y. Mikami, M. Hashimoto, T. Nara, Y. Hara, T. Aoki, Molecular cloning and characterization of ouabain-insensitive $Na(^+)$-ATPase in the parasitic protist, *Trypanosoma cruzi*, Biochim. Biophys. Acta 1758 (2006) 738–746.

[23] M.A. Rocafull, F.J. Romero, L.E. Thomas, J.R. Del Castillo, Isolation and cloning of the $K(^+)$-independent, ouabain-insensitive $Na(^+)$-ATPase, Biochim. Biophys. Acta 1808 (2011) 1684–1700.

[24] E.E. De Almeida-Amaral, C. Caruso-Neves, V.M. Pires, J.R. Meyer-Fernandes, *Leishmania amazonensis*: characterization of an ouabain-insensitive Na^+-ATPase activity, Exp. Parasitol. 118 (2008) 165–171.

[25] M. Vieira, P. Rohloff, S. Luo, N. Cunha-e-Silva, W. de Souza, R. Docampo, Role for a P-type H^+-ATPase in the acidification of the endocytic pathway of Trypanosoma cruzi, Biochem. J. 392 (2005) 467–474.

[26] K.J. Saliba, R.E. Martin, A. Bröer, R.I. Henry, C.S. McCarthy, M.J. Downie, R.J.W. Allen, K.A. Mullin, G.I. McFadden, S. Bröer, K. Kirk, Sodium-dependent uptake of inorganic phosphate by the intracellular malaria parasite, Nature 443 (2006) 582–585.

[27] H. Murer, J. Biber, Phosphate transport in the kidney, J. Nephrol. 23 (2010) S145–S151.

[28] A. Cuba-Cuba, Review of the biologic and diagnostic aspects of *Trypanosoma* (Herpetosoma) *rangeli*, Rev. Soc. Bras. Med. Trop. 31 (1998) 207–220.

[29] K. Kirk, S.M. Howitt, S. Bröer, K.J. Saliba, M.J. Downie, Purine uptake in *Plasmodium*: transport versus metabolism, Trends Parasitol. 25 (2009) 246–249.

[30] B.L. Persson, J. Petersson, U. Fristedt, R. Weinander, A. Berhe, J. Pattison, Phosphate permeases of *Saccharomyces cerevisiae*: structure, function and regulation, Biochim. Biophys. Acta 1422 (1999) 255–272.

[31] R.A. Zvyagilskaya, F. Lundh, D. Samyn, J. Pattison-Granberg, J.M. Mouillon, Y. Popova, J.M. Thevelein, B.L. Persson, Characterization of the Pho89 phosphate transporter by functional hyperexpression in *Saccharomyces cerevisiae*, FEMS Yeast Res. 8 (2008) 685–696.

[32] W.K. Versaw, R.L. Metzenberg, Repressible cation-phosphate symporters in *Neurospora crassa*, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 3884–3887.

[33] N. Van Der Heyden, R. Docampo, Proton and sodium pumps regulate the plasma membrane potential of different stages of *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 120 (2002) 127–139.

[34] C. Ghezzi, H. Murer, I.C. Forster, Substrate interactions of the electroneutral Na^+-coupled inorganic phosphate cotransporter (NaPi-IIc), J. Physiol. 587 (2009) 4293–4307.

[35] C. Caruso-Neves, M. Einicker-Lamas, C. Chagas, M.M. Oliveira, A. Vieyra, A.G. Lopes, Ouabain-insensitive Na^+-ATPase activity in *Trypanosoma cruzi* epimastigotes, Z. Naturforsch. C 54 (1999) 100–104.

[36] Y.V. Balnokin, L.G. Popova, I.M. Andreev, Electrogenicity of the Na^+-ATPase from the marine microalga *Tetraselmis (Platymonas) viridis* and associated H^+ countertransport, FEBS Lett. 462 (1999) 402–406.

[37] R. Serrano, A. Ruiz, D. Bernal, J.R. Chambers, J. Ariño, The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signaling, Mol. Microbiol. 46 (2002) 1319–1333.

[38] S. Haile, B. Papadopoulou, Developmental regulation of gene expression in trypanosomatid parasitic protozoa, Curr. Opin. Microbiol. 10 (2007) 569–577.

[39] S. Martínez-Calvillo, J.C. Vizuet-de-Rueda, L.E. Florencio-Martínez, R.G. Manning-Cela, E.E. Figueroa-Angulo, Gene expression in Trypanosomatid parasites, J. Biomed. Biotechnol. 2010 (2010) 1–15.

[40] L.B. Rangel, A.G. Lopes, L.S. Lara, T.L. Carvalho, I.V. Silva, M.M. Oliveira, M. Einicker-Lamas, A. Vieyra, L. Nogaroli, C. Caruso-Neves, PI-PLCβ is involved in the modulation of the proximal tubule Na^+-ATPase by angiotensin II, Regul. Pept. 127 (2005) 177–182.

[41] S. Khoshniat, A. Bourgine, M. Julien, P. Weiss, J. Guicheux, L. Beck, The emergence of phosphate as a specific signaling molecule in bone and other cell types in mammals, Cell. Mol. Life 68 (2011) 205–218.

[42] B.L. Persson, J.O. Lagerstedt, J.R. Pratt, J. Pattison-Granberg, K. Lundh, S. Shokrollahzadeh, F. Lundh, Regulation of phosphate acquisition in *Saccharomyces cerevisiae*, Curr. Genet. 43 (2003) 225–244.