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Ca^{2+} and H^+ homeostasis in fission yeast: a role of Ca^{2+}/H^+ exchange and distinct V-H^+-ATPases of the secretory pathway organelles

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Abstract We determined the H^+ and Ca^{2+} uptake by fission yeast membranes separated on sucrose gradient and found that (i) Ca^{2+} sequestering is due to Ca^{2+}/H^+ antiporter(s) localized to secretory pathway organelles while Ca^{2+}-ATPase activity is not detectable in their membranes; (ii) immunochemically distinct V-H^+-ATPases acidify the lumen of the secretory pathway organelles. The data indicate that the endoplasmic reticulum, Golgi and vacuole form a network of Ca^{2+} and H^+ stores in the single cell, providing favorable conditions for such key processes as protein folding/sorting, membrane fusion, ion homeostasis and Ca^{2+} signaling in a differential and local manner. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ca^{2+} and H^+ homeostasis; V-H^+-ATPase; Ca^{2+}/H^+ antiporter; *Schizosaccharomyces pombe*

1. Introduction

Ca^{2+} homeostasis in lower eukaryotes and plants is believed to be dependent on the precisely coordinated activities of (i) high affinity and low capacity Ca^{2+}-ATPases, (ii) low affinity and high capacity Ca^{2+}/H^+ antiporter of vacuolar membrane and (iii) Ca^{2+} release channels [1,2]. Genes of the putative Ca^{2+}-ATPases were revealed in the genome of the fission yeast *Schizosaccharomyces pombe* [3], suggesting that they are involved in Ca^{2+} homeostasis. Ca^{2+} uptake by *S. pombe* cells after permeabilization of the plasma membrane was attributed to the Ca^{2+}-ATPase activity [4]. Surprisingly, up to now there have been no reports on the Ca^{2+} uptake driven by Ca^{2+}-ATPase(s) or by Ca^{2+}/H^+ antiporter(s) in membrane vesicles isolated from *S. pombe*. Also, the expression of foreign genes of Ca^{2+}-ATPases in *S. pombe* has not been reported. This fact is in contrast with our knowledge on Ca^{2+} transporters in *Saccharomyces cerevisiae* where their activities have been demonstrated both in the vacuolar [5,6,28,29] and in the Golgi membranes [7–10]. We show here for the first time that (i) sequestering of Ca^{2+} in fission yeast is carried out with the Ca^{2+}/H^+ antiporter(s) of intracellular membranes while Ca^{2+}-ATPase activity is not detectable, (ii) the secretory pathway (SP) organelles are equipped with functionally active and immunochemically distinct V-H^+ ATPases and (iii) the endoplasmic reticulum (ER) and Golgi besides vacuole are the Ca^{2+} and H^+ sequestering compartments which all together form the dynamic network of the ion stores in the yeast cell.

2. Materials and methods

2.1. Yeast strains and medium

The fission yeast *S. pombe* haploid strain *972h*$^-$ was grown at 30°C in YPD medium containing 1% yeast extract, 2% bactopeptone and 2% glucose.

2.2. Cell fractionation and biochemical assays

Yeast cells were grown up to 3–7 OD_{600}/ml (beginning of log phase) or 11–15 OD_{600}/ml (mid-log phase). After incubation in 1.2 M sorbitol and 30 mM mercaptoethanol at pH 8.5 for 10 min at 25°C they were washed with 1.2 M sorbitol and 50 mM NaH_2PO_4 adjusted with citric acid to pH 5.8. Spheroplasts were then isolated by incubation of the cells with lytic enzymes from *Tritrichoderma* at 30°C in the same buffer. Spheroplast lysis and isolation of membranes followed published procedures [9]. The resuspended total membranes were loaded onto a step gradient formed of 56, 52, 48, 45, 42, 39, 36, 33, 30 and 25% sucrose (w/w) [9]. After centrifugation at $140\,000 \times g$ for 2 h 45 min, the membrane fractions were collected and stored at −70°C. Activities of organellar marker enzymes as well as the protein determination followed published procedures cited in [9]. $^{45}Ca^{2+}$ uptake by membrane vesicles was measured by the filtration method [9]. To measure H^+ transport membrane vesicles were added to 1930 μl of incubation medium containing 20 mM MOPS-Na pH 7.4, 2.5 mM $MgCl_2$, 20 mM KCl, 12.5% sucrose and 1 μM 9-amino-6-chloro-2-methoxyacridine (ACMA). H^+ transport was initiated by addition of ATP-Na (0.5 mM) and was monitored by the fluorescence quenching of ACMA [6]. Subsequent addition of bafilomycin A_1 or carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP) was used to show recovery of the fluorescence which reflects a collapse of the preliminarily formed H^+ gradient. F_{max} reflects a steady-state amplitude of the ΔpH formation achieved after 10 min of H^+ transport; it was calculated as $\Delta F/F$ and was expressed as a percentage.

2.3. Immunoblot

Yeast membranes from the sucrose gradient fractions (20 μl) were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with antibodies. Rabbit polyclonal antibodies specific for either subunit A or subunit B of V-ATPase from mung bean (*Vigna radiata*) were kindly provided by Dr. M. Maeshima. The blots were developed with peroxidase-conjugated secondary antibody.

3. Results and discussion

Vesicles of the total membranes isolated from *S. pombe* showed an ATP-dependent ΔpH formation and Ca^{2+} uptake (Fig. 1). The Ca^{2+} uptake by total membranes was inhibited by bafilomycin A_1, an inhibitor of V-H^+-ATPases [11], and

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone; SP, secretory pathway

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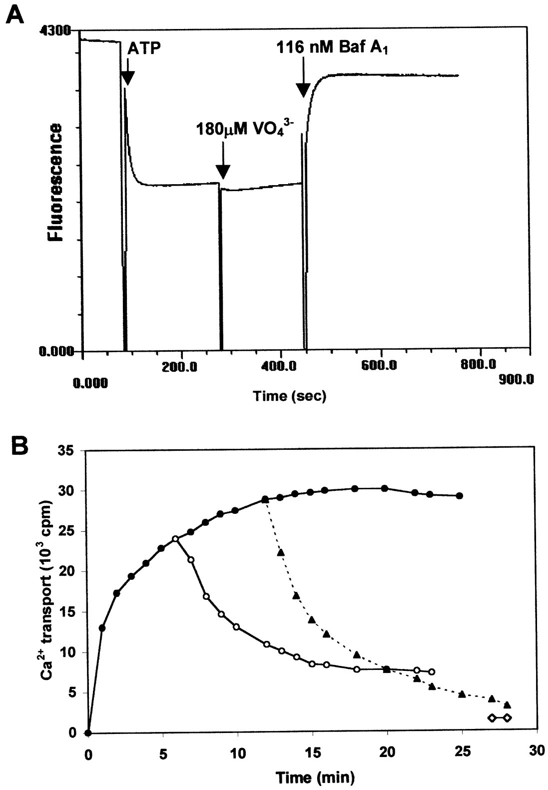

Fig. 1. ATP-dependent and bafilomycin A$_1$-sensitive formation of ΔpH and Ca^{2+} transport across the total membranes. A: H$^+$ transport. B: ^{45}Ca^{2+} uptake in the presence (●) or absence of ATP (◇) and after addition of 100 nM bafilomycin A$_1$ (▲) or 1 μM ionomycin (○).

protonophore FCCP (Fig. 2). Bafilomycin A$_1$ collapsed ΔpH and released pre-accumulated Ca^{2+} even more efficiently than ionomycin, the Ca^{2+}/H$^+$ ionophore (Fig. 1). These facts point to a crucial role of ΔpH both for Ca^{2+} accumulation and for the Ca^{2+} retention by membrane vesicles. The inhibitor of P-type ATPases vanadate (up to 800 μM) and the inhibitor of intracellular Ca^{2+}-ATPases thapsigargin (1–5 μM) [12] did not block Ca^{2+} uptake (not shown). Thus the results indicate that Ca^{2+} uptake by total membranes of *S. pombe* does not depend on Ca^{2+}-ATPases and P-type H$^+$-ATPase. We conclude therefore that Ca^{2+} uptake by the membrane vesicles was due to the Ca^{2+}/H$^+$ exchange driven by V-H$^+$-ATPase.

To our knowledge this is a first report describing ATP-dependent Ca^{2+} and H$^+$ transport activities by membrane vesicles isolated from fission yeast. In *S. cerevisiae*, the functionally active V-H$^+$-ATPase and Ca^{2+}/H$^+$ antiporter were shown to be localized to the vacuolar membrane [5,6,13]. The pleiotropic phenotypes of the V-H$^+$-ATPase mutants in *S. cerevisiae* were also interpreted only on the basis of vacuolar localization of the enzyme [13]. However, the existence of an additional compartment equipped with V-H$^+$-ATPase was recently proposed for the correct targeting of the plasma membrane H$^+$-ATPase in *S. cerevisiae* [14].

Previously we have assumed that the SP organelles of *S. cerevisiae* are equipped with distinct V-H$^+$-ATPases and Ca^{2+}/H$^+$ antiporters [8]. The supposition was based on the finding of the bafilomycin A$_1$-sensitive activities of ATPase and Ca^{2+} uptake in the ER, Golgi and vacuolar membrane vesicles ([8] and in preparation). However, those activities have not been determined in the isolated membrane vesicles of the fission yeast until now.

The present work shows that Ca^{2+}/H$^+$ exchange activity is a characteristic of different membranes derived from various

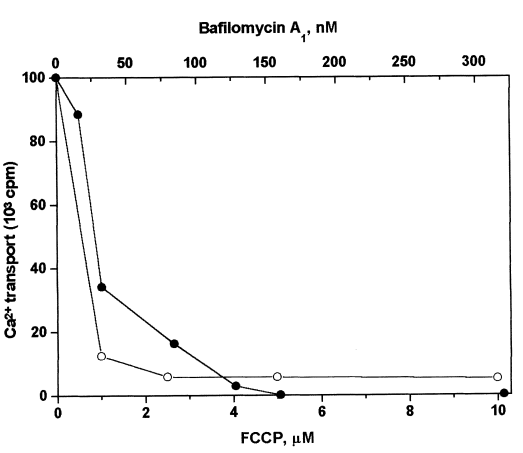

Fig. 2. ^{45}Ca^{2+} uptake by total membranes of *S. pombe 972h$^-$* is blocked by protonophore FCCP (○) and by bafilomycin A$_1$ (●), an inhibitor of V-H$^+$-ATPases. The 10 min ^{45}Ca^{2+} uptake by membrane vesicles (25 μg) was measured without preincubation with inhibitors.

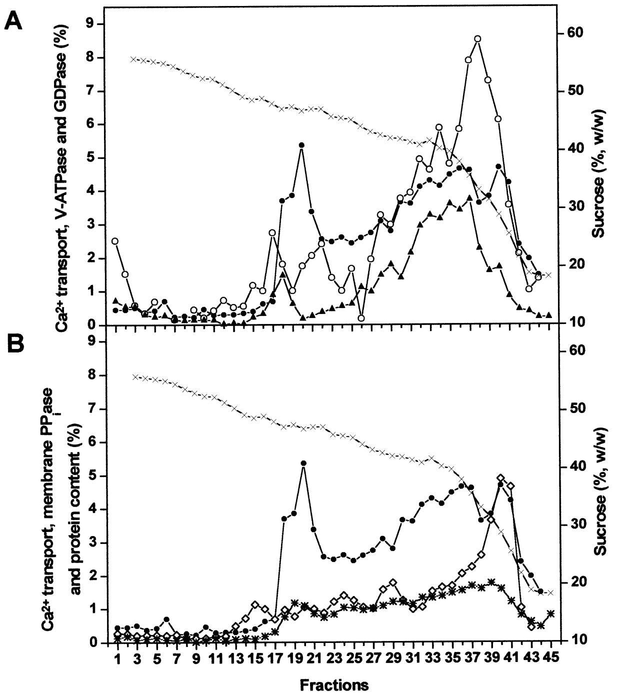

Fig. 3. Membrane vesicles of different organelles display ΔpH-dependent Ca^{2+} uptake. Total membranes were isolated from yeast harvested at the beginning of the log phase of growth (5 OD$_{600}$/ml) and fractionated on sucrose density gradient. Fractions were collected from the bottom of the centrifuge tubes, frozen and then subjected to determination of the 10 min Ca^{2+} uptake (●), GDPase (▲), ATPase sensitive to 150 mM nitrate (○), membrane-bound inorganic pyrophosphatase (PP$_i$ase, ◇), protein (*) and sucrose content (×). Data are presented as a contribution of each membrane fraction (%) in the activity of all fractions (100%); the corresponding values of the protein content and the PP$_i$ase activity were accordingly divided by 3 and 2 to fit the same scale of the ordinate.

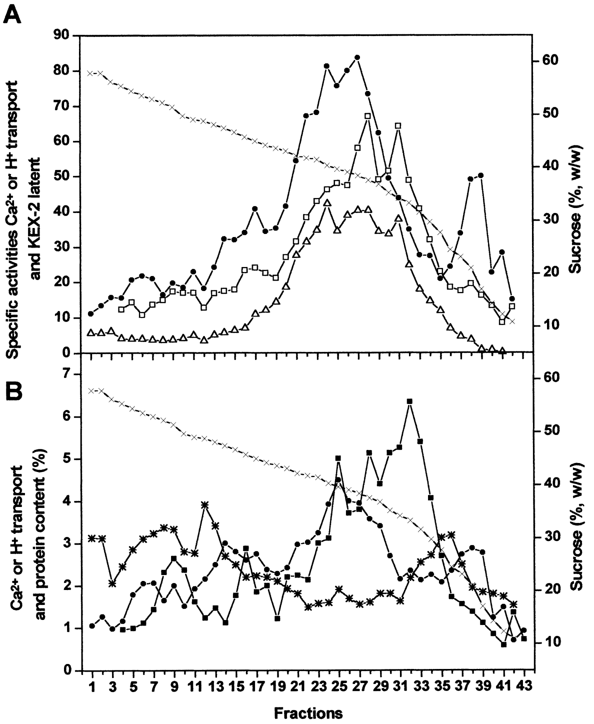

Fig. 4. Membrane vesicles of different organelles of the yeast SP show the V-ATPase-mediated H^+ transport and the ΔpH-dependent Ca^{2+} transport. Membranes were isolated from yeast harvested in the mid-log phase (11 OD_{600}/ml) and fractionated on sucrose density gradient. A: Specific activities of the KEX2 protease (fluorescence units/60 min mg protein, △) and of Ca^{2+} (cpm/10 min mg protein, ●) or H^+ transport (% of the fluorescence quenching after 10 min/mg protein, □); the original specific activities were divided by factors of 12, 400 and 1.5, respectively, to fit the same scale of the ordinate; sucrose content (×). B: Ca^{2+} transport activity (10 min, ●), initial velocity of the H^+ transport activity (■) and protein content (*) of each membrane fraction are expressed as its contribution (%) in the total activity or protein content of all fractions (%).

compartments of *S. pombe* (Fig. 3). One peak of Ca^{2+} transport activity (fractions 38–44) comigrated with the activity of membrane-bound pyrophosphatase, the marker of the tonoplast [9]. Fractions 17–26 and 26–38 were enriched with the ER and Golgi, accordingly (Fig. 3). The Golgi markers GDPase and KEX2 [15,16] migrated at 30–42% sucrose (Figs. 3 and 4) while the marker of ER membranes, NADPH cytochrome *c* oxidoreductase [17], was found mainly at 42–49% sucrose (not shown). We failed to detect Ca^{2+}-ATPase in any membrane fractions, since Ca^{2+} uptake was not sensitive to vanadate, but was completely blocked by bafilomycin A_1 at 60–120 nM (not shown). It is noteworthy that in the same experimental conditions both the Ca^{2+}-ATPases and Ca^{2+}/H^+ antiporters of different compartments of *S. cerevisiae* were detectable and contributed to Ca^{2+} uptake almost equivalently ([8,9]; in preparation). Therefore, our work indicates that Ca^{2+} homeostasis in fission yeast may be carried out exclusively by Ca^{2+}/H^+ antiporters while Ca^{2+}-ATPases are silent. The question why Ca^{2+}-ATPases are not functionally expressed or not detectable in *S. pombe* needs further investigation.

Since a main part of Ca^{2+} in the organelle lumen is bound [18,19] we assume that Ca^{2+} transport capacity may reflect a potential contribution of each organelle in Ca^{2+} sequestering. A contribution of membranes derived from the ER, Golgi and vacuoles to Ca^{2+} transport by all membranes depended on the phase of yeast growth. It was equal to 37, 38, 18% and to 22, 44, 18% in the beginning and the middle of the log phase, respectively (Figs. 3 and 4). It looks likely that during the transition from the beginning to the middle of the log phase the Ca^{2+} uptake activity was redistributed between the ER and the membranes heavier than ER which contributed 16% (Figs. 3 and 4, fractions 1–11). These membranes are believed to be derived from a nuclear envelope or from the complex of nuclear envelope and ER. Our data propose that Ca^{2+}/H^+ antiporter of these membranes is distinct from the antiporter(s) of other membranes or it is regulated differently during yeast growth.

It is noteworthy that the contribution of the vacuole in Ca^{2+} sequestering did not exceed 20%, while the ER and Golgi contributed up to 66–75% (Figs. 3 and 4). We conclude that the ER and Golgi of fission yeast cooperate with vacuoles in Ca^{2+} sequestering and that they are principal contributors in this process. The existence of several physically separated Ca^{2+} pools may underlie the occurrence of localized and spatially distinct patterns in Ca^{2+} signaling and therefore its specificity [2].

V-H^+-ATPase was previously found in the Golgi, lysosomes, endosomes and in clathrin-coated vesicles of mammalian cells [14,20] and in the vacuoles and Golgi of plants [21,22]. It was widely accepted that in these organisms the enzyme is assembled in the ER but becomes active only in such downstream organelles of the SP as the Golgi and vacuole [13,14,20–23]. It has been assumed that in yeast the pump is functionally active only in tonoplast [5,6,13,20].

We show here that V-H^+-ATPase operates in the organelles of the SP including the ER and Golgi of fission yeast (Figs. 3 and 4). The finding of the H^+ translocase activity of V-type ATPase in various organelles indicates that the enzyme is already functionally assembled in the ER and then transported to the Golgi and vacuoles in active form. The detection of the enzyme which acidifies a lumen of the SP organelles and forms ΔpH and a potential difference across their membranes provides a possibility to investigate the role of these factors in such key processes as ion homeostasis, protein folding/sorting, membrane budding/fusion and metabolite transport across membranes distinct from the tonoplast [13,14,24,25].

To address the question whether the same V-H^+-ATPase services different Ca^{2+} compartments we used a polyclonal antibody raised against the regulatory subunit B of the tonoplast V-ATPase of mung bean. It recognized a single protein in membranes derived from ER, in membranes heavier than ER and in membranes enriched with tonoplast (Fig. 4, fractions 11–19, fractions 4–11, fractions 37–41, respectively). It is of note that the immunoreactivity did not correlate with the H^+ transport activity. For example, fractions 8–12 and 14–18 showing similar initial velocities of H^+ transport (Fig. 4B) presented detectable differences in the immunoreactivity (not shown). Surprisingly, the Golgi-enriched membranes displaying higher H^+ transport activity in comparison with other membranes (Fig. 4, fractions 24–35) exhibited weak or undetectable levels of subunit B (not shown). Additionally, maximum activities of nitrate-sensitive ATP hydrolysis and bafilomycin A_1-sensitive H^+ transport were found in the same light sub-population of the Golgi membranes which migrated at

33.3% sucrose (Fig. 3A, fraction 38 and Fig. 4B, fraction 32, respectively) and presented no immune response.

Interestingly, membrane fractions 19–20 exhibiting maximum Ca^{2+}/H^+ exchange activity (Fig. 3) also displayed the maximum content of catalytic subunit A whereas fractions 22–23 presented a high content of subunit B (not shown). It points to additional differences between V-H^+-ATPases of various membranes. Taken together, the data indicate that immunochemically distinct V-H^+-ATPases serve different Ca^{2+} sequestering compartments of the fission yeast.

Moreover, the results are in agreement with the supposition that V-H^+-ATPases of some organelles might be regulated differently during yeast growth. For example, the activity of H^+ transport as well as the content of subunit B increased in membranes heavier than the ER during the transition from the beginning to the middle of the log phase (Figs. 3 and 4, fractions 1–11 and not shown). A plausible molecular basis for the differences between V-H^+-ATPases of various compartments needs further investigation, but it may lie in such modifications of subunits as phosphorylation, glycosylation or limited proteolysis. A different ratio of various subunits [21] and the existence of isoforms of the same subunit should also be considered [26,27].

A comparison of profiles of Ca^{2+} and H^+ transport activities showed that some membrane fractions contributed more to Ca^{2+} transport by all membranes than to H^+ transport (Fig. 4B, fractions 12–19, 20–26 and 36–41, enriched with the ER, 'heavy-type' Golgi and vacuolar membranes, respectively) and vice versa (fractions 28–35, enriched with the 'light-type' Golgi membranes). A preliminary explanation could be a different number of copies of Ca^{2+}/H^+ antiporters and/or of V-H^+-ATPases, a variation of coupling of H^+ transport and ATP hydrolysis by distinct forms of V-H^+-ATPases in different compartments, a changeable stoichiometry of Ca^{2+}/H^+ exchange, catalyzed by different antiporters, as well as combinations of those possibilities.

Our work raises the question whether Ca^{2+}/H^+ exchange activity of the *S. pombe* membranes is due to distinct antiporters, as was assumed for *S. cerevisiae* [8], or a homologue of the Chx1/Hum1 [28,29] is present in all organelles of the SP. We believe that the existence of several antiporters is likely and could underlie their differential regulation providing favorable features for Ca^{2+} signaling and homeostasis. The idea that each organelle of the SP of eukaryotes could be equipped with its own form of the same transporter [8] needs further verification. By now the growing body of evidence supports this idea both for Ca^{2+}-ATPases [1,2,9,10] and for V-type H^+-ATPases ([22,27] and this work).

Our data make it possible to consider a SP as a machinery of ion homeostasis and a functionally continuous network [30] of Ca^{2+} and H^+ store organelles. Thus each step of the membrane trafficking may be supplied with Ca^{2+} and H^+ gradients across membranes as well as with relatively high concentrations of the ions in the lumen of organelles. Our data are consistent with earlier reports linking the importance of those ions and their gradients for protein sorting and membrane fusion [13,14,24,25,31,32]. There, however, it was not resolved whether the main organelles of the SP are equipped with functionally active V-H^+-ATPases and Ca^{2+} transporters.

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