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Ca^{2+} transport into an intracellular acidic compartment of *Candida parapsilosis*

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Abstract In this report, we study Ca^{2+} transport in permeabilized *Candida parapsilosis* spheroplasts prepared by a new technique using lyticase. An intracellular non-mitochondrial Ca^{2+} uptake pathway, insensitive to orthovanadate and sensitive to the V-H^+-ATPase inhibitor bafilomycin A_1, nigericin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was characterized. Acidification of the compartment in which Ca^{2+} accumulated was followed using the fluorescent dye acridine orange. Acidification was stimulated by the Ca^{2+} chelator EGTA and inhibited by Ca^{2+}. These results, when added to the observation that Ca^{2+} induces alkalization of a cellular compartment, provide evidence for the presence of a Ca^{2+}/nH^+ antiporter in the acid compartment membrane. Interestingly, like in acidocalcisomes of trypanosomatids, the antioxidant 3,5-dibutyl-4-hydroxytoluene inhibits the V-H^+-ATPase. In addition, the antifungal agent ketoconazole promoted a fast alkalization of the acidic compartment. Ketoconazole effects were dose-dependent and occurred in a concentration range close to that attained in the plasma of patients treated with this drug. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: *Candida parapsilosis*; Ca^{2+} transport; Ketoconazole; Lyticase; Intracellular Ca^{2+}

1. Introduction

The incidence of severe fungal infections has increased during the last decades [1,2] and fungal infections have become a major cause of mortality among imunocompromised patients, including those with AIDS [2–4]. Disturbingly, frequent reports that fungi are developing resistance to antifungal agents have surfaced [4,5]. In oncological patients, *Candida albicans* accounted for 54% and *Candida parapsilosis* for 7% of all systemic *Candida* infections [4]. Current chemotherapy against these parasites has many flaws, including low specificity, toxicity and drug resistance [1]. It is, therefore, very important to search for biological targets in these parasites that could be exploited for the rational development of improved therapies. In this search, understanding of the mechanisms involved in fungal control of Ca^{2+} homeostasis is very important, since disruption of Ca^{2+} homeostasis leads to cell death [8]. Cell death is also intimately linked to energetic metabolism, and we have recently characterized aspects of *C. parapsilosis* energy conservation systems [7].

Intracellular Ca^{2+} ions play a crucial role in controlling many biological processes [9] which are dependent on Ca^{2+} compartmentalization, governed by several transport systems operating in a highly regulated fashion [9]. Cells have developed a variety of molecular devices, such as channels, pumps and transporters, to regulate Ca^{2+} influx and efflux across the plasma membrane and between intracellular stores, adjusting cytoplasmic Ca^{2+} concentrations [9]. Mammalian cells contain very active Ca^{2+}-ATPases and Ca^{2+}/Na^+ antiporters in their plasma membrane, able to promote the fast exchange of intracellular Ca^{2+} with the extracellular pool. In contrast, most Ca^{2+} transport into and out of the cytoplasm of fungal cells is mediated by their vacuolar membranes [13]. Vacuoles are the largest compartments in yeast cells and are postulated to function as lysosomes and storage compartments [10]. In most plant and fungal cells, the vacuoles occupy over 50% of the cell volume and their membrane contains numerous carriers, transporters, channels and enzymes. Little is known about the mechanisms involved in Ca^{2+} homeostasis in *C. parapsilosis*, which is assumed to transport Ca^{2+} in a manner similar to *Saccharomyces cerevisiae* [11].

In this report we demonstrate that *C. parapsilosis* possesses a non-mitochondrial, nigericin-sensitive Ca^{2+} compartment that functions in a manner dependent on the pH gradient formed by a bafilomycin A_1-sensitive H^+-ATPase. A Ca^{2+}/nH^+ antiporter apparently mediates Ca^{2+} uptake and release by this acidic compartment. Furthermore, ketoconazole, an antimycotic drug, promotes a fast and extensive alkalization of the acidic compartment, followed by Ca^{2+} release.

2. Materials and methods

2.1. C. parapsilosis culture

C. parapsilosis CCT 3834 (ATCC 22019) cells were cultured at 37°C under vigorous aeration in complete liquid medium (2% glycerol, 2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Difco) until the mid-exponential phase.

2.2. Spheroplast preparation

Cultured *C. parapsilosis* cells were harvested by centrifugation from a liter of culture media, and cells were washed with cold water, followed by a wash using buffer A (1 M sorbitol, 10 mM $MgCl_2$ and 50 mM Tris–HCl, pH 7.8). Cells were resuspended in buffer A (3 ml/g of cells) containing 30 mM dithiothreitol (DTT). After 15 min incubation at room temperature with shaking, cells were harvested by centrifugation, resuspended in buffer A containing lyticase (1 mg/g of

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; AO, acridine orange; BHT, 3,5-dibutyl-4-hydroxytoluene

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cells) and 1 mM DTT and incubated at 30°C until approximately 90% of the cells converted to spheroplasts (60–80 min). The digestion was stopped by the addition of an equal volume of ice-cold buffer A, and spheroplasts were washed twice with the same buffer. Protein concentration of the final suspension was determined using the biuret assay [14] in the presence of 0.2% deoxycolate.

2.3. Measurement of Ca^{2+} movements

Variations in free Ca^{2+} concentrations were followed by measuring the changes in the absorbance spectrum of arsenazo III [15], using a SLM Aminco DW 2000 spectrophotometer at the wavelength pair 675–685 nm [16,17]. The concentrations of the ionic species and complexes at equilibrium were calculated by employing an interactive computer program as described before [18].

2.4. Proton pump activity

Acidification of permeabilized cells was followed by measuring the changes in the absorbance or fluorescence spectrum of acridine orange (AO) [19], using a SLM Aminco DW2000 spectrophotometer at the wavelength pair 493–530 nm or a Hitachi F4500 fluorescence spectrophotometer at the wavelength pair 510–550 nm.

Each experiment was repeated at least three times with different cell preparations, and the figures show representative experiments.

2.5. Chemicals

ATP, calcium ionophore A_{23187}, sodium orthovanadate, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), succinate, arsenazo III, EGTA, nigericin, antimycin A, myxothiazol, oligomycin, ammonium chloride and lyticase were purchased from Sigma. AO was from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade.

3. Results

3.1. Ca^{2+} uptake by C. parapsilosis spheroplasts: inhibition by nigericin and bafilomycin A_1

Fig. 1 shows that, when *C. parapsilosis* spheroplasts were added to a reaction medium containing MgATP, succinate and 8 μM free Ca^{2+}, most of the Ca^{2+} present was taken up and retained by the spheroplasts. The subsequent addition of the Ca^{2+} ionophore A_{23187} promoted a release of the accumulated cation [trace (a)]. Nigericin, a K^+/H^+ exchanger, was also able to release part of the accumulated Ca^{2+} [trace (c)]. The simultaneous presence of antimycin A, oligomycin and myxothiazol (see [7]), which prevent mitochondrial energization and Ca^{2+} uptake [21] or sodium orthovanadate, an inhibitor of the P-type Ca^{2+}-ATPase, caused no difference either on the rate or extent of Ca^{2+} uptake (the trace is superimposable with trace (a)). The presence of a V-H^+-PPase [22] in these vacuoles was also ruled out in our experiments.

Acidic compartments may possess an ATP-driven H^+ pump that maintains their interior at a pH lower than that of the cytoplasm [20]. These H^+ pumps are bafilomycin A_1-sensitive [21,23]. Indeed, trace (b) shows that when *C. parapsilosis* spheroplasts were added to reaction medium containing nigericin or bafilomycin A_1, Ca^{2+}-transport activity was completely inhibited (the traces with bafilomycin A_1 and nigericin are superimposable). The sensitivity of this Ca^{2+}-transporting activity to bafilomycin A_1 confirms that Ca^{2+} is being taken up by a compartment acidified by a V-type proton ATPase [24].

3.2. ATP-dependent vacuolar acidification in C. parapsilosis spheroplasts: effects of nigericin, NH_4Cl and bafilomycin A_1

In order to confirm the presence of a V-H^+-ATPase in these cells we used AO, a tertiary amine that becomes concentrated in acidic compartments and changes both its absorbance and fluorescence properties as a consequence of its stacking [19] as a probe. Trace (a) shows that the addition of ATP to a reaction medium containing antimycin A, myxothiazol, oligomycin, EGTA and *C. parapsilosis* spheroplasts caused a significant time-dependent decrease in the absorbance of AO compatible with its accumulation in acidic compartments. This uptake was inhibited by bafilomycin A_1 [trace (c)]. The addition of bafilomycin A_1 also reversed the decrease in AO absorbance [trace (b)]. EGTA, present in trace a, decreased the concentration of Ca^{2+} from 8 μM to less than 1 nM and stimulated both the rate and extent of AO absorbance decrease, when compared to trace (b). Under all experimental conditions tested, nigericin, FCCP or NH_4Cl reversed the decrease in AO absorbance induced by ATP, confirming that the decrease in AO absorbance was caused by its accumulation into acidic compartments.

3.3. Calcium-induced AO release from C. parapsilosis acidic vacuoles

We also investigated the effect of Ca^{2+} on the *C. parapsilosis* ATP-dependent compartment acidification, in a reaction medium containing antimycin A, myxothiazol and oligomycin. Fig. 3 shows that 40 μM $CaCl_2$ [trace (b)] reversed the decrease in AO absorbance promoted by the spheroplasts.

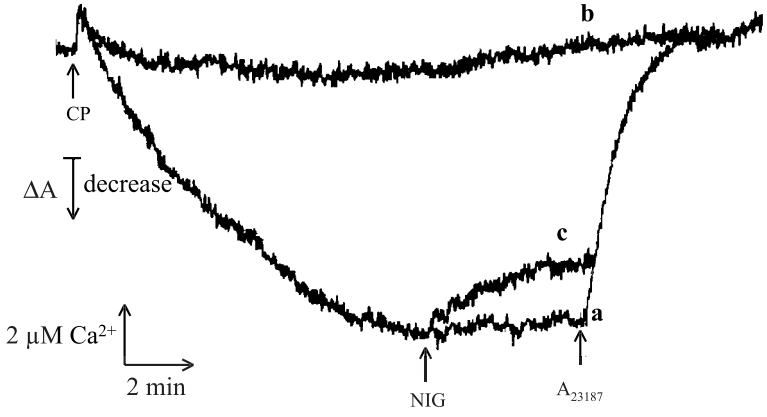

Fig. 1. Ca^{2+} uptake by *C. parapsilosis* spheroplasts. The *C. parapsilosis* spheroplasts (1 mg of protein/ml) were added to standard reaction media (125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2.5 mM potassium phosphate, 1 mM Mg Cl_2) containing 5 mM succinate, 40 μM arsenazo III and 0.5 mM ATP. Trace (a), control; trace (b), 1.3 μM nigericin and/or 2.5 μM bafilomycin A_1 present from the beginning of the experiment and trace (c), 1.3 μM nigericin added where indicated. Calcium ionophore A_{23187} (1 μM) was added where indicated by the arrow.

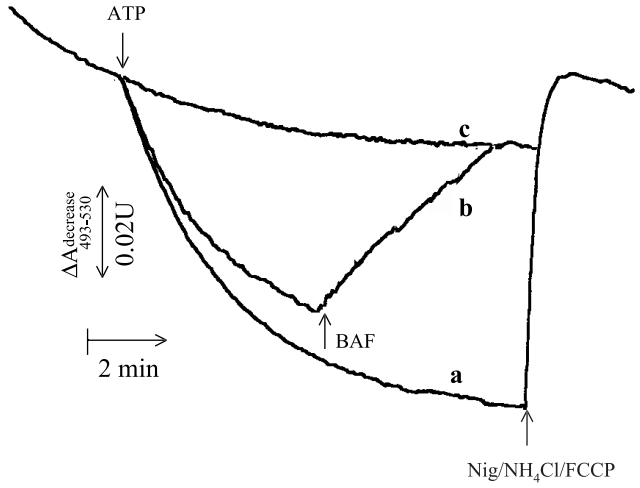

Fig. 2. ATP-driven AO accumulation by *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to the standard reaction media containing 2 μM antimycin A, 2 μg/ml oligomycin, and 2 μg/ml myxothiazol. AO (3.3 μM) was added 3 min after the cells. Trace (a), EGTA (1 mM) present in the reaction media; trace (b), 4 μM bafilomycin A_1 added where indicated by the arrow and trace (c), bafilomycin A_1 (4 μM, from the beginning of the experiment) or absence of ATP (traces were superimposable). ATP (0.5 mM), 20 mM NH_4Cl, 1 μM FCCP and 1.3 μM nigericin were added where indicated.

Addition of 20 mM NH_4Cl completed the release of AO. This AO release induced by Ca^{2+} also occurs when vanadate was present in the incubation medium, indicating that this process is totally independent of cation transport by a P-type Ca^{2+}-ATPase [trace (a)]. Furthermore, the presence of 20 μM EGTA, which removes most of the contaminant Ca^{2+} present in the reaction medium (about 8 μM), increased the extent of ATP-dependent vacuolar acidification in the permeabilized cells [trace (a)], indicating a close relationship between Ca^{2+} uptake and vacuolar acidification. Both the insensitivity of Ca^{2+} uptake to vanadate (Fig. 1) and the vacuolar alkalization promoted by this cation (Fig. 2 traces (a) and (b)) suggest the presence of a Ca^{2+}/nH^+ antiporter in the vacuoles of these cells [11,12]. The stimulation of AO uptake by EGTA (Fig. 2) and its release by Ca^{2+} (Fig. 3) indicate that this dye and Ca^{2+} are, in fact, being accumulated in the same compartment.

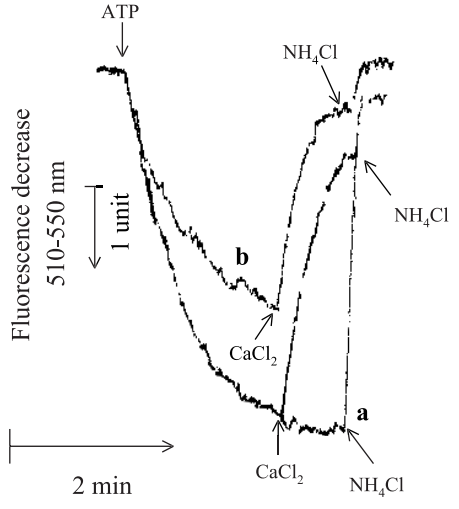

Fig. 3. Ca^{2+} effect on ATP-driven AO accumulation in *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to standard reaction media containing 2 μM antimycin A, 2 μg/ml oligomycin, 2 μg/ml myxothiazol and 20 μM EGTA. AO (3.3 μM) was added 3 min after the cells. ATP (0.5 mM), 40 μM $CaCl_2$ and 20 mM NH_4Cl were added where indicated. Trace (a), 400 μM vanadate present from the beginning of the experiment and trace (b), control.

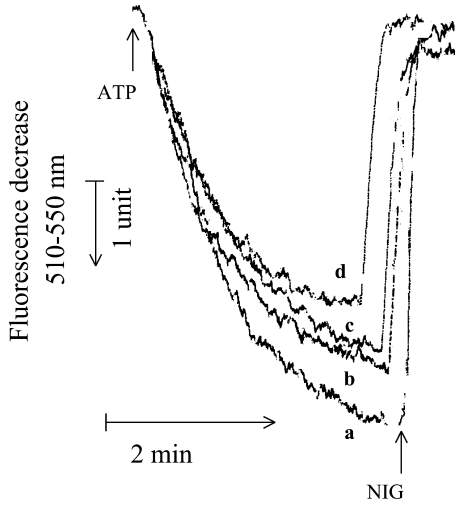

Fig. 4. Inhibition by BHT of ATP-driven AO accumulation in *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to the standard reaction medium containing 2 μM antimycin A, 2 μg/ml oligomycin, 2 μg/ml myxothiazol and 1 mM EGTA. AO (3.3 μM) was added 3 min after the cells. ATP (0.5 mM) and 1.3 μM nigericin were added where indicated. BHT was added 30s before ATP. Trace (a), control; trace (b), 5 μM BHT; trace (c), 10 μM BHT; trace (d), 20 μM BHT.

3.4. *Effect of 3,5-dibutyl-4-hydroxytoluene (BHT) on ATP-driven AO accumulation by C. parapsilosis spheroplasts*

We have previously demonstrated [29] that BHT, at commonly used antioxidant concentrations [25], has an inhibitory effect on both V-H^+-ATPases and Na^+/H^+ antiporters of the acidocalcisomes present in *Trypanosoma brucei* procyclic trypomastigotes. At the same concentration, and depending on the preincubation time, BHT presented a stimulatory or inhibitory effect on the vacuolar H^+-ATPase present in those trypanosomes [29].

In Fig. 4 we studied the effects of different concentrations of BHT on AO uptake in *C. parapsilosis* spheroplasts. When ATP was added 30 s after BHT was included in the medium, a progressive inhibition of AO accumulation was detected with increasing BHT concentrations (5–20 μM) [traces (b–d)].

3.5. *Vacuole alkalization by the antimycotic drug ketoconazole*

Ketoconazole (R41.400; Janssen Pharmaceutica) is an imidazole derivative with broad-spectrum activity against fungi and pathogenic yeast [26]. It is proposed that ketoconazole and other azole-based antimycotic agents (fluconazole, itraconazole) act by inhibiting the synthesis of ergosterol, the predominant component of the fungal cell membrane [27].

Since Ca^{2+}-containing vacuoles are not present in most mammalian cells, they can be exploited as important targets for drugs against diseases caused by fungi. In this regard, we studied the effect of the antimycotic drug ketoconazole [28] on the retention of AO by the acidic vacuoles in *C. parapsilosis* (Fig. 5). Ketoconazole (16–128 μg/ml, 75–600 nM) induced a progressive alkalization of the acidocalcisome [traces (b–e)]. As expected, this was followed by Ca^{2+} release (not shown).

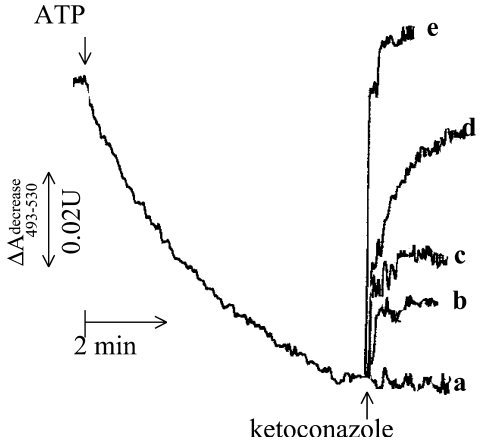

Fig. 5. Effect of ketoconazole on ATP-driven AO accumulation in *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to the standard reaction medium containing 2 μM antimycin A, 2 μg/ml oligomycin, 2 μg/ml myxothiazol and 0.5 mM EGTA. AO (3.3 μM) was added 3 min after the cells. Ketoconazole was added at the following concentrations: Trace (a), control; trace (b), 16 μg/ml; trace (c), 32 μg/ml; trace (d), 64 μg/ml and trace (e), 128 μg/ml.

The concentration range studied was the same used in drug susceptibility tests, with the difference that the cell concentration used in these experiments was at least three orders of magnitude higher than that used in these tests [6]. Other azole-based antimycotic agents, such as fluconazole and itraconazole, had no effect on the retention of AO by the acidic vacuoles.

4. Discussion

This study demonstrates that lyticase can be used to prepare *C. parapsilosis* spheroplasts, permeable to ions, nucleotides and respiratory substrates, without affecting the functional integrity of their internal vacuoles. Previous results [7] demonstrated that the functional properties of their mitochondria are also preserved after this treatment.

Our experiments provide strong evidence for the presence of acidic Ca^{2+} storage vacuoles in *C. parapsilosis*. First, we demonstrate inhibition and release of Ca^{2+} by the K^+/H^+ ionophore nigericin and the vacuolar H^+-ATPase inhibitor bafilomycin A_1 (Fig. 1). Also using permeabilized spheroplasts, we show that Ca^{2+} induces alkalization of the intracellular vesicles, as measured by shifts in AO fluorescence (Fig. 2). The mitochondrial inhibitors used (antimycin A, myxothiazol and oligomycin) had no effect on Ca^{2+} uptake by these permeabilized cells, suggesting that mitochondria, although able to carry out oxidative phosphorylation and other energy-linked functions [7], are not actively participating in Ca^{2+} homeostasis. The inhibition of Ca^{2+} uptake by the vacuolar H^+-ATPase inhibitor bafilomycin A_1 (Fig. 1) indicates that the internal acidic pH generated by the vacuolar proton pump promotes Ca^{2+} uptake and retention. Furthermore, EGTA, which decreases medium Ca^{2+} concentration (Fig. 2), increased the rate of ATP-dependent vacuolar acidification in *C. parapsilosis* spheroplasts, again indicating a close relationship between Ca^{2+} uptake and vacuolar acidification. The insensitivity of Ca^{2+} uptake to orthovanadate indicates that this transport is not mediated by a P-type Ca^{2+}-ATPase and that this enzyme is not present in these vacuoles where Ca^{2+} movements seem to be mediated by a Ca^{2+}/nH^+ antiporter, dependent on Ca^{2+} and pH gradients.

The possible physiological roles of this integrated system for Ca^{2+} and H^+ transport (H^+-ATPase and Ca^{2+}/nH^+) could be many: regulation of cytosolic Ca^{2+} concentration, a large-scale Ca^{2+} storage system to provide a homeostatic reserve, or a detoxification system to prevent the effects of excess free Ca^{2+} in the cytosol when the cells are submitted to an unfavorable environment. Alternatively, the regulation of cytosolic pH could be very important in many physiological situations. In many microorganisms, relatively small increases in cytosolic pH halt cell division and activate the expression of different genes [37]. On the other hand, increases in cytosolic Ca^{2+}, mediated by Ca^{2+} influx through the plasma membrane, could induce H^+ release from the acidic vacuoles, protecting the cells against alkaline pH.

We have previously reported [29] that the antioxidant BHT has effects on both H^+-ATPases and Na^+/H^+ antiporters from *T. brucei*. These effects are not related to alterations in the fluidity or viscosity of the bilayer, as expected with a hydrophobic molecule [35]. Using *C. parapsilosis* spheroplasts, we also demonstrated an inhibitory effect of BHT (Fig. 4), which is probably due to its effect on the H^+-ATPase, since we could not detect a Na^+/H^+ antiporter in these cells.

Acidic vacuoles containing Ca^{2+} have been previously described in *Dictyostelium discoideum* [30], trypanosomatids [31,34,38] and some mammalian cells [32,33], although this finding is controversial [36]. The acidic vacuole in *C. parapsilosis* could represent a potential target for the chemotherapy against this fungus. In this regard, the antimycotic drug ketoconazole promoted a fast alkalization of these vacuoles (Fig. 5) that, in addition to its effect on the synthesis of ergosterol [39], could be involved in the mechanism of its antimycotic action. This is a new and singular effect described for ketoconazole, since the other two azole-based antimycotic agents tested (fluconazole and itraconazole) had no effect on the *C. parapsilosis* acidic vacuole. Although all these compounds inhibit ergosterol synthesis [39], there is a heterogeneity of action among them and ketoconazole is the only drug known to have effects on several membrane-bound enzymes [39], a property which seems to be in agreement with its unique effect on the acidic vacuole of *C. parapsilosis*. In this regard, μM concentrations of ketoconazole can be detected in the plasma of patients using therapeutic doses of this drug [40]. The Ca^{2+}-containing *C. parapsilosis* acid vacuoles seem to be similar to those present in plant cells [24] and differ from those present in most trypanosomatids [22] because they lack Ca^{2+}-ATPase, V-H^+-PPase and Na^+/H^+ exchanger.

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