Presence of a Na^+/H^+ exchanger in acidocalcisomes of *Leishmania donovani* and their alkalization by anti-leishmanial drugs

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Abstract Acidocalcisomes are acidic vacuoles present in trypanosomatids that contain most of the cellular calcium. The data presented here demonstrate that *Leishmania donovani* acidocalcisomes possess a Na^+/H^+ exchanger. 3,5-Dibutyl-4-hydroxytoluene, in the concentration range of 0–20 μM, inhibited the Na^+/H^+ exchanger, and strongly stimulated the activity of the vacuolar H^+-ATPase responsible for vacuolar acidification. As occurs with Na^+, the cationic anti-leishmanial drugs pentamidine, WR-6026, and chloroquine promoted a fast and extensive alkalization of the *L. donovani* acidocalcisomes.

Key words: Acidocalcisome; Na^+/H^+ exchanger; 3,5-Dibutyl-4-hydroxytoluene; Volutin; *Leishmania donovani*

1. Introduction

Na^+/H^+ exchangers are ubiquitous membrane proteins found throughout the eukaryotic and prokaryotic kingdoms. They catalyze the electroneutral countertransport of Na^+ and H^+ across both cytoplasmic and organellar membranes [1]. Na^+/H^+ exchangers of animal cells that localize to the plasma membrane use the prevailing Na^+ gradient generated by a Na^+/K^+-ATPase to drive H^+ equivalents from the cell. As such, these exchangers are involved in the regulation of intracellular pH, cell volume control, and intracellular Na^+ movement [2]. The first intracellular Na^+/H^+ exchanger identified in eukaryotic cells was found in the mitochondria [3]. Intracellular Na^+/H^+ exchangers were also described in endosomal preparations from kidney [4] and liver [5], in zymogen granules of pancreatic acinar cells [6], in chromaffin granules of adrenal glands [7], and in intracellular vacuoles of yeast, algae and plants [1]. In these organelles Na^+ flows through a Na^+/H^+ exchanger by using the electrochemical proton gradient generated by vacuolar proton pumps, such as the vacuolar H^+-ATPase (V-H^+-ATPase) in endosomes, secretory granules and yeast vacuoles, and a H^+-pyrophosphatase (V-H^+-PPase) in plant vacuoles, driving protons to the cytoplasm. The consequence is Na^+ sequestration in these organelles.

Trypanosomatids and apicomplexan parasites possess acidic vacuoles with a high content of sodium in addition to other elements such as calcium, magnesium, zinc, and phosphorus, that were termed acidocalcisomes and that are similar to what was described before as volutin granules in different microorganisms [8]. The acidic pH of this compartment is generated and sustained by a V-H^+-ATPase and/or a V-H^+-pyrophosphatase [8]. The acid pH inside these vacuoles favors Ca^{2+} retention since a Ca^{2+}/nH^+ antiporter mediates Ca^{2+} release [9]. This model of Ca^{2+} handling by the acidocalcisomes is supported by findings that interior alkalization by nigericin, NH_4Cl, and V-H^+-ATPase or V-H^+-PPase inhibitors [9–15] trigger Ca^{2+} release from the acidocalcisomes. Evidence has been provided [9,10,15] that both V-H^+-ATPase- and V-H^+-PPase-containing acidocalcisomes of *Trypanosoma brucei* procyclic trypomastigotes possess a Na^+/H^+ exchanger that mediates vacuole alkalization followed by Ca^{2+} release. This Na^+/H^+ exchanger has been shown to be inhibited by 3,5-dibutyl-4-hydroxytoluene (BHT) [10].

In this report we provide evidence that *Leishmania donovani* acidocalcisomes also possess a Na^+/H^+ exchanger that is inhibited by BHT. BHT was also shown to strongly stimulate the V-H^+-ATPase activity responsible for acidification of these organelles. As occurs with Na^+, addition of cationic anti-leishmanial drugs results in a fast and extensive alkalization of the acidocalcisomes.

2. Materials and methods

L. donovani promastigotes (S-2 strain) were grown at 28°C in medium SDM-79 [16] supplemented with 10% (v/v) heat-inactivated fetal calf serum. At 2–3 days after inoculation, cells were collected by centrifugation and washed twice in a buffer (buffer A) containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose and 50 mM HEPES, pH 7.2, and resuspended in the same buffer. The protein concentration was measured by using the Bio-Rad Coomassie blue method.

ATP, acridine orange (AO), antimycin A, BHT, chloroquine, digitonin, EGTA, 2-hydroxyethyl-1-piperazine-ethanesulfonic acid (HEPES), nigericin, oligomycin, and pentamidine were purchased from Sigma (St. Louis, MO, USA). WR6026 (lepidine) was a gift from the Walter Reed Army Institute (Washington, DC, USA) through the courtesy of Dr. M. Grogl. All other reagents were of analytical grade.

Acidification of digitonin-permeabilized cells was followed by measuring the changes in the absorbance spectrum of AO [9,17], using a SLM Aminco DW2000 spectrophotometer at the wavelength pair 493–530 nm. Cells (0.4 mg/ml) were incubated at 30°C in 2.5 ml of standard reaction medium containing 125 mM sucrose, 65 mM KCl, 250 μM EGTA, 2 mM potassium phosphate, 2 mM $MgCl_2$, 10 mM HEPES buffer, pH 7.2, 1 μg/ml antimycin A, 2 μg/ml oligomycin and digitonin (16 μM) for 2 min before addition of AO (1.0 μg/ml). Each experiment was repeated at least three times with different cell preparations, and the figures show representative experiments.

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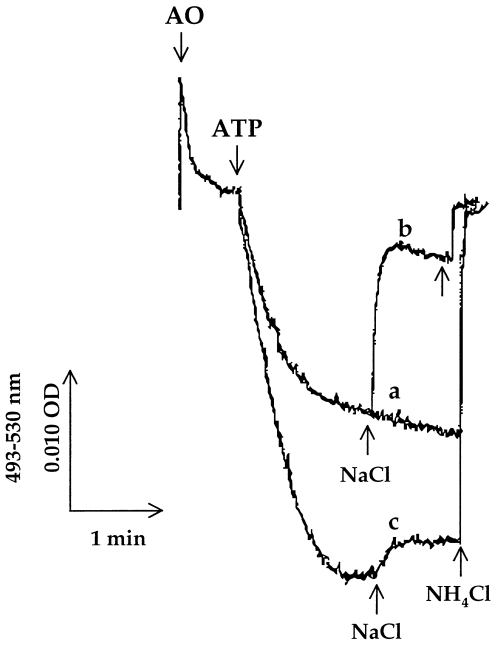

Fig. 1. BHT stimulates ATP-driven AO accumulation and inhibits Na^+-induced AO release from acidocalcisomes of permeabilized *L. donovani*. Cells (0.4 mg/ml) were added to the standard reaction medium containing 16 μM digitonin and 1.0 μg/ml AO (traces a and b). In trace c 10 μM BHT was added 20 s before ATP. ATP (0.5 mM), 40 mM NaCl and 10 mM NH_4Cl were added where indicated.

3. Results and discussion

To investigate whether the *L. donovani* acidocalcisomes possess a Na^+/H^+ exchanger we used the dye AO, whose distribution between two compartments varies with the pH gradient; its accumulation increases and its absorbance decreases when the compartment becomes more acidic [17]. In this regard, Fig. 1 (trace a) shows that, in agreement with previous results [9–15], ATP drives AO accumulation by the permeabilized *L. donovani*, presumably into the acidocalcisomes. The addition of NH_4Cl, which alkalinizes the vacuoles, promoted a very fast increase in AO absorbance, compatible with its release to the incubation medium. A partial release of AO (trace b) could also be induced by the inclusion of 40 mM NaCl, while 40 mM KCl had no effect (data not shown). The subsequent addition of NH_4Cl was followed by completion of AO release suggesting that both, NaCl and NH_4Cl, induced AO release from the same compartment. To ascertain whether a Na^+/H^+ exchanger mediated this Na^+-induced alkalization similar to that present in *T. brucei* procyclics [9] we tested its sensitivity to BHT. Therefore, 10 μM BHT was included in the incubation medium containing the permeabilized *L. donovani* 20 s before ATP. Interestingly, the AO accumulation was much faster and extensive (trace c) than that observed in the control experiment (trace a). In contrast, the Na^+-induced AO release was strongly inhibited as it happens in *T. brucei* procyclic trypomastigotes [10]. The experiment of Fig. 2 shows that the stimulation of acidocalcisome acidification by BHT was dose-dependent and attained a maximum at 20 μM BHT. No further stimulation was detected at higher concentrations of BHT (data not shown). The faster acidification of the acidocalcisome is certainly mediated by activation of the V-H^+-ATPase,

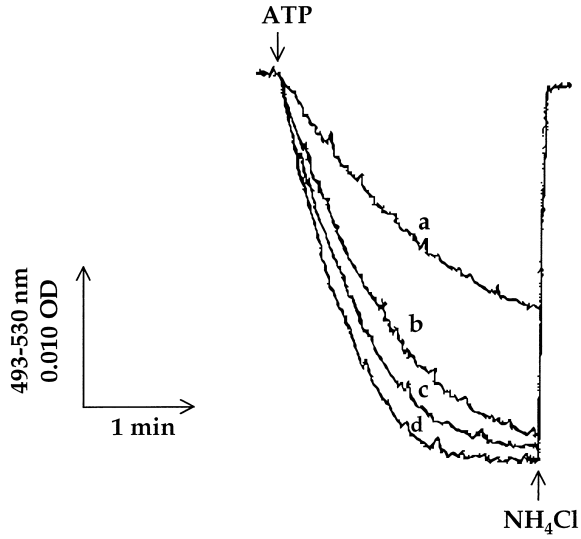

Fig. 2. Stimulation by BHT of AO accumulation by acidocalcisomes of permeabilized *L. donovani*. Cells (0.4 mg/ml) were added to the standard reaction medium containing 16 μM digitonin, 1.0 μg/ml AO and BHT at different concentrations (0, 5, 10, 20 μM, a–d, respectively). ATP (0.5 mM) and 10 mM NH_4Cl were added where indicated.

in contrast to the results observed with *T. brucei* procyclics [10]. Indeed, it is known that BHT can either activate or inhibit the (Ca^{2+}-Mg^{2+})-ATPase of the sarcoplasmic reticulum depending on its concentration [19]. In the concentration range used here, it has been shown that the (Ca^{2+}-Mg^{2+})-ATPase stimulation by BHT is strictly correlated with the presence of cholesterol and the chemical structure of the surrounding phospholipids [18]. In reconstituted systems, increased activity is always observed when phospholipids with

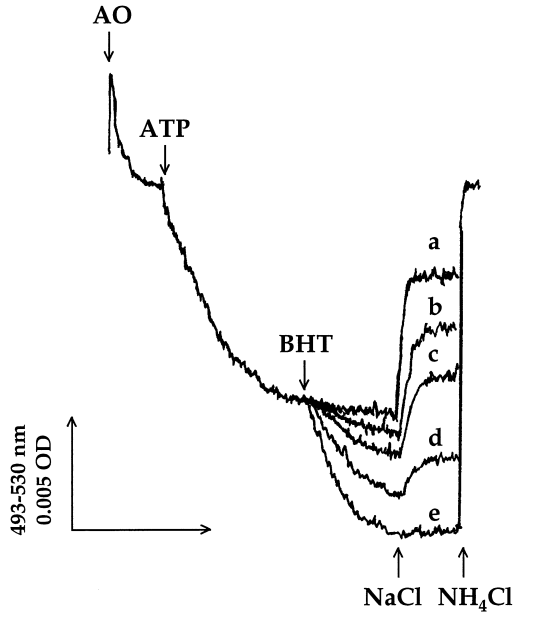

Fig. 3. BHT increases the H^+-driven force of the V-H^+-ATPase. Cells (0.4 mg/ml) were added to the standard reaction medium containing 16 μM digitonin and 1.0 μg/ml AO. ATP (0.5 mM), different concentrations of BHT (0, 2.5, 5, 10, 20 μM, a–e, respectively), 40 mM NaCl and 10 mM NH_4Cl were added where indicated.

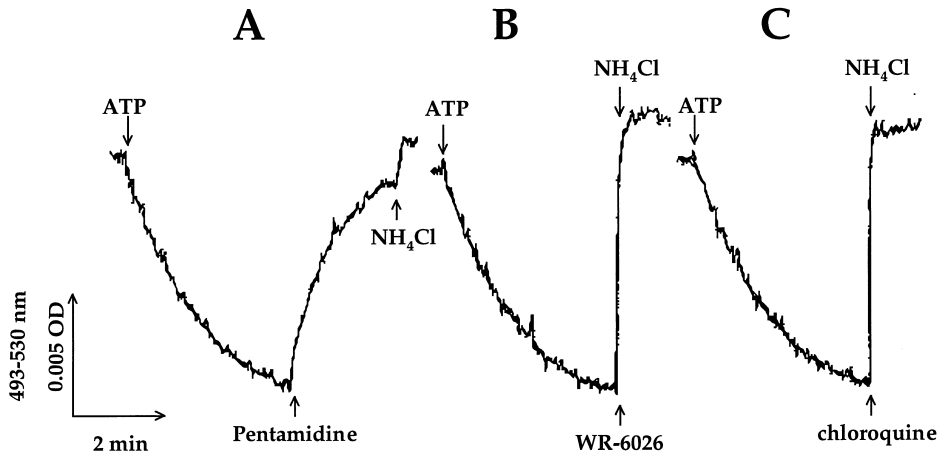

Fig. 4. Pentamidine, WR-6026, and chloroquine induce AO release from acidocalcisomes of permeabilized *L. donovani*. Cells (0.4 mg/ml) were added to the standard reaction medium containing 16 μM digitonin and 1.0 μg/ml AO. ATP (0.5 mM) and 10 mM NH_4Cl were added in all experiments where indicated. Pentamidine (200 μM, A), WR-6026 (200 μM, B) and chloroquine (100 μM, C) were added where indicated.

shorter fatty acid chains are used [18]. This implies that the opposite effects of BHT on the activity of the V-H^+-ATPases present in the acidocalcisomes of *L. donovani* and *T. brucei* procyclics could be due to differences in the phospholipid composition of their membranes. Indeed, the higher sensitivity of the *L. donovani* than the *T. brucei* procyclic membranes to permeabilization by digitonin (see [19,20] for comparison), suggest that the first parasite possesses more cholesterol in its membranes.

Since stimulation or inhibition of ATPases by BHT is also a function of the preincubation period [18] we analyzed the effect of this compound on the activities of the V-H^+-ATPase and the Na^+/H^+ exchanger when BHT was added after the AO accumulation was essentially complete. It can be observed that in the same range of concentrations used in Fig. 2, BHT promoted a dose-dependent increment in AO accumulation compatible with changes in the equilibrium constant of the V-H^+-ATPase reaction, an effect also observed on the (Ca^{2+}-Mg^{2+})-ATPase [18]. The subsequent addition of 40 mM NaCl to the experiment of Fig. 3 provided evidence that the Na^+/H^+ exchanger was inhibited in the same concentration range in which the H^+-ATPase was activated. The opposite effects of BHT on the activity of these membrane proteins, under the same experimental conditions, support the notion that the BHT action in these experiments is mediated by direct interactions with the proteins rather than causing changes in membrane permeability to H^+ or to membrane fluidity [18].

The detection of a Na^+/H^+ exchanger in the acidocalcisomes of *L. donovani*, a carrier that in many cells is known to be regulated by diverse signaling mechanisms such as protein kinases [21,22], supports the notion that the acidocalcisomes play an important role in the physiology of *L. donovani*. Since these vacuoles are not present in mammalian cells, they can be explored as important targets for drugs against diseases caused by this parasite. In this regard, we have studied the effect of the anti-leishmanial cationic drugs pentamidine [23] and WR-6026 [24] on the retention of AO by the acidocalcisomes (Fig. 4). Chloroquine, which is known to be accumulated in acidic organelles of *T. brucei* procyclic trypomastigotes and to slow down the growth rate of these trypanosomes in vitro [25] was also used. An extensive alkalization of the acidocalcisomes was induced by all these drugs, an effect which is certainly mediated by their common property to be lipophilic weak bases, thus rapidly accumulating in acidic compartments. Although these drugs may have other effects in *L. donovani* [19], the alkalization promoted in the acidocalcisomes could also be involved in their anti-leishmanial action.

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