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Calcium homeostasis in yeast cells exposed to high concentrations of calcium

Roles of vacuolar H^+-ATPase and cellular ATP

D. Halachmi and Y. Eilam

Department of Bacteriology, Hebrew University – Hadassah Medical School, Jerusalem 91010, Israel

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Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) were determined in haploid and diploid cells of *Saccharomyces cerevisiae*, loaded with indo-1 and exposed to media containing a range of Ca^{2+} concentrations. $[Ca^{2+}]_i$ homeostasis was maintained at the 100–150 nM level in cells which were pre-incubated with glucose and exposed to 0.1 μM–10 mM Ca^{2+} in the medium. Slightly higher levels of $[Ca^{2+}]_i$ were determined in cells exposed to 50 mM Ca^{2+}. Pre-incubation with metabolic inhibitors instead of glucose caused a reduction in cellular ATP levels and an impaired $[Ca^{2+}]_i$ homeostasis; $[Ca^{2+}]_i$ reached 800 nM in cells exposed to 10 mM $CaCl_2$. Cells of the *Δvma1* mutant strain, with no functional vacuolar H^+-ATPase, had elevated levels of $[Ca^{2+}]_i$, reaching 1.8 μM when pre-incubated with glucose and exposed to 10 mM $CaCl_2$. Higher levels of $[Ca^{2+}]_i$ were measured in the mutant cells which were pre-incubated with metabolic inhibitors. This result indicates the central role of the vacuoles in maintaining $[Ca^{2+}]_i$-homeostasis and suggests the presence of an additional non-vacuolar ATP-requiring mechanism which contributes to keeping $[Ca^{2+}]_i$ at low levels.

Saccharomyces cerevisiae; Cytosolic calcium; $[Ca^{2+}]_i$ homeostasis; ATP; Vacuolar H^+-ATPase

1. INTRODUCTION

In animal and plant cells, cytosolic free calcium ($[Ca^{2+}]_i$) is a major signal transducing element by which extracellular factors induce a variety of physiological responses, such as proliferation, muscle contraction, neurotransmitter release and responses to hormones [1,2]. These cellular functions are mediated by temporal and spacial fluctuations in $[Ca^{2+}]_i$, while the basal level of $[Ca^{2+}]_i$ is regulated at a constant low level [1,3]. Understanding the mechanism of signal transduction by calcium depends on the availability of non-disruptive methods of measuring $[Ca^{2+}]_i$ in intact cells. Calcium-sensitive fluorescent dyes, which penetrate into the cells as acetoxymethylester derivatives and become de-esterified within the cytosol, provide an important tool for studying $[Ca^{2+}]_i$-signalling functions in animal cells [4,5]. In yeast cells, this method could not be applied since it was impossible to load the cells with the acetoxymethylester derivatives of the fluorescent dyes. Methods for measuring $[Ca^{2+}]_i$ in yeast cells have been developed only recently. In our previous work, we have described a method for loading pentapotassium indo-1 into yeast cells, incubated in a medium at an acidic pH [6]. Accumulation of indo-1 within the cells is dependent on the ΔpH across yeast cell membranes. When indo-1 is dissolved in an acidic medium, the dye is in its uncharged, un-dissociated form and penetrates through cell membrane. Once inside a cell, the dye is exposed to the higher cytosolic pH; it dissociates and becomes trapped intracellularly. Using the low-pH loading method, the values of $[Ca^{2+}]_i$ in a diploid strain of *Saccharomyces cerevisiae* was determined [6].

In contrast to mammalian cells, yeast cells can grow in media containing very high concentrations of calcium which may reach 200 mM. The mechanisms responsible for this remarkable capacity to sustain high concentrations of Ca^{2+} in the medium ($[Ca^{2+}]_o$), which is outstanding among eukaryotic cells, have not yet been elucidated. It is not known whether cells which have been exposed to high $[Ca^{2+}]_o$ maintain low $[Ca^{2+}]_i$ levels, or alternatively, yeast cells are adapted to sustain high $[Ca^{2+}]_i$ levels. If low $[Ca^{2+}]_i$ levels are maintained, is it through low permeability of cell membranes to calcium or by the action of efficient energy dependent mechanisms for Ca^{2+} extrusion and/or sequestration?

The aim of the present work is to clarify the problems listed above. We determined the level of $[Ca^{2+}]_i$ in yeast cells exposed to increasing concentrations of calcium and examined the roles of cellular ATP levels and the functions of vacuolar H^+-ATPase in maintaining Ca^{2+} homeostasis.

Correspondence address: Y. Eilam, Department of Bacteriology, Hebrew University – Hadassah Medical School, Jerusalem 91010, Israel. Fax: (972) (2) 784010.

2. MATERIALS AND METHODS

2.1. *Yeast strains*

The following strains of *S. cerevisiae* were used: N123 (MAT a/α, *his1*); 834 (MAT a, *his1*); 934 (MAT α, *ura3, ade6, arg4, aro7, asp5, met14, lys2, trp1, pet17*); W303-1B (MAT α, *ade2, ura3, leu2, his3, trp1, rho*$^+$); *Δ vma4* (MAT α, *ura3, ade2, leu2, his3, trp1, rho*$^+$, *vma4*::URA3) [7] provided by F. Foury (Université de Louvain, Belgium).

2.2. *Growth media*

The *S. cerevisiae* strains N123 and 834 were grown at 30°C for 24 h in YPD medium (1% yeast extract (Difco), 2% glucose, 2% bactopeptone (Difco)). The strain 934 was grown in the same medium for 48 h. The strains W303-1B and *Δvma4* were grown at 30°C for 48 h in SD medium (0.3% yeast nitrogen base without amino acids and ammonium sulfate (Difco) plus the required amino acids; 0.3% ammonium sulfate; 2% glucose; and MES/dimethylglutaric acid (DMG) buffer containing 5 mM MES, 5 mM DMG, brought to pH 5.2 with NaOH). Solid media were supplemented with 2% bactoagar.

2.3. *Measurements of* $[Ca^{2+}]_i$

Cells were collected from growth media by centrifugation, washed 3 times with distilled water by centrifugation and resuspension, and suspended (10^9 cells/ml) in loading solution containing 10 mM DMG, pH 4.5, 50 mM KCl, and 20 μM pentapotassium indo-1. When indicated, glucose (100 mM) was added to the loading solution for the entire loading period (90 min), or the metabolic inhibitors antimycin A (15 μM) and 2-deoxy-D-glucose (5 mM), were added to the loading solution 20 min before the termination of the incubation. The cells were then spun down, washed 3 times with buffer D containing DMG (10 mM, pH 4) and EDTA (0.1 mM), suspended (10^9 cells/ml) in the same buffer and kept at 0°C in the dark until measurement (between 15 min and 3 h). Control samples were treated similarly, but without indo-1. Small aliquots were removed from the loaded and unloaded cell suspensions and the cell numbers per ml were determined and equilibrated. Samples of 0.2 ml were removed from the loaded cell suspension and added to a cuvette containing 3 ml buffer D (10 mM DMG, pH 4, 0.1 mM EDTA) to which the indicated concentration of $CaCl_2$ was added. The suspension was mixed for 5 min in the dark and the emission spectra at 410 nm and 480 nm were measured (excitation at 355, slit 2.5 nm) using a Perkin Elmer Fluorescence Spectrophotometer.

Immediately after measurement, the cell suspension was filtered through a membrane filter (0.2 μM pore size) and the filtrate was collected into another cuvette. An aliquot of the unloaded cell suspension was then added to the filtrate, to yield the same cell concentration as in the loaded cell suspension, which had been measured. The emission spectra were measured at 410 nm and 480 nm (excitation as above). The values obtained were subtracted from the respective values obtained with loaded cells. This procedure provides a one-step correction for both cellular autofluorescence and the fluorescence of indo-1 in the solution as the result of a slight leakage of indo-1 from the loaded cells during measurement. Care was taken to maintain all indo-1-containing solutions and cell suspensions in the dark throughout the procedure. Preliminary experiments have established that after 5 min incubation with the different external calcium concentrations (up to 50 mM) the levels of $[Ca^{2+}]_i$ did not change by more than 5% during the following 10 min of incubation (not shown).

2.4. *Calculation of* $[Ca^{2+}]_i$

Values of $[Ca^{2+}]_i$ were determined from the ratio of fluorescence intensities at 410/480 nm according to Grynkiewicz et al. [6]. R_{min} and R_{max} were determined in a solution of ionic concentration as close as possible to the intracellular one, containing 170 mM KCl, 35 mM NaCl, 25 mM Mg^{2+} and 10 mM MES/Tris buffer, pH 6.2, to which indo-1 (0.1 μM) had been added. R_{min} was measured after the addition of NaOH to adjust the pH to 8 and EGTA (1 mM). R_{max} was determined after the addition of $CaCl_2$ (3 mM).

2.5. *Determination of cellular ATP content*

Cells of the different strains were incubated as for indo-1 loading, but without indo-1, in solution containing glucose, (100 mM) for the entire period, or without glucose and with metabolic inhibitors (antimycin A (15 μM) and 2-deoxy-D-glucose (5 mM)) added 20 min before the termination of incubation. The cells were then spun down, washed 3 times with buffer D, suspended at 5×10^7 cells/ml in the same buffer and divided into test tubes. $CaCl_2$ was added in small aliquots to the yeast suspensions in the test tubes to yield a range of concentrations of $CaCl_2$ between 0.1 μM and 50 mM as indicated. The suspensions were incubated for 5 min at room temperature; then samples (0.1 ml) were removed, diluted in 2 ml boiling distilled water and maintained at 100°C for 5 min. Additional samples were removed and the viability was determined by the Methylene blue exclusion method. The samples which had been boiled were cooled, 0.2 ml of these solutions were added to 2.8 ml of the reaction mixture in scintillation vials. The reaction mixture contained 1 ml solution A, 1 ml solution B and 0.8 ml H_2O. Solution A contained sodium arsenate (0.2 M) and $MgSO_4$ (40 mM), and was brought to pH 7.4 with H_2SO_4; solution B contained phosphate buffer pH 7.4 (10 mM) and $MgSO_4$ (4 mM). The reaction was initiated by the addition of 75 μl of a solution of luciferase-luciferin (4 mg/ml, Sigma) followed by immediate mixing and the placing of the vial into the well of a liquid scintillation counter that had been set for maximum sensitivity with the coincidence circuit off. Each sample was immediately counted for 10 s. Calibration curves were prepared in every experiment by adding different volumes of a solution containing 5×10^{-7} M ATP dissolved in solution B to the reaction mixture, in which 0.2 ml water replaced the sample volume, and the volume of solution B was adjusted according to the volume of the ATP solution.

2.6. *Determination of cell viability*

Aliquots of cell suspensions were taken at the indicated times and were mixed with equal volumes of a solution containing Methylene blue (0.01%) and sodium citrate (2%). The mixtures were sonicated briefly and the numbers of Methylene blue stained and unstained cells were immediately determined microscopically. Viability (Methylene blue unstained cells) was expressed as a percentage of the total number of cells at each time point.

3. RESULTS

3.1. *Levels of* $[Ca^{2+}]_i$ *in* Saccharomyces cerevisiae

To determine the value of $[Ca^{2+}]_i$ in yeast cells exposed to high $[Ca^{2+}]_o$ it was necessary to modify the method described by us previously [6] by introducing a correction for the slight leakage of indo-1 from the cells during measurement. In the presence of high $[Ca^{2+}]_o$, even a slight leakage of indo-1 may lead to a gross overestimation of $[Ca^{2+}]_i$. The modification, described in Section 2, enabled us to obtain values of $[Ca^{2+}]_i$ in cells exposed to $[Ca^{2+}]_o$ between 0.1 μM and 50 mM. It was found that the level of $[Ca^{2+}]_i$ depends on previous pre- incubation with glucose. In diploid cells pre-incubated with 100 mM glucose during 90 min, and exposed to 2 mM $CaCl_2$, the value of $[Ca^{2+}]_i$ was found to be between 100–150 nM, whereas in similar cells which were pre-incubated without glucose and exposed to 2 mM $CaCl_2$, $[Ca^{2+}]_i$ value reached 400 nM. These results may indicate that the level of metabolic energy is important in the maintenance of low values of $[Ca^{2+}]_i$. Therefore, in the following group of experiments we compared the values of $[Ca^{2+}]_i$, as a function of $[Ca^{2+}]_o$, in cells pre- incubated with glucose, and in cells pre-incu-

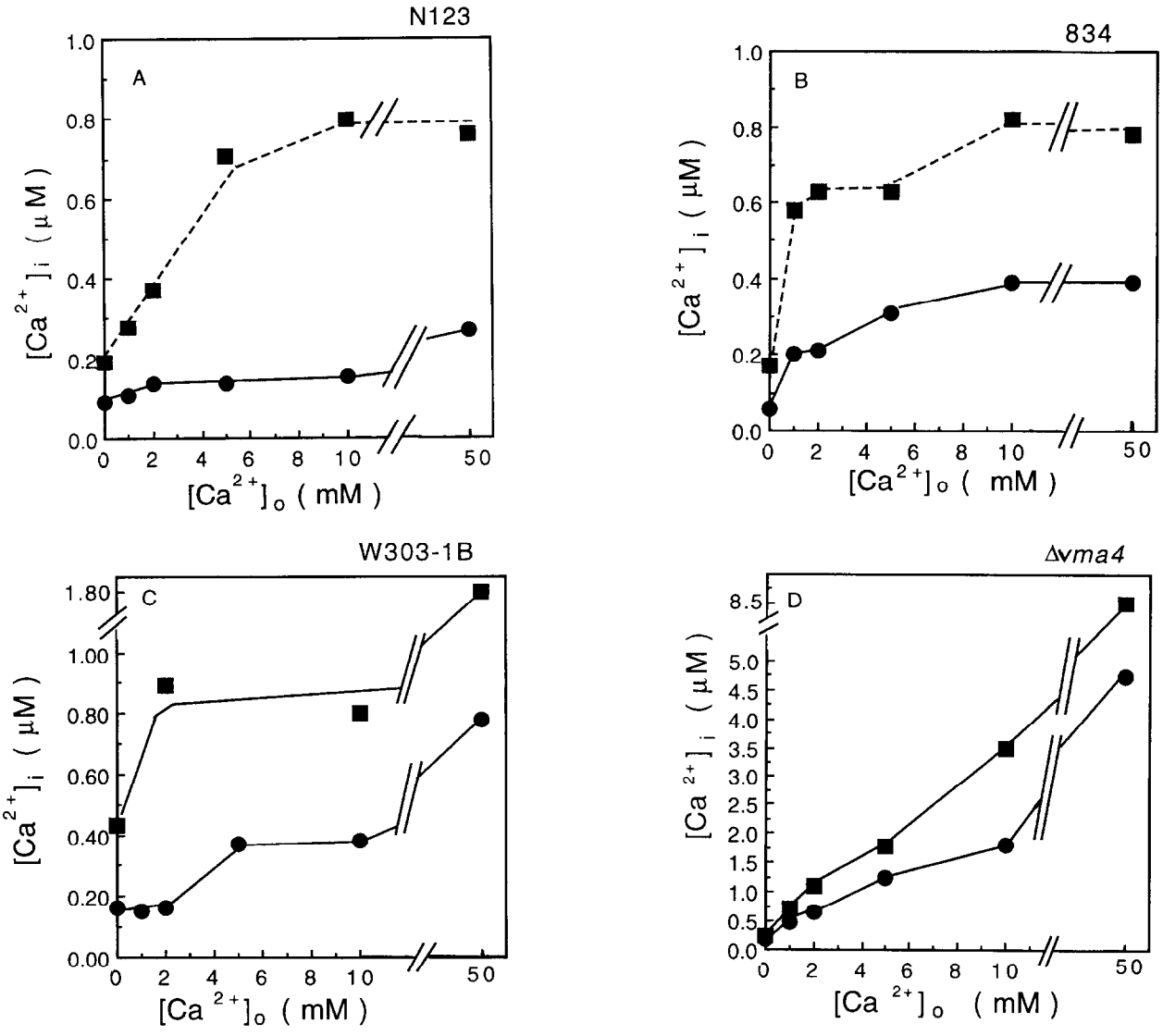

Fig. 1. The concentrations of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in yeast cells exposed to different concentrations of Ca^{2+} in the medium ($[Ca^{2+}]_o$). Cells of the indicated yeast strain were pre-incubated with glucose during 90 min (●) or without glucose during 70 min followed by incubation with metabolic inhibitors (antimycin A (15 μM) and deoxy-D-glucose (5 mM)) during 20 min (■). $[Ca^{2+}]_i$ was measured 5 min after exposure to the indicated concentration of $[Ca^{2+}]_o$. Representative experiments are shown. Similar results were obtained in 5 different experiments. The standard deviation between the values obtained did not exceed ±15%.

bated with the metabolic inhibitors antimycin A and 2-deoxyglucose. Cellular ATP levels in these cells was determined under similar conditions as for $[Ca^{2+}]_i$ measurement.

3.2. *Levels of $[Ca^{2+}]_i$ in cells exposed to increasing concentrations of $[Ca^{2+}]_o$*

Cells of the diploid strain N123 which were pre-incubated with 100 mM glucose, maintain $[Ca^{2+}]_i$ homeostasis at levels between 100 nM and 150 nM, when suspended in media containing between 0.1 μM and 10 mM calcium. An increase in $[Ca^{2+}]_o$ to 50 mM caused an elevation in $[Ca^{2+}]_i$ to 270 nM (Fig. 1A). The haploid parental strain 834 showed lower capacity to maintain $[Ca^{2+}]_i$-homeostasis. The values of $[Ca^{2+}]_i$ ranged between 70 nM in cells exposed to 0.1 μM calcium to 350 nM and 390 nM in cells suspended in media containing 10 mM and 50 mM calcium, respectively (Fig. 1B). The cellular ATP levels were not affected by $[Ca^{2+}]_o$ between 0.1 μM and 10 mM, but decreased when the cells were exposed to 50 mM calcium. Similar ATP levels were measured in cells of the diploid and haploid strains (Fig. 2). Cells pre-incubated with the metabolic inhibitors antimycin A and 2-deoxyglucose instead of glucose, had markedly reduced ATP levels (Fig. 2). Impaired $[Ca^{2+}]_i$ homeostasis was observed in the two strains; their

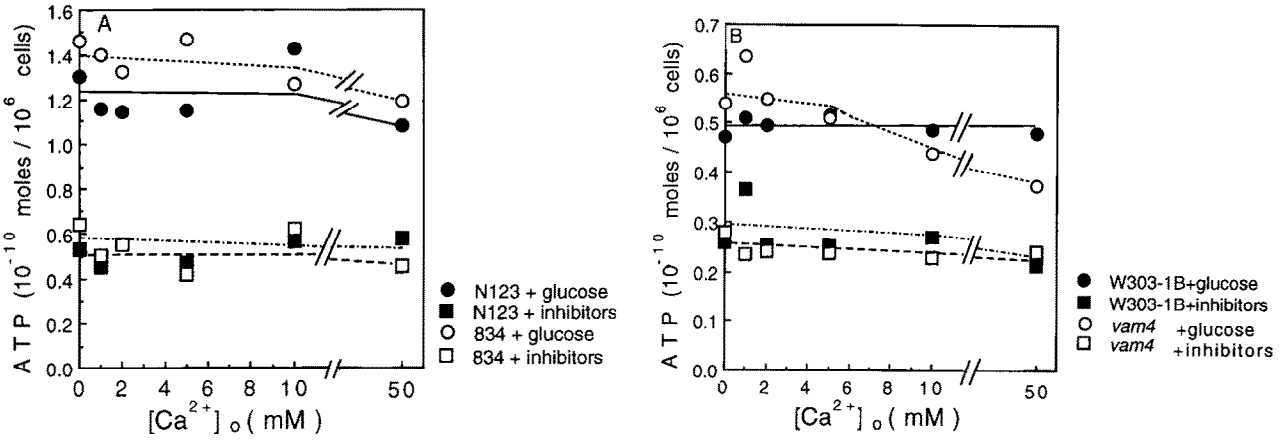

Fig. 2. ATP concentrations in yeast cells exposed to different concentrations of calcium in the medium. Cells of the indicated strains were pre-incubated with glucose during 90 min (●, ○) or without glucose during 70 min followed by incubation with the metabolic inhibitors as in Fig. 1 (■, □). ATP was determined 5 min after exposure to the indicated concentration of $[Ca^{2+}]_o$. The concentration of ATP was calculated per viable cells. Representative experiments are shown. Similar results were obtained in 5 different experiments. The standard deviation between the values obtained did not exceed ±10%.

$[Ca^{2+}]_i$ values reached 800 nM when the cells were exposed to 10 mM calcium (Fig. 1). The diploid strain displayed somewhat lower values of $[Ca^{2+}]_i$ than the haploid cells when exposed to 1 and 2 mM $CaCl_2$. Since pre-incubation with metabolic inhibitors caused only a modest decrease in cellular ATP levels (about 60% decrease), it was attempted to obtain a more drastic ATP depletion by starving for glucose cells of a respiratory deficient (*pet17*) strain. Cells of a strain 934 were incubates in indo-1 loading solution without glucose during 70 min followed by the addition of deoxy-D-glucose (5 mM) for 20 min. $[Ca^{2+}]_i$ and cellular ATP levels were determined 5 min after incubating the cells with 0.1 μM and 10 mM $CaCl_2$. The ATP level in cells incubated with 0.1 μM $CaCl_2$ was 0.10 ± 0.02 × 10^{-10} mol/10^6 cells (mean ± S.D., n = 4) whereas in cells incubated with 10 mM $CaCl_2$ the level of ATP was undetectable (below 0.02 × 10^{-10} mol/10^6 cells). The mean levels of $[Ca^{2+}]_i$ in cells incubated with 0.1 μM and with 10 mM $CaCl_2$ were 52 nM ± 8 and 634 mM ± 100 (mean ± S.D., n = 9).

3.3. *Role of vacuolar H^+-ATPase in $[Ca^{2+}]_i$-homeostasis*

$[Ca^{2+}]_i$ homeostasis was examined in the haploid strain, W303-1B and in its *vma4* null mutant strain in which no bafilomycin-sensitive vacuolar H^+-ATPase activity was detected. The values of $[Ca^{2+}]_i$ in cells of W303-1B, exposed to 0.1 μM and 10 mM calcium, were similar to the corresponding values in strain 834 (Fig. 1C). Pre-incubation with metabolic inhibitors reduced the capacity to maintain $[Ca^{2+}]_i$ homeostasis in W303-1B as well as in strain 832, but when exposed to 50 mM calcium, W303-1B cells were less capable of maintaining $[Ca^{2+}]_i$ homeostasis at low values as compared with the cells of strain 834. Evidently, some differences exist between the strains in the capacity to maintain $[Ca^{2+}]_i$ homeostasis at low levels while exposed to high $[Ca^{2+}]_o$.

Loss of the function of vacuolar H^+-ATPase in the mutant *Δvma4* caused an impaired calcium homeostasis. An almost linear increase in the values of $[Ca^{2+}]_i$ was observed in cells of the mutant strain which were pre-incubated with glucose, when $[Ca^{2+}]_o$ was elevated from 0.1 μM to 50 mM; $[Ca^{2+}]_i$ values reached 1.8 μM and 4.7 μM when the cells were exposed to 10 mM and 50 mM $[Ca^{2+}]_o$, respectively (Fig. 1D). Much higher values of $[Ca^{2+}]_i$ were determined in cells pre-incubated with metabolic inhibitors; $[Ca^{2+}]_i$ reached 3.6 and 8.5 μM in cells exposed to 10 mM and 50 mM $[Ca^{2+}]_o$, respectively (Fig. 1D). Reduced cellular ATP levels were observed in the *Δvma4* strain, but the levels were not much lower than those observed in the parental strain W303-1B after pre-incubation with metabolic inhibitors.

3.4. *Effects of elevated $[Ca^{2+}]_i$ on cell viability*

The effects of high $[Ca^{2+}]_i$ levels on cell viability in strain *Δvma4* was examined by comparing the viability of cells of this strain to that of its parental strain, W303-1B, when suspended in solutions containing DMG buffer, pH 4, and a range of $CaCl_2$ concentrations (0.1 μM–50 mM). Samples were taken during incubation at 30°C and the viability was determined by the Methylene blue exclusion method. The results in Fig. 3 show that while the cell viability in strain W303-1B was not affected by incubation in high $[Ca^{2+}]_o$ (50 mM) during 10 h, the mutant strain showed considerable loss of viability starting 2 h after the initiation of incubation with 50 mM Ca^{2+}. Interestingly, incubation with low $[Ca^{2+}]_o$ also caused considerable reduction of viability in the

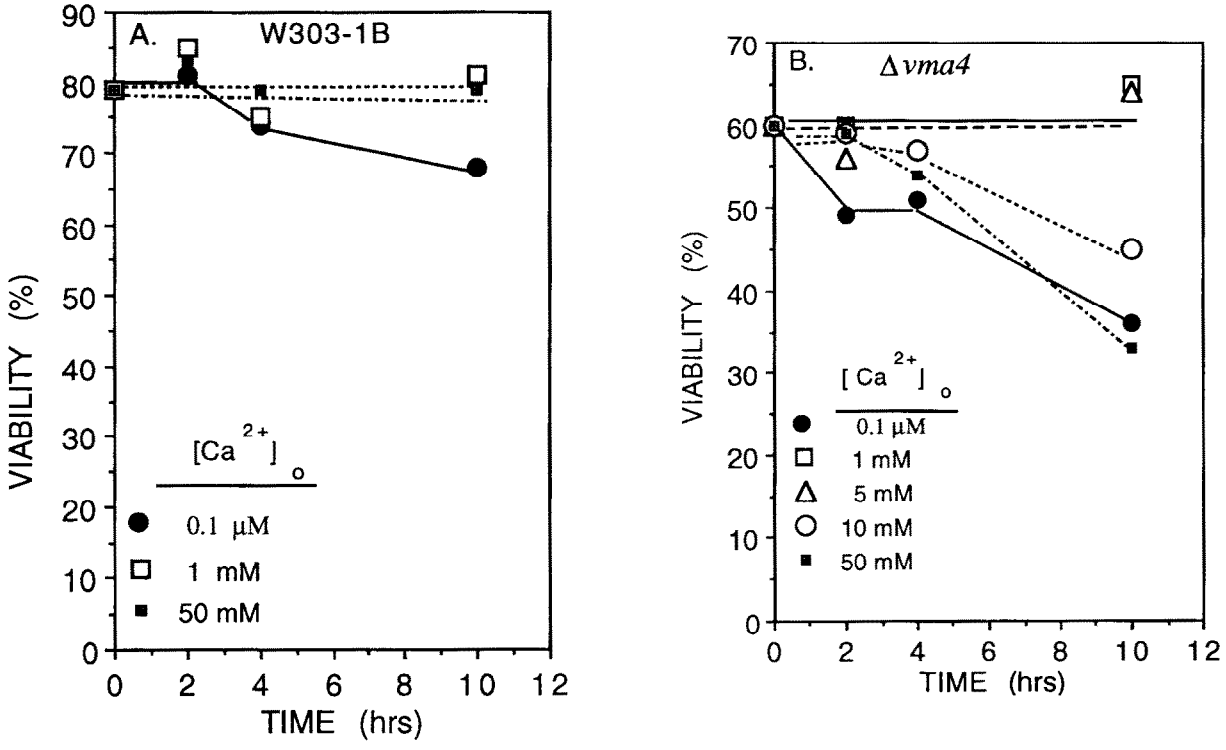

Fig. 3. Viability of cells of strains *Δvma4* and W303-1B exposed to different concentrations of calcium in the medium. Cells of the indicated strains were suspended (10^7/ml) in solution containing DMG buffer, pH 4 (10 mM); EDTA (0.1 mM) and a range of $CaCl_2$ concentrations (0.1 μM–50 mM). The suspensions were maintained at 30°C on a shaker. At different times, samples were removed and the viability was determined by the Methylene blue exclusion method. Representative experiments are shown. Similar results were obtained in 3 different experiments. The standard deviation between the values obtained did not exceed ±10%.

mutant strain, and to a lesser extent, also in strain W303-1B (Fig. 3). A 24 h incubation in all Ca^{2+} concentrations caused a reduction in viability to 7–16% in cells of strain *Δvma4* and to 60–87% in strain W303-1B (not shown).

4. DISCUSSION

The results presented in this paper show that cells of *S. cerevisiae* capable of maintaining $[Ca^{2+}]_i$ values of 100–150 nM when exposed to $[Ca^{2+}]_o$ up to 10 mM, and slightly higher $[Ca^{2+}]_i$ values when exposed to 50 mM $CaCl_2$. The exact $[Ca^{2+}]_i$ value is dependent on the yeast strain (haploid or diploid), pre-incubation with glucose and the concentration of calcium in the media.

Iida et al. [8], have measured $[Ca^{2+}]_i$ in individual *S. cerevisiae* cells loaded with fura-2 by electroporation. Average $[Ca^{2+}]_i$ values of 116 ± 90 nM [8] and 162 ± 86 nM [9] were determined in 2 haploid yeast strains suspended in a medium containing 0.68 mM $CaCl_2$. Similar values of $[Ca^{2+}]_i$ (between 100 and 200 nM) were determined in the present work in 3 wild-type yeast strains pre-incubated with glucose and exposed to 1 mM $CaCl_2$. This similarity confirmed the validity of the methods of loading and the values determined. Pre-incubation with glucose strongly affects the value of $[Ca^{2+}]_i$. The higher $[Ca^{2+}]_i$ values reported by us previously (364 nM) [6], originate from variation in the experimental procedure which involves exposure of the cells to medium without glucose before measurement (unpublished data).

Measurements of $[Ca^{2+}]_i$ values in cells exposed to high $[Ca^{2+}]_o$ became possible only after the introduction of a procedure which corrected for the slight leakage of indo-1 during measurement. The leakage of fluorescent dyes from plant and fungal cells has been reported to complicate $[Ca^{2+}]_i$ determination [10]. It is found in the present work that the maintenance of low $[Ca^{2+}]_i$ values in cells exposed to high $[Ca^{2+}]_o$ is roughly correlated with cellular ATP levels. This finding strongly suggests that the ability of yeast cells to maintain low $[Ca^{2+}]_i$ values is mediated by ATP-dependent calcium transport mechanisms. However, a drastic decrease in ATP level in the respiratory deficient strain, starved for glucose, and exposed to 10 mM Ca^{2+}, did not lead to elevation of $[Ca^{2+}]_i$ levels above those found in the wild-type strains which were pre-incubated with metabolic inhibitors. The lower ATP levels in the respiratory deficient cells incubated with 10 mM Ca^{2+} as compared with ATP levels in those cells incubated with 0.1 μM Ca^{2+}, may suggest that ATP has been used for maintaining the observed $[Ca^{2+}]_i$ levels in cells exposed to 10 mM $[Ca^{2+}]_o$.

The results of the present work indicate that $[Ca^{2+}]_i$

homeostasis at low levels is not maintained by low permeability of cell membranes to Ca^{2+}, since incubation with glucose increases the rates of calcium influx and efflux in yeast cells, thus increasing membrane permeability to calcium, but decreasing $[Ca^{2+}]_i$ levels. Therefore, there is no correlation between membrane permeability to Ca^{2+} and the ability to maintain low $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ homeostasis is grossly impaired in cells of the *Δvma4* strain in which the vacuolar H^+-ATPase is not active. In yeast cells, vacuoles are the main storage compartment for Ca^{2+} [13]. The transport of Ca^{2+} into the vacuole is mediated by Ca^{2+}/nH^+ antiporter, which is driven by the electrochemical proton gradient generated by the vacuolar H^+-ATPase [14]. The vacuolar H^+-ATPase is a multimeric complex; a range of mutants in the function of this enzyme have been described [7,15–18]. The *VMA1*, *VMA2* and *VMA3* disrupted mutants completely lost vacuolar membrane H^+-ATPase activity, vacuolar Ca^{2+}-uptake activity, and the ability to acidify the vacuole in vivo [15,16]. The *VMA4* gene, isolated by Foury [7], encodes a hydrophobic polypeptide which is essential for the activity of the vacuolar H^+-ATPase. Null haploid *vma4* mutant shares in vivo and in vitro phenotypes with null mutants of *VMA1–VMA3* genes [7,16,18]. Ohya et al. [18], reported values of $[Ca^{2+}]_i$ between 900 and 1,000 nM in a range of mutants defective in the functions of vacuolar H^+-ATPase, grown in SD medium which contained 0.68 mM $CaCl_2$. In the present work, this observation was extended; we examined $[Ca^{2+}]_i$ values in cells of the strain *Δvma4* exposed to a range of concentrations of $CaCl_2$ between 0.1 μM and 50 mM. The effects of cellular ATP levels on the values of $[Ca^{2+}]_i$ in this strain were also examined. Whereas $[Ca^{2+}]_i$ values in cells exposed to 0.1 μM Ca^{2+} were in the normal range, increasing $[Ca^{2+}]_o$ to 50 mM, resulted in the elevation of $[Ca^{2+}]_i$ to 4.7 μM in cells pre-incubated with glucose (with high ATP levels), and 8.8 μM in cells pre-incubated with metabolic inhibitors (with low ATP levels). The finding that cellular ATP levels contribute to lowering the values of $[Ca^{2+}]_i$ in *Δvma4* strains indicates that apart from the vacuolar Ca^{2+} transport system, an additional ATP dependent Ca^{2+} transport mechanism(s) functions in maintaining $[Ca^{2+}]_i$ homeostasis in yeast. This transport system is probably Ca^{2+}-ATPase. Our group has recently described an intracellular ATP-dependent Ca^{2+} pump within the yeast *Schizosaccharomyces pombe*, encoded by the gene *cta3* [19,20]. A null mutation of *cta3* leads to higher levels of $[Ca^{2+}]_i$. In *S. cerevisiae*, two genes for P-type ATPase, PMR1 and PMR2, were identified [21]. The results of the present work suggest that in *S. cerevisiae*, cytosolic Ca^{2+} homeostasis is maintained at a low level by the function of both the vacuolar Ca^{2+} transport system and the putative Ca^{2+}-ATPase.

It has been reported by Ohya et al. [18], that cells of a *vma* mutant lost viability rapidly when exposed to growth medium containing 200 mM Ca^{2+}. In the present study, we examined the viability of non-growing cells of strain *Δvma4* suspended in buffers containing a range of Ca^{2+} concentrations between 0.1 μM and 50 mM. A decrease in viability was observed in this strain after 2 h exposure to 50 mM Ca^{2+}, and after 10 h exposure to 10 mM Ca^{2+}. These results show that non-growing yeast cells can sustain μM-levels of $[Ca^{2+}]_i$ for relatively long periods of time.

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