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Elevated cytosolic free Ca^{2+} concentrations and massive Ca^{2+} accumulation within vacuoles, in yeast mutant lacking *PMR1*, a homolog of Ca^{2+}-ATPase

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Abstract The Ca^{2+}-ATPase homolog of *Saccharomyces cerevisiae*, *PMR1*, cloned by Rudolph et al. (Cell 58 (1989) 133–145) is required for normal Golgi functions. We have investigated the role of Pmr1-protein in maintaining homeostasis of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). It was found that exposure to moderately high Ca^{2+} concentrations led to elevated levels of $[Ca^{2+}]_i$ in cells of *pmr1* null mutant, in comparison with cells of *pmr2* isogenic mutant (defective in cell-membrane Na^+-ATPase) and of an isogenic wild type. In addition, we showed that *PMR1* deletion causes massive accumulation of Ca^{2+} in the vacuoles and affects the rates of Ca^{2+} influx and efflux.

Key words: *Saccharomyces cerevisiae*; Cytosolic Ca^{2+} homeostasis; Ca^{2+}-ATPase; Vacuolar Ca^{2+}; Golgi; *PMR1* gene

1. Introduction

The *PMR1* gene of *Saccharomyces cerevisiae,* cloned by Rudolph et al. [1], encodes a putative Ca^{2+}-ATPase. The deduced amino acid sequence of Pmr1-protein (Pmr1p) demonstrates 50% homology with mammalian Ca^{2+}-ATPase of the type localized in the Golgi and secretory granules [2,3]. In *S. cerevisiae*, Pmr1p was localized to Golgi-like organelles by indirect immunofluorescence staining and co-migration with Golgi markers in sub-cellular fractionation experiments [4]. The phenotype of *pmr1* mutant strains implies a functional role of Pmr1p in the transport and processing of secretory proteins: *pmr1* mutant strains are defective in the addition of an outer-chain mannose residue to secreted invertase and display impaired proteolytic processing of pro-α factor. In addition, *pmr1* mutant strains cannot grow in low-Ca^{2+} medium and are supersensitive to EGTA. These defects are reversed by the addition of 10–20 mM Ca^{2+} to the medium [1,4].

The results described above are consistent with a working hypothesis which assumes that the functional role of Pmr1p is to pump Ca^{2+} from the cytosolic compartment into the lumen of one of the Golgi compartments, in which Ca^{2+}-mediated protein processing [5,6] and exocytosis [7,8] take place. The Ca^{2+} pumping function of Pmr1p has been suggested on the basis of sequence homology to mammalian Ca^{2+} pumps. Genetic data have strongly suggested a role of Pmr1p in maintaining the homeostasis of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$): overexpression of Pmr1p suppresses defects associated with loss of vacuolar Ca^{2+} pump Pmc1p. Non-viable *pmr1 pmc1* double mutants become viable upon mutation in calcineurin [9,10]. However, a direct evaluation of the role of Pmr1p in $[Ca^{2+}]_i$ homeostasis, by measurments of $[Ca^{2+}]_i$ levels in *pmr1* mutants exposed to a range of external Ca^{2+} concentrations, has not as yet been reported. It is also not known whether deletion of the *PMR1* gene affects cellular Ca^{2+} handling, and how the addition of Ca^{2+} reverses the defects in protein secretion.

Using a method recently developed in our laboratory [11], we determined $[Ca^{2+}]_i$ levels as a function of Ca^{2+} concentrations in the suspension solution ($[Ca^{2+}]_{out}$), in the *pmr1* mutant, in comparison with the *pmr2* isogenic mutant which is defective in cell-membrane Na^+-ATPase [12–14] and with the isogenic wild type (WT). In addition, we showed that *PMR1* deletion causes massive accumulation of Ca^{2+} in the vacuoles and affects the rates of Ca^{2+} influx and efflux.

2. Materials and methods

2.1. Yeast strains, media and growth conditions

The following strains of *S. cerevisiae* were used: YR98 (MAT α, *ade 2, his3-Δ200, leu2-3,112, lys2-Δ201, ura3-52*); YR93 (MAT α, *ade 2, his3-Δ200, leu2-3,112, lys2-Δ201, ura3-52, pmr2::HIS3*); YR122 (MAT α, *ade 2, his3-Δ200, leu2-3,112, lys2-Δ201, ura3-52, PMR1-Δ1::LEU2*). The strains were provided by Dr. Hans K. Rudolph (Institut für Biochemie der Universität Stuttgart, Germany). The yeast cells were grown in standard YPD medium (2% Difco yeast extract, 1% bacto-peptone, 2% glucose) at 30°C, to late logarithmic phase or as indicated.

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

$[Ca^{2+}]_i$ was measured as described previously [11]. Cells were harvested from growth media, washed 3 times with distilled water by centrifugation and suspended (10^9 cells/ml) in loading solution containing 10 mM dimethylglutaric acid (DMG), pH 4.5, 50 mM KCl, 100 mM glucose and 20 μM pentapotassium indo-1. The cells were incubated in the loading solution for 90 min, at 30°C in the dark, 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 1 h). Control samples were treated similarly, but without indo-1. Aliquots were removed from the loaded and unloaded cell suspensions and the cell densities 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 to which the indicated concentration of $CaCl_2$ was added. The suspensions were mixed for 5 min in the dark and the emission spectra at 410 and 480 nm were

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Abbreviations: $[Ca^{2+}]_i$, concentration of free cytosolic calcium; $[Ca^{2+}]_{out}$, concentration of Ca^{2+} in the suspending solution; DMG, dimethylglutaric acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2[*N*-morpholino]ethanesulfonic acid; ER, endoplasmic reticulum

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measured (excitation at 355 nm, 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 density as in the loaded cell suspension which had been measured. The emission spectra were measured at 410 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 established that after 5 min incubation with the different external calcium concentrations the levels of $[Ca^{2+}]_i$ did not change by more than 5% during the following 10 min of incubation (not shown).

2.3. 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. [15]. R_{min} (free dye fluorescence ratio) and R_{max} (Ca^{2+} saturated dye fluorescence ratio) were determined in a calibration solution containing 170 mM KCl, 35 mM NaCl, 25 mM Mg^{2+}, 10 mM MES/Tris-HCl buffer, pH 6.2, and 0.1 μM pentapotassium indo-1. Ethanol (20%) was included in the calibration solution to correct for the lower polarity of the cytosol, compared with the polarity of an aqueous calibration solution [16]. (Calibration without the addition of ethanol yielded higher values of $[Ca^{2+}]_i$.) The fluorescence intensity ratio at 410 : 480 nm of the free dye (R_{min}) was measured in the calibration solution after the addition of NaOH (to adjust the pH to 8) and EGTA (1 mM); that of the Ca^{2+} saturated dye (R_{max}) was determined after the addition of $CaCl_2$ (3 mM).

2.4. Measurements of whole-cell Ca^{2+} content

To determine the whole-cell Ca^{2+} content (free, bound and precipitated) yeast cells were grown in YPD medium labeled with $^{45}CaCl_2$ (0.2 μCi/ml). The cells were collected by centrifugation and washed 3 times by resuspension in distilled water. Aliquots were immediately collected on membrane filters (0.45 μm pore size, 25 mm diameter), pre-washed with $MgCl_2$, 20 mM, (10^8 cells/filter), and washed 4 times with cold 20 mM $MgCl_2$ to remove the cell-wall-bound Ca^{2+}. The filters were dried and the radioactivity was determined in toulene-based scintillation fluid. The amounts of Ca^{2+} in the cells were calculated from the specific activity of the growth medium and the cell-associated counts.

To determine the content of whole-cell free Ca^{2+} the yeast cells were grown in YPD medium without added $^{45}Ca^{2+}$, collected by centrifugation, and washed 4 times by centrifugation and resuspension in distilled water. The cells were then resuspended in distilled water, transfered into flasks (3 ml in each flask) and incubated at 100°C for 15 min, then precipitated, and the concentration of Ca^{2+} in the suspension was determined using a Perkin Elmer atomic absorption spectrometer.

2.5. Ca^{2+} efflux measurements

The cells were grown in YPD medium labeled with $^{45}Ca^{2+}$ (0.2 μCi/ml) and washed with water as above. The washed cells were resuspended (10^8 cells/ml) in a solution containing 20 mM glucose and 25 mM MES/DMG buffer, pH 5.0, or HEPES/Tris-HCl buffer, pH 7.5, and incubated at 30°C with shaking. At zero time and at the indicated time intervals, 1 ml samples were removed, collected on membrane filters pre-washed with $MgCl_2$ and washed 4 times with 2 ml of cold 20 mM $MgCl_2$. The filters were dried and the radioactivity determined. The amounts of cellular Ca^{2+} were calculated from cell-associated radioactivity and the specific activity of the growth medium. Aliquots of cell suspensions were taken at the times of efflux measurements and viability was determined by methylene blue exclusion.

2.6. Measurements of Ca^{2+} influx

The cells of the *pmr1* mutant and the WT strain were harvested from the growth medium, washed 4 times with distilled water and resuspended (2×10^8 cells/ml) in buffer-glucose solution containing MES/DMG buffer (25 mM, pH 5.0) and glucose (20 mM). The cell suspensions were divided into portions of 1 ml in each flask and the flasks were incubated at 30°C on a shaker. Influx was initiated by the addition of $^{45}Ca^{2+}$ (1 or 10 μM, 1 μCi) to each flask. At the indicated times, influx was stopped by the addition of 1 ml of cold washing solution, containing $MgCl_2$ (20 mM) and $LaCl_3$ (0.2 mM), to each flask. The cells in the flask were immediately collected on membrane filters (0.45 μm pore size, 25 mm diameter), pre-washed with $MgCl_2$, 20 mM, and washed 4 times with cold washing solution. The filters were dried and the radioactivity was determined in toulene-based scintillation fluid.

3. Results

3.1. Growth rates of the strains YR98 (WT), YR93 (pmr2) and YR122 (pmr1) exposed to a range of Ca^{2+} concentrations

The *pmr1* mutant cells, lacking a putative Ca^{2+}-ATPase [1,4], the isogenic *pmr2* mutant cells, lacking a cell-membrane Na^+-ATPase [12–14] and the isogenic WT cells grew well in YPD medium containing 0.3 mM Ca^{2+}. The growth rate of the *pmr1* mutant cells was slightly stimulated by the addition of 20 mM Ca^{2+} to the medium, markedly inhibited by reducing external Ca^{2+} by EGTA, as previously reported [1,4], but was reduced by only 12% (which is no more than the decrease in the growth rate of the WT cells) by increasing $[Ca^{2+}]_{out}$ to 200 mM (Fig. 1). The growth of the *pmr2* mutant was inhibited by the addition of 10 mM LiCl to the medium, but not by very low or very high $[Ca^{2+}]_{out}$. The growth of the *pmr1* and the WT strains was not inhibited by 10 mM LiCl (not shown).

3.2. Levels of $[Ca^{2+}]_i$ in the strains YR98 (WT), YR93 (pmr2) and YR122 (pmr1) exposed to a range of Ca^{2+} concentrations

Cells of the three strains were loaded with indo-1 and exposed to buffer-glucose solutions containing different concentrations of Ca^{2+}, between 0.1 and 200 mM. After stabilization of $[Ca^{2+}]_i$ levels, approximately 5 min after exposure to high Ca^{2+}, the levels of $[Ca^{2+}]_i$ were determined as a function of

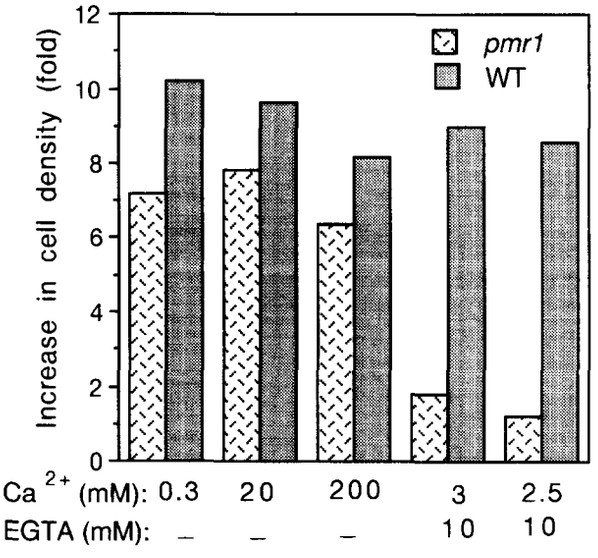

Fig. 1. Increase in cell density of cultures of the *pmr1* mutant and the WT strain after 24 h of growth in YPD medium containing various concentrations of Ca^{2+}. Cells of the indicated strains were suspended (4×10^6 cells/ml) in YPD medium containing the indicated concentrations of Ca^{2+} and EGTA, and incubated at 30°C on a shaker for 24 h. Cell densities were then determined.

$[Ca^{2+}]_{out}$, in cells of the three strains. A moderate increase in $[Ca^{2+}]_i$ levels was found in the WT and *pmr2* mutant cells (from 86 ± 6 nM to 380 ± 15 and 420 ± 75 nM, respectively) as $[Ca^{2+}]_{out}$ was raised from 0.1 to 10 mM. In contrast, cells of the *pmr1* mutant displayed a marked increase in $[Ca^{2+}]_i$ with the increase in $[Ca^{2+}]_{out}$, reaching 933 ± 37 nM when exposed to 10 mM Ca^{2+} (Fig. 2a). This result indicates that Pmr1p participates in maintaining cytosolic Ca^{2+} homeostasis in cells exposed to moderate Ca^{2+} concentrations. However, when the cells were exposed to very high Ca^{2+} concentrations (150 and 200 mM) the level of $[Ca^{2+}]_i$ in the cells of the WT strain reached 1.45 ± 0.15 and 1.56 ± 0.17 μM respectively, while $[Ca^{2+}]_i$ levels in the cells of *pmr1* mutant (1.55 ± 0.17 and 1.60 ± 0.16 μM) did not differ much from the corresponding levels in the WT cells (Fig. 2b). This result indicates that Pmr1p's contribution to the maintenance of $[Ca^{2+}]_i$ homeostasis in cells exposed to very high $[Ca^{2+}]_{out}$ is small. This result is in accordance with our observation that the growth rate of *pmr1* mutant cells is not inhibited more than that of the WT cells in medium containing 200 mM Ca^{2+} (Fig. 1).

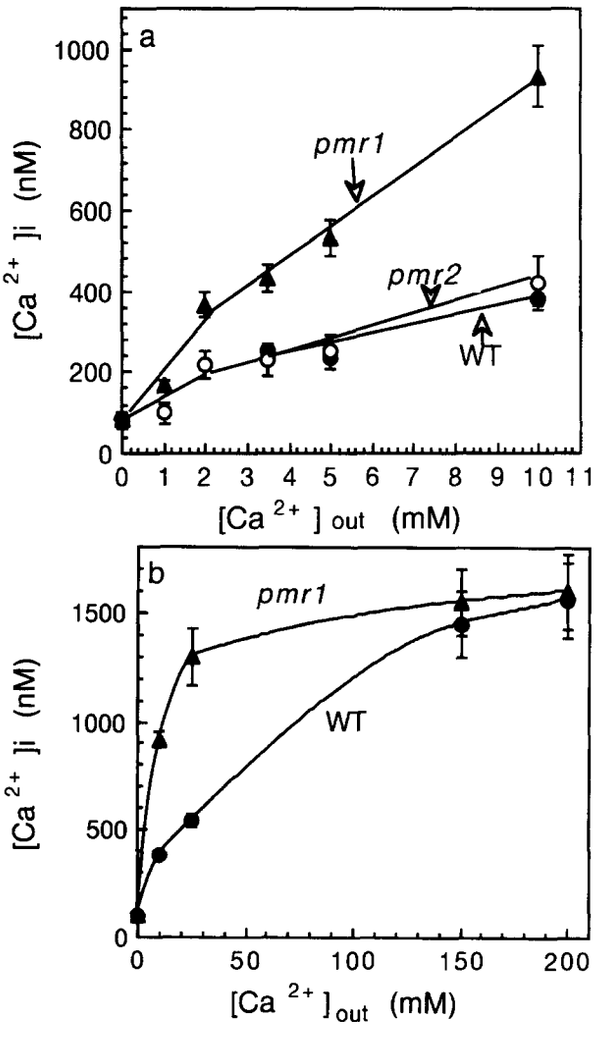

Fig. 2. Levels of $[Ca^{2+}]_i$ in cells of the indicated strains exposed to a low (a) and a high (b) concentration range of Ca^{2+}. Experiments were done as described in Section 2.

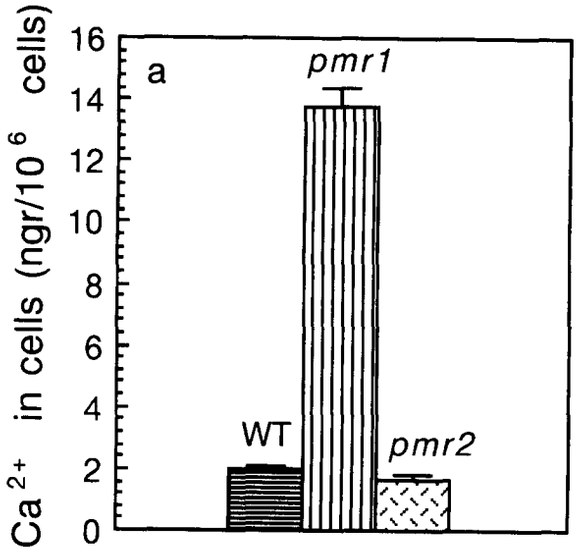

Fig. 3. Whole-cell Ca^{2+} content in cells of the *pmr1*, *pmr2* and WT strains. Cells of the indicated strains were grown in YPD medium labeled with $^{45}Ca^{2+}$ to the stationary phase. The cells were then washed and aliquots were collected on filters. The cells on the filters were washed, the radioactivity was determined and the amounts of whole-cell Ca^{2+} were calculated as described in Section 2.

3.3. Accumulation of Ca^{2+} in intracellular organelles in cells of the pmr1 mutant strain

If we assume that Pmr1p is a Ca^{2+}-ATPase, the main function of which is to maintain $[Ca^{2+}]_i$ homeostasis by pumping Ca^{2+} into intracellular organelles, it would be expected that deletion of *PMR1* would diminish the amount of whole-cell Ca^{2+} which is localized within the vacuoles and other organelles. To examine this assumption we measured total cellular Ca^{2+} in cells of the three strains after growth for 24 h in YPD medium. Surprisingly, we found much higher amounts of whole-cell Ca^{2+} in the *pmr1* mutant cells as compared with the amounts in the WT cells and in cells of the *pmr2* mutant (Fig. 3). The same differences were obtained when we measured total cellular Ca^{2+}, including bound and precipitated Ca^{2+} (Fig. 3) or when we determined only free cellular Ca^{2+} (which is concentrated predominantly in the vacuoles and in other organelles) (not shown). In the next group of experiments we measured whole-cell free Ca^{2+} levels after growth for 24 h in YPD media supplemented with various concentrations of Ca^{2+} between 0.3 mM (medium without addition of Ca^{2+}) and 200 mM. An increase in whole-cell Ca^{2+} was observed in WT cells with the increase in $[Ca^{2+}]_{out}$ from 0.3 to 100 mM but there was no further increase when $[Ca^{2+}]_{out}$ was raised from 100 to 200 mM. Much higher accumulation of Ca^{2+} in the *pmr1* mutant cells, as compared with the WT cells, was observed after growth in media containing all the examined Ca^{2+} concentrations (Fig. 4). The amount accumulated in cells of the *pmr1* mutant was 5.2-fold that accumulated in cells of the WT when the two strains were exposed to 10 mM Ca^{2+}, but this ratio decreased to 2.4-fold in cells exposed to 100 and 200 mM Ca^{2+}.

3.4. Rates of Ca^{2+} influx in cells of the pmr1 and pmr2 mutants and in the WT cells

The time-course of $^{45}Ca^{2+}$ influx into yeast cells is composed of two components.

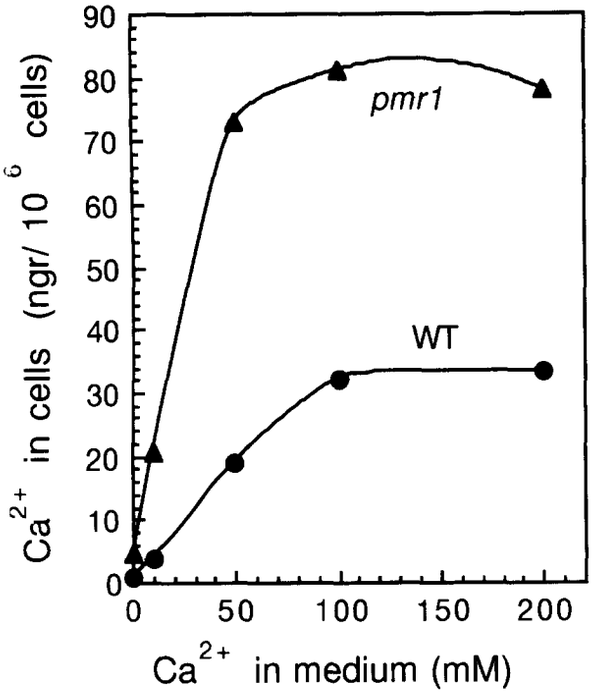

Fig. 4. Whole-cell free Ca^{2+} contents in cells of the *pmr1* mutant and WT strain grown in YPD medium containing the indicated Ca^{2+} concentrations. See Section 2 for details.

(1) An initial fast component which represents $^{45}Ca^{2+}$ binding to extracellular sites and transport across the plasma membrane. It was found in our laboratory that rates of transport across the cell membrane in *S. cerevisiae* could be determined by measuring the 'initial rate' of $^{45}Ca^{2+}$ influx during short time intervals up to 20 s, and subtracting the 'zero-time' value which represents binding [17].

(2) The second component represents uptake of $^{45}Ca^{2+}$ into intracellular organelles. This component may be determined by measuring $^{45}Ca^{2+}$ uptake during longer time intervals [17]. Under normal conditions, uptake of $^{45}Ca^{2+}$ into the vacuole is the rate limiting step for cellular Ca^{2+} accumulation [18]. However, under specific conditions, increased Ca^{2+} accumulation may result from increased transport of Ca^{2+} across the plasma membrane, as found previously in yeast cells exposed to substances which disrupt the integrity of the plasma membrane [19]. We therefore examined the initial rate of $^{45}Ca^{2+}$ influx into cells of the *pmr1* mutant and the WT to determine which component is regulated by Pmr1p. Cells of the two strains were suspended in buffer-glucose solution containing 1 μM Ca^{2+} labeled with $^{45}Ca^{2+}$. Influx was determined at 'zero-time' and at various time intervals up to 30 s. The 'zero-time' values were subsequently subtracted from all corresponding influx values. Ca^{2+} influx across the plasma membrane was linear with time during 30 s, The slopes of the lines, determined by linear regression, are 0.92 and 1.01 pgr Ca^{2+}/10^8 cells ×s, for the WT and *pmr1* cells, respectively (regression coefficients are 0.98 and 0.97, respectively) (Fig. 5). The difference between the slopes is not significant. In additional experiments cells were suspended in buffer-glucose solution containing 10 μM Ca^{2+} labeled with $^{45}Ca^{2+}$. Influx was determined at 'zero-time' and at 30 s, and the 'zero-time' values were subtracted from the corresponding 30 s values. In these experiments, influx values at 30 s were 0.30 ± 0.06 and 0.428 ± 0.036 ngr $Ca^{2+}/10^8$ cells ×30 s in cells of the WT and *pmr1* mutant, respectively, compared with 0.025 ± 0.004 and 0.034 ± 0.012 ngr $Ca^{2+}/10^8$ cells×30 s in the WT and *pmr1* cells suspended in a solution containing 1 μM Ca^{2+}. The results show substantial increases in transport rates in both strains upon increasing the concentration of Ca^{2+} from 1 to 10 μM. This result indicates that 1 μM Ca^{2+} is below the saturation concentration of the transport systems in cells of the two strains, and may be used to compare rates of Ca^{2+} transport between these strains.

The second component of Ca^{2+} transport, uptake into intracellular organelles, was determined in the two strains by measuring $^{45}Ca^{2+}$ uptake during different time intervals up to 30 min. The results shown in Fig. 6 indicate a much higher rate of Ca^{2+} uptake into cells of the *pmr1* mutant as compared with cells of the WT strain. Similar differences between the strains were observed when the cells were suspended in buffer-glucose solution containing 1 or 10 μM Ca^{2+}. The results of the two groups of experiments indicate that deletion of the *PMR1* gene does not affect Ca^{2+} transport systems in the plasma membrane but affects those in membranes enclosing some intracellular organelles.

3.5. Rates of Ca^{2+} efflux from cells of the pmr1 and pmr2 mutants and the WT cells

In order to identify the organelles which over-accumulate Ca^{2+} in the *pmr1* mutant cells, efflux experiments were conducted. The rates of Ca^{2+} efflux from the vacuole into medium without Ca^{2+} are extremely slow [18]. This is in contrast to faster Ca^{2+} efflux rates from other organelles which accumulate Ca^{2+}. It was recently reported that the protein Csg2p is required for the regulation of Ca^{2+} accumulation in a fast-exchangeable Ca^{2+} pool, presumably in the endoplasmic reticulum (ER) [20,21]. To determine whether the lack of function

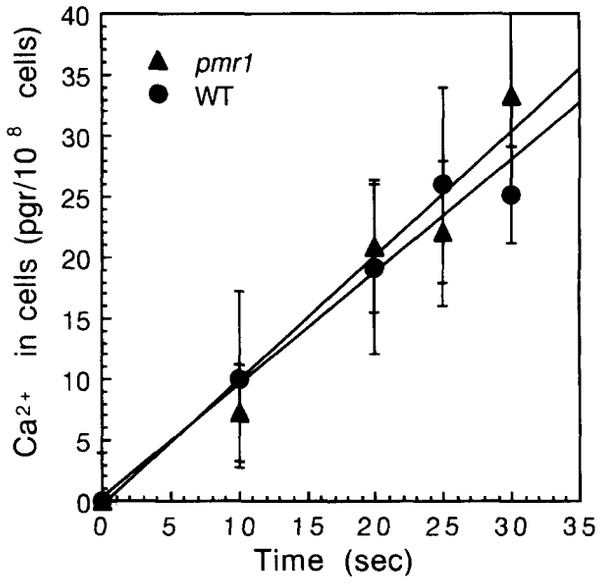

Fig. 5. 'Initial rates' of Ca^{2+} influx into WT and *pmr1* mutant cells. Cells of the two strains were suspended in buffer-glucose solution (25 mM MES/DMG buffer, pH 5.0, and 20 mM glucose), containing 1 μM Ca^{2+} labeled with 1 μCi/ml $^{45}Ca^{2+}$. Influx was determined at 'zero-time' and at various time intervals up to 30 s. The 'zero-time' values were subtracted from all corresponding influx values. See Section 2 for details. Data are means+SEM, $n = 6$.

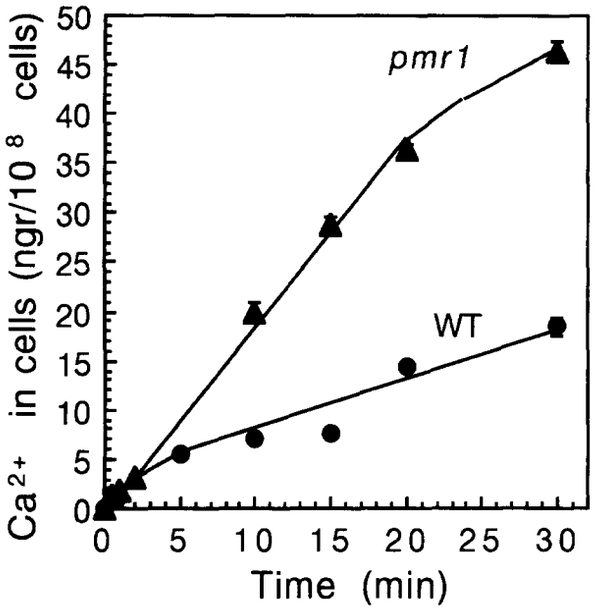

Fig. 6. Ca^{2+} uptake by cells of the *pmr1* mutant and the WT strain. Cells were suspended in buffer-glucose solution (25 mM MES/DMG buffer, pH 5.0, and 20 mM glucose), which contained $^{45}Ca^{2+}$ (1 μM, 1 μCi/ml). Ca^{2+} uptake was measured as described in Section 2.

of Pmr1p causes over-accumulation of Ca^{2+} in the slow-releasing Ca^{2+} pool or in the fast-releasing Ca^{2+} pool, cells of the two mutants and the WT strain were equilibrated with $^{45}Ca^{2+}$ by growing them in $^{45}Ca^{2+}$ containing YPD medium. The cells were then harvested, washed and diluted in buffer-glucose solution. The cell suspensions were incubated at 30°C on a shaker. Immediately after dilution (zero-time) and at the indicated times, aliquots were removed, filtered through membrane filters, washed 4 times with $MgCl_2$ solution, dried, the radioactivity was determined and the amounts of Ca^{2+} in the cells were calculated. Due to the very low efflux rates, experiments continued up to 24 h. There was no decrease in the viability of the cells during the experiments as determined by methylene blue exclusion. Results are expressed as log percentage of the amount of Ca^{2+} at zero-time, remaining in the cells at each sampling time, versus time. Single efflux rates were obtained in the three strains. Whereas WT and *pmr2* mutant cells lost 40–50% of cellular Ca^{2+} during 24 h of incubation (rates were -0.92×10^{-2} and -1.04×10^{-2} h^{-1}, respectively) the *pmr1* mutant cells did not show any Ca^{2+} efflux at all. In *pmr1* mutant cells there was even a small increase in cellular $^{45}Ca^{2+}$ (rate was $+0.19\times10^{-2}$ h^{-1}). This $^{45}Ca^{2+}$ was probably liberated from cell walls after cell wash and before the zero-time sampling. We have measured a concentration of 0.5 μM of Ca^{2+} in the suspension solutions; this Ca^{2+} was labelled with $^{45}Ca^{2+}$ at the same specific activity as cellular Ca^{2+}. The increase in $^{45}Ca^{2+}$ was reduced by the addition of EGTA to the buffer-glucose solution (rate was reduced to $+0.08\times10^{-2}$ h^{-1}) (Fig. 7a).

Addition of unlabeled Ca^{2+} to the buffer-glucose solution caused substantial release of $^{45}Ca^{2+}$ from the *pmr1* mutant cells (Fig. 7c). The release was much larger than that from the WT cells. These results were obtained when Ca^{2+} efflux was expressed as quantities of Ca^{2+} (Fig. 7c). Since the amounts of whole-cell Ca^{2+} were very large in the *pmr1* mutant cells, the percentage of the initial amount of Ca^{2+} which was released from the cells of *pmr1* mutant was smaller than that released from the WT cells (rates were -0.20×10^{-2} and -0.98×10^{-2} h^{-1}, respectively) (Fig. 7b). The release of $^{45}Ca^{2+}$ from the *pmr1* mutant after the addition of unlabeled Ca^{2+} was due to $^{45}Ca^{2+}$–Ca^{2+} exchange, probably mediated by the vacuolar Ca^{2+}/H^{+} exchanger [18,22]. Efflux from the WT and the *pmr2* mutants cells showed a very small component of $^{45}Ca^{2+}$–Ca^{2+} exchange, since addition of unlabeled Ca^{2+} increased $^{45}Ca^{2+}$ efflux only slightly (rates were increased from -0.92×10^{-2} to -0.98×10^{-2} and from -1.04×10^{-2} to -1.11×10^{-2} h^{-1}, respectively). Similar patterns of Ca^{2+} efflux from the examined strains were observed in experiments curried out at pH 5.0 (Fig. 7) and at pH 7.5 (not shown).

3.6. *Inhibition of Ca^{2+} accumulation by Mg^{2+}*

Our results indicate that the over-accumulation of Ca^{2+} in *pmr1* mutant cells is into a slow-releasing (non-exchangeable) Ca^{2+} pool. The next experiments were designed to characterize the non-exchangeable Ca^{2+} pool in pmr1 cells. Beeler et al. [20] has found that whereas the vacuolar Ca^{2+} pool in yeast cells is non-exchangeable, another cellular Ca^{2+} pool, controlled by the ER protein Csg2p, is exchangeable. In contradiction, Tanida et al. [23] reported that the Csg2p/Cls2p Ca^{2+} pool is non-exchangeable. To determine whether the accumulation of Ca^{2+} in pmr1 cell is into the vacuoles or also into the ER — Csg2p/Cls2p Ca^{2+} pool, we have used the procedure reported by Beeler et al. [20], who showed that Ca^{2+} accumulation into the vacuole is blocked by 5 mM Mg^{2+} while accumulation of Ca^{2+} into the ER — Csg2p/Cls2p pool remains unaffected. Cells of the WT and the *pmr1* mutant were grown in YPD medium for 24 h, then diluted 5-fold into YPD medium containing 50 mM $CaCl_2$ without or with 5 or 10 mM $MgCl_2$. The amounts of whole-cell free Ca^{2+} were determined immediately upon dilution (zero-time) and after 2 h incubation at 30°C as described in Section 2 (Section 2.4). Whole-cell free Ca^{2+} contents after 24 h growth in YPD medium were 2.3 and 15.5 ngr $Ca^{2+}/10^6$ cells in the WT and *pmr1* cells respectively. During 2 h incubation in medium containing 50 mM Ca^{2+}, whole-cell free Ca^{2+} increased by 1.89 and 4.5 ngr/10^6 cells in the WT and *pmr1* cells, respectively. Mg^{2+} (5 mM) blocked the increase in whole-cell Ca^{2+} completely in the *pmr1* cells, but only partially in the WT cells. The Ca^{2+} accumulation in the WT cells was further inhibited by increasing the concentration of Mg^{2+} to 10 mM (Fig. 8). Our results indicate that Ca^{2+} accumulation in *pmr1* cells is exclusively into the vacuoles.

4. Discussion

The results of the present study indicate that Pmr1p participates in maintaining $[Ca^{2+}]_i$ homeostasis when the cells are exposed to moderately elevated $[Ca^{2+}]_{out}$. When the cells were exposed to very high $[Ca^{2+}]_{out}$ (150–200 mM) the activity of Pmr1p in lowering $[Ca^{2+}]_i$ appeared to be very small, since the levels of $[Ca^{2+}]_i$ in the *pmr1* mutant cells did not differ much from the levels in the WT cells. Indeed, the growth rate of *pmr1* mutant cells was only slightly reduced in medium containing 200 mM Ca^{2+}.

Massive accumulation of Ca^{2+} within intracellular organelles was observed in cells of the *pmr1* mutant. This accumulation was not mediated by increased Ca^{2+} influx across cell

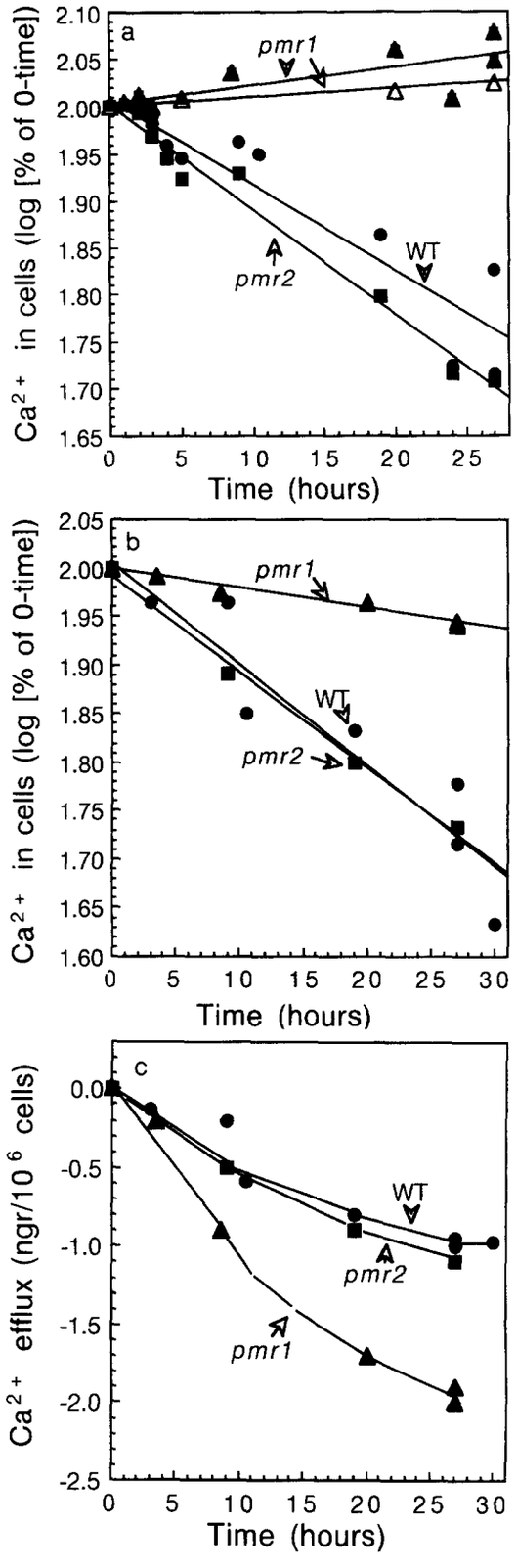

Fig. 7. Rates of Ca^{2+} efflux from cells of the *pmr1*, *pmr2* and WT strains. Cells were grown in $^{45}Ca^{2+}$ containing YPD medium, then washed and incubated in a solution containing (a): MES/DMG buffer (25 mM, pH 5.0) and glucose (20 mM) (●, ■, ▲) or with the addition of EGTA (△), or (b,c): buffer and glucose as in (a), with the addition of $CaCl_2$ (2 mM). Efflux was measured as described in Section 2. Lines are drawn by linear regression. All regression coefficient (r) values were larger than 0.94 except *pmr1* (o) in (a) in which $r = 0.8$.

membranes but by stimulation of Ca^{2+} uptake into some intracellular organelles. The pattern of efflux of $^{45}Ca^{2+}$ from *pmr1* mutant cells strongly suggests that the organelles which over-accumulate Ca^{2+} are the vacuoles. It was shown that the $^{45}Ca^{2+}$ pool in yeast vacuoles is released extremely slowly into medium without added Ca^{2+}. Addition of Ca^{2+} to the medium induces a release of lumen $^{45}Ca^{2+}$ due to $^{45}Ca^{2+}$–Ca^{2+} exchange, which is probably mediated by the vacuolar Ca^{2+}/H^+ exchanger [18]. In *pmr1* mutant cells there was no $^{45}Ca^{2+}$ efflux into medium without added Ca^{2+} but enhanced Ca^{2+} efflux into medium containing unlabeled Ca^{2+}. In contrast, in the *pmr2* mutant and WT cells, efflux of $^{45}Ca^{2+}$ into medium without added Ca^{2+} was observed, and the addition of unlabeled Ca^{2+} increased $^{45}Ca^{2+}$ efflux only slightly. Further characterization of the *pmr1* intracellular Ca^{2+} pool was based on the effect of Mg^{2+}. It was reported that the accumulation of Ca^{2+} into the vacuoles is blocked by 5 mM Mg^{2+} whereas accumulation into the Cls2p/Csg2p-regulated ER Ca^{2+} pool remains unaffected [20]. We have found that accumulation of Ca^{2+} into intracellular organells in *pmr1* cells is completely blocked by 5 mM Mg^{2+}, while accumulation of Ca^{2+} into intracellular organelles in the WT is only partially inhibited by 5 mM Mg^{2+} and further inhibited by 10 mM Mg^{2+}. Thus, the results show that deletion of the *PMR1* gene causes changes in the distribution of the accumulated Ca^{2+} among the various intracellular Ca^{2+} pools. In *pmr1* mutant cells Ca^{2+} is accumulated exclusively into the vacuoles.

Yeast cells are unique among eukaryotes in being able to grow in media containing very high concentrations of Ca^{2+} such as 200 mM. When yeast cells were exposed to low $[Ca^{2+}]_{out}$, $[Ca^{2+}]_i$ levels between 100 and 350 nM were measured, depending on levels of cellular ATP [11,24]. An increase in $[Ca^{2+}]_i$ was observed in cells of various yeast strains as a function of the increase in $[Ca^{2+}]_{out}$ [11]. In cells of the *pmr1* mutants and the WT, $[Ca^{2+}]_i$ values of 1.5–1.6 μM were measured when exposed to 200 mM of $[Ca^{2+}]_{out}$. The huge Ca^{2+} gradient across the plasma membranes (more than 5 orders of magnitude) is maintained by the activity of several Ca^{2+} transport systems which sequester Ca^{2+} into intracellular organelles. The major Ca^{2+} sequestering organelles are the vacuoles [25]. Ca^{2+} is transported into the vacuoles by the Ca^{2+}/H^+ exchanger which depends on the electrochemical proton gradient ($\Delta\mu_{H^+}$) formed by the vacuolar H^+-ATPase [22]. Mutants lacking vacuolar H^+-ATPase activity cannot grow in high Ca^{2+} medium and show marked increase in $[Ca^{2+}]_i$ levels when exposed to moderate $[Ca^{2+}]_{out}$ [11,26]. The high levels of $[Ca^{2+}]_i$ in cells of such a mutant strain (Δ*vma4*) increased even further after depletion of cellular ATP [11]. This finding indicates that apart from the Ca^{2+}/H^+ exchanger, ATP-dependent, $\Delta\mu_{H^+}$ independent transport systems contribute to the maintenance of $[Ca^{2+}]_i$ at low levels. Several putative transport systems may fulfil this function. Recently, a *PMC1* gene which encodes a vacuole membrane protein with high homology to plasma membrane Ca^{2+}-ATPase has been cloned [9,10]. Null mutants of *pmc1* did not grow in high Ca^{2+} media. When grown in low Ca^{2+} medium it sequestered less Ca^{2+} into the vacuole than the WT. The results suggest that Pmc1p is a vacuolar Ca^{2+}-ATPase which sequesters Ca^{2+} into the

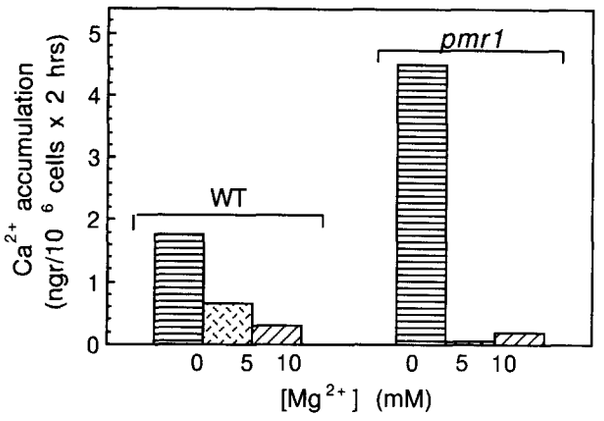

Fig. 8. Inhibition of Ca^{2+} accumulation by Mg^{2+} in the WT and *pmr1* mutant cells. WT and *pmr1* mutant cells were grown in YPD medium for 24 h, then diluted 5-fold into YPD medium containing 50 mM $CaCl_2$ and the indicated Mg^{2+} concentration. Whole-cell free Ca^{2+} contents were determined immediately upon dilution and after 2 h incubation at 30°C. The initial values were subtracted from the corresponding 2 h values. See Section 2 for details.

vacuoles [9]. A second putative Ca^{2+} transporter may be present in the ER membrane. This transporter has not yet been identified but a gene, *CLS2/CSG2*, has recently been cloned [20,21]. Cls2p was localized in the ER by immunofluorescence staining of yeast cells expressing epitope tagged *CLS2*. Cls2p/Csg2p appears to regulate Ca^{2+} sequestration into the ER, since *cls2* null mutant cells do not grow in high Ca^{2+} media and when grown in low Ca^{2+} medium sequester huge amounts of Ca^{2+} into a non-vacuolar Ca^{2+} pool, probably the ER [20,21]. The third Ca^{2+} transporter is Pmr1p [1] which was localized in Golgi-like particles by immunofluorescence staining of yeast cells expressing epitope tagged *PMR1* [4]. It was shown in the present study that Pmr1p participates in lowering $[Ca^{2+}]_i$ in cells exposed to moderately high $[Ca^{2+}]_{out}$, probably by pumping Ca^{2+} into the Golgi. Our results are consistent with a recent report showing that the double mutant *pmc1 pmr1* is not viable but the single mutant *pmc1* is viable at low $[Ca^{2+}]_{out}$ [9]. When exposed to medium containing 150–200 mM Ca^{2+}, the contribution of Pmr1p to lowering $[Ca^{2+}]_i$ is very small. In accordance with this result it was recently found in our laboratory that the expression of *PMR1* is suppressed by very high $[Ca^{2+}]_{out}$ (Halachmi and Eilam, manuscript in preparation).

Two genes for P-type ATPase, *PMR1* and *PMR2*, were cloned by Rudolph et al. [1] *PMR2* was found later to encode a plasma membrane Na^+-ATPase responsible for resistance to high concentrations of Na^+ and Li^+ [12–14]. It was suggested that *PMR1* encodes a Ca^{2+}-ATPase. This suggestion was based on sequence homology to mammalian Ca^{2+}-ATPase, inhibition of the growth of the *pmr1* mutant in very low Ca^{2+} medium, and reversal of the defects in Golgi function in the *pmr1* mutant by the addition of extracellular Ca^{2+} [1,4]. In the present study we showed that deletion of *PMR1* but not of *PMR2* causes elevation of $[Ca^{2+}]_i$. This indicates that Pmr1p is indeed a Ca^{2+}-pump since it lowers $[Ca^{2+}]_i$ by transporting it from the cytosol. The localization of Pmr1p by immunofluorescence and the defects in Golgi function in the *pmr1* mutant [1,4] indicate that the transport is into the Golgi.

Why do cells of the *pmr1* mutant accumulate high quantities of Ca^{2+} in the vacuoles? We suggest that this accumulation of Ca^{2+} is a direct consequence of the elevated $[Ca^{2+}]_i$ levels in *pmr1* mutant cells. It was shown in the present study that when $[Ca^{2+}]_{out}$ is elevated increased $[Ca^{2+}]_i$ is observed together with increased whole-cell Ca^{2+} (which is a measure of the amounts of Ca^{2+} sequestered in organelles). This result may indicate that the increase in Ca^{2+} uptake into organelles is mediated by the increase in $[Ca^{2+}]_i$. Since $[Ca^{2+}]_i$ is higher in cells of the *pmr1* mutant than in WT cells, particularly at low and moderate $[Ca^{2+}]_{out}$, more Ca^{2+} will be transported into the organelles of the *pmr1* mutant cells than in the WT cells. We show that Ca^{2+} uptake under these conditions is exclusively into the vacuoles. The suggested model is consistent with possible increase in gene expression or any other sort of activation of vacuolar Ca^{2+} transporter(s) by the high levels of $[Ca^{2+}]_i$ in the *pmr1* mutant.

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