Extracellular Ca^{2+} sensing contributes to excess Ca^{2+} accumulation and vacuolar fragmentation in a *pmr1Δ* mutant of *S. cerevisiae*

Richard Kellermayer[1], David P. Aiello[1], Attila Miseta[2] and David M. Bedwell[1,*]

[1]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[2]Department of Clinical Chemistry, University Medical School, 7624 Peçs, Hungary
*Author for correspondence (e-mail: dbedwell@uab.edu)

Accepted 16 January 2003
Journal of Cell Science 116, 1637-1646 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00372

Summary

Previous studies have suggested that yeast strains lacking the Ca^{2+}-ATPase Pmr1p are unable to maintain an adequate level of Ca^{2+} within the Golgi apparatus. It is thought that this compartmental store depletion induces a signal that causes an increased rate of Ca^{2+} uptake and accumulation in a manner similar to the capacitative Ca^{2+} entry (CCE) response in non-excitable mammalian cells. To explore this model further, we examined cellular Ca^{2+} uptake and accumulation in a *pmr1Δ* strain grown in the presence of a reduced level of divalent cations. We found that the level of Ca^{2+} uptake and accumulation in a *pmr1Δ* strain increased as the concentration of divalent cations in the growth medium decreased. These results are inconsistent with a model in which cellular Ca^{2+} uptake and accumulation are determined solely by the depletion of Ca^{2+} in an intracellular compartment. Instead, our results suggest that a second regulatory mechanism couples cellular Ca^{2+} uptake to the availability of Ca^{2+} in the extracellular environment. Furthermore, we found that various conditions that increase the level of cytosolic Ca^{2+} correlate with vacuolar fragmentation in wild-type (WT), *pmr1Δ* and *pmr1Δ/pmc1Δ* yeast strains. This suggests that vacuolar fragmentation might function as a normal physiological response to Ca^{2+} stress that increases the vacuolar surface/volume ratio, thereby maximizing the sequestration of this important signaling molecule.

Key words: Yeast, Pmr1p, Ca^{2+} homeostasis, Vacuole

Introduction

Changes in the cytosolic Ca^{2+} concentration are thought to participate in a variety of physiological processes in yeast, including cell-cycle control (Hartley et al., 1996; Iida et al., 1990a); escape from G_0/G_1 arrest following carbon source limitation (Eilam and Othman, 1990; Eilam et al., 1990; Kaibuchi et al., 1986); glucose and galactose sensing (Tokes-Fuzesi et al., 2002); the mating response (Nakajima-Shimada et al., 1991; Withee et al., 1997); protein processing in the secretory pathway (Durr et al., 1998); and adaptation to environmental stress (Batiza et al., 1996; Denis and Cyert, 2002; Mori et al., 1998). The basic features of Ca^{2+} signaling in *Saccharomyces cerevisiae* appear to be remarkably similar to the mechanisms used in mammalian cells. Ca^{2+} acts as a signaling ion in yeast through the activation of the calmodulin/calcineurin pathway, which leads to alterations in the transcription of a large number of genes (Matheos et al., 1997; Stathopoulos and Cyert, 1997; Yoshimoto et al., 2002).

A relatively small number of Ca^{2+} transporters appear to maintain cellular Ca^{2+} homeostasis in yeast. Among these are the vacuolar Ca^{2+}-ATPase Pmc1p (Cunningham and Fink, 1994b); the vacuolar Ca^{2+}/H^+ exchanger Vcx1p/Hum1p (Cunningham and Fink, 1996; Pozos et al., 1996); the endoplasmic reticulum (ER) Ca^{2+}-ATPase Cod1p/Spf1p (Bonilla et al., 2002; Cronin et al., 2000; Cronin et al., 2002; Suzuki and Shimma, 1999); and the Golgi Ca^{2+}-ATPase Pmr1p (Antebi and Fink, 1992; Rudolph et al., 1989; Sorin et al., 1997). Remarkably, the action of this small group of Ca^{2+} transporters maintains the resting cytosolic Ca^{2+} level between 50 and 200 nM when environmental Ca^{2+} concentrations range from <1 μM to >100 mM (Batiza et al., 1996; Iida et al., 1990b; Miseta et al., 1999b). When the level of environmental Ca^{2+} is elevated, the calmodulin/calcineurin signaling pathway activates the expression of many genes (including those encoding Pmr1p and Pmc1p) by the transcription factor Tcn1p/Crz1p. By contrast, the level of Vcx1p is slightly reduced by high environmental Ca^{2+}, and its activity is further repressed by calcineurin activation through a post-translational mechanism (Cunningham and Fink, 1996; Matheos et al., 1997; Stathopoulos-Gerontides et al., 1999; Yoshimoto et al., 2002).

The vacuole is the principle Ca^{2+} storage organelle in yeast, and normally contains >95% of the total cellular Ca^{2+} (Dunn et al., 1994; Eilam et al., 1985). However, it has been shown that the loss of the Golgi-localized Ca^{2+} transporter Pmr1p causes an increased sensitivity to high environmental Ca^{2+} when vacuolar Ca^{2+} transport is compromised, indicating that the Golgi apparatus also plays an important role in Ca^{2+} sequestration (Miseta et al., 1999b; Tanida et al., 1995). Furthermore, Pmr1p has also been reported to be involved in maintaining the resting Ca^{2+} concentration within the ER (Strayle et al., 1999), whereas both Pmr1p and Pmc1p

influence Ca^{2+}-dependent functions within the secretory pathway such as protein degradation in the ER and protein sorting in the Golgi apparatus (Durr et al., 1998). The ability of these transporters to influence Ca^{2+}-dependent processes in multiple organelles might be due to their movement through these compartments during the transit to their final cellular destinations. However, it has been reported that the distribution of Pmc1p in Golgi fractions increases in a *pmr1Δ* strain, suggesting that its abundance in compartments of the secretory pathway might be influenced by the lumenal Ca^{2+} concentration (Marchi et al., 1999). These findings illustrate both the complexity and the sophisticated regulatory mechanisms that control cellular Ca^{2+} homeostasis in yeast.

In mammalian cells, free Ca^{2+} located in the ER serves as a mobilizable pool that can be released into the cytosol in response to an appropriate stimulus. The resulting increase in cytosolic Ca^{2+} can then activate signaling pathways that alter the expression of many genes in a coordinated manner (Putney, 1992). In many non-excitable cells, the release of ER Ca^{2+} can also induce a store depletion signal that results in the influx of Ca^{2+} ions across the plasma membrane in a process termed capacitative Ca^{2+} entry (CCE). Recent studies have provided evidence that yeast cells might also utilize a mechanism that couples intracellular store depletion to Ca^{2+} uptake across the plasma membrane. A *pmr1Δ* mutant has been shown to exhibit a higher rate of Ca^{2+} uptake than the WT strain (Antebi and Fink, 1992; Halachmi and Eilam, 1996; Rudolph et al., 1989; Sorin et al., 1997). This led to the model that the depletion of Golgi Ca^{2+} stores can stimulate Ca^{2+} uptake into yeast cells in a manner analogous to CCE in mammalian cells (Csutora et al., 1999; Durr et al., 1998; Locke et al., 2000). In contrast to the *pmr1Δ* mutant, a *pmc1Δ* strain exhibits a reduced level of total cellular Ca^{2+} (Cunningham and Fink, 1994b). Since Pmc1p is the only known vacuolar Ca^{2+}-ATPase in yeast and the vacuole normally contains the bulk of total cellular Ca^{2+}, this suggests that Ca^{2+} uptake across the plasma membrane is coupled to the ability of the cell to remove it efficiently from the cytosol.

In the current study, we examined how a reduced level of divalent cations in the growth medium influences cellular Ca^{2+} homeostasis in the *pmr1Δ* strain. We found a large increase in Ca^{2+} uptake and accumulation under these conditions, which led to activation of the calcineurin signaling pathway. Consistent with this high level of Ca^{2+} uptake, we found that the *PMC1* gene was required for growth of the *pmr1Δ* mutant under these growth conditions. Our observation that cellular Ca^{2+} uptake increases as the concentration of environmental Ca^{2+} decreases suggests that an extracellular Ca^{2+} sensor is capable of coupling Ca^{2+} uptake to extracellular Ca^{2+} levels. Finally, we found that conditions of cellular Ca^{2+} stress result in a vacuolar fragmentation phenotype in both WT and mutant yeast strains. This might serve as an adaptive mechanism to maintain cellular Ca^{2+} homeostasis under these stress conditions.

Materials and Methods

Strains and plasmids used

The yeast strains used in this study are described in Table 1. The Pmc1p-GFP fusion plasmid was constructed as follows. A 4.9 kb *Hin*dIII fragment containing the *PMC1* gene was isolated from pKC44 (gift from Kyle Cunningham) and ligated into pBlueKS+. Site-directed mutagenesis was performed according to the QuickChange Site Directed Mutagenesis (Stratagene) protocol to generate a *Bss*HII site at the 3′ end of *PMC1* (5 codons upstream of the stop codon) using the oligonucleotides DB571 (5′-CTGATAGTCC TTGGCGCGCC AACTTTTATT AATAGACGC-3′) and DB572 (5′-GCGTCTATTA ATAAAAGTTG GCGCGCCAAG GACTATCAG-3′). A 635 bp *Bgl*II/*Sna*BI fragment was isolated from the resulting plasmid and cloned back into pBlueKS+/*PMC1* for DNA sequencing. A *Bss*HII DNA fragment carrying the GFP gene was generated using primers DB573 (5′-CGATAAGGAT TGGCGCG-CCA AATTCATGAG-3′) and DB574 (5′-CGATAAGGAT TGGCG-CGCCA AATTCATGAG-3′) and the template pRSETB-GFP (gift from Kelly Thatchell), and ligated into the *Bss*HII site of pBlueKS+/*PMC1.* The correct GFP coding sequence was confirmed by automated DNA sequencing and a *Hin*dIII fragment carrying the *PMCI*-GFP construct was ligated into the low-copy yeast shuttle vector pSEYC58 (Emr et al., 1986). This final plasmid was then transformed into the *pmc1Δ* (YDB0224) and the *pmr1Δ/pmc1Δ* (YDB0276) strains. The *Hin*dIII fragment carrying the complete *PMC1-GFP* open reading frame was also inserted into the high-copy shuttle vector pSEY8 (Emr et al., 1986), digested with *Apa*I, and the digested linear DNA was transformed into the *pmc1Δ* (YDB0224) and the *pmr1Δ/pmc1Δ* (YDB0276) strains to integrate it into the genome of each strain. With both approaches, the construct reversed the EGTA-sensitive phenotype of the *pmr1Δ/pmc1Δ* strain and the high Ca^{2+}-sensitive phenotype of the *pmc1Δ* strain.

Culture media

Bacterial strains were grown on standard media (Miller, 1992). Yeast strains were maintained on YP medium containing 2% D-glucose (YPD) or synthetic minimal medium containing 2% D-glucose (SMD) and other supplements as required (Burke et al., 2000). Culture media were routinely buffered with 40 mM Mes-Tris, pH 5.5.

Total cellular Ca^{2+} measurements

Total cellular Ca^{2+} was measured using flame photometry as described previously (Miseta et al., 1999a). Cells were grown in 30-40 ml of YPD or the same medium supplemented with either 1 mM EGTA or 50 mM $CaCl_2$. Cultures were harvested at a cell density of ~1 OD_{600}/ml, harvested by centrifugation and washed with 20 ml of YPD. Cells were then transferred to previously weighed microfuge tubes and harvested by centrifugation in a micro-centrifuge (16,000 ***g***) for 5 minutes. After aspirating the supernatant, a second spin was

Table 1. Yeast strains used in this study

Strain	Genotype	Source
SEY6210	*MATα, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9*	S. Emr, UCSD, San Diego, CA
YDB224	*MATα, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, pmc1Δ::TRP1*	Miseta et al., 1999a
YDB225	*MATα, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vcx1Δ::URA3*	Miseta et al., 1999a
YDB279	*MAT**a**, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, pmr1Δ::LEU2*	Miseta et al., 1999a
YDB254	*MATα, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, pmc1Δ::TRP1, vcx1Δ::URA3*	Miseta et al., 1999a
YDB276	*MAT**a**, trp1-Δ901, ura3-52, his3-Δ200, leu2-3,112, pmc1Δ::TRP1, pmr1Δ::LEU2*	This study
YDB289	*MAT**a**, trp1-Δ901, ura3-52, his3-Δ200, leu2-3,112, lys2-80, vcx1Δ::URA3, pmr1Δ::LEU2*	This study

conducted for 3 minutes. Samples were weighed, then dried in a Speedvac. The dried samples were weighed again and resuspended in HCl for flame photometric measurements.

Ca^{2+} uptake

Ca^{2+} uptake measurements were similar to a method described previously (Halachmi and Eilam, 1996). Cells were grown in YPD to 0.7-1.0 OD_{600}/ml, harvested, washed twice with ddH_2O, then re-suspended in buffer containing 25 mM Mes-Tris, pH 6.0 supplemented with 20 mM glucose to 1 OD_{600}/ml and incubated at 30°C for 10 minutes. Uptake was initiated by the addition of 1 μCi/ml $^{45}Ca^{2+}$. At the indicated time-points, 1 ml aliquots were collected by filtration through 0.45 μm membrane filters (Gelman Sciences) using a vacuum manifold. Membranes were immediately washed with two 5 ml aliquots of ice-cold wash buffer (20 mM $MgCl_2$, 0.2 mM $LaCl_3$), dried, and the cell-associated radioactivity was measured by liquid scintillation counting.

Northern analysis

RNA extraction and northern blot analysis were carried out as described previously (Bonetti et al., 1995). Strains were grown overnight in YPD medium or YPD supplemented with either 1 mM EGTA or 50 mM $CaCl_2$ to ~1 OD_{600}/ml. A 0.44 kb probe for the *PMC1* mRNA was generated by PCR using primers DB490 (5′-ATGTCTAGACAAGACGAAAA-3′) and DB491 (5′-ATACTGTG-GAGGTTGCATCC-3′). As control, an *ACT1* probe was generated using the primers DB154 (5′-GCGCGGAATTCAACGTTCCAG-CCTTCTACG-3′) and DB155 (5′-GGATGGAACAAAGCTTCTGG-3′). Probes were labeled with [α-^{32}P]dATP using the random hexamer method (Sambrook et al., 1989). The specific band representing the *PMC1* mRNA was confirmed by its absence in RNA extracted from the *pmc1Δ* strain YDB0224. Gels were quantitated by PhosphorImager analysis (Molecular Dynamics). The relative mRNA levels were normalized using the *ACT1* mRNA as internal control after background correction.

Aequorin assay

Aequorin assays were carried out as described earlier (Miseta et al., 1999a). The two-micron-based plasmid pDB617 expressing a functional apoaequorin gene (*pAEQ*) was transformed into yeast. Cells containing pAEQ were grown in SMD medium and harvested in the early logarithmic growth phase (0.5-1.0 A_{600} units/ml). 10 A_{600} units of cells were harvested and re-suspended in 0.2 ml of aequorin test medium, which consists of SMD medium (which normally contains 1 mM Ca^{2+}) supplemented with 2 mM EGTA and 40 mM MES-Tris, pH 6.5. To convert the apoaequorin to aequorin, 20 μl of 590 μM coelenterazine (dissolved in methanol) was added, and the cells were incubated for 20 minutes at room temperature. The cells were then briefly centrifuged, and the supernatant containing excess coelenterazine was removed. The cell pellets were washed again in 0.5 ml of aequorin test medium, re-suspended to a cell density of 1 OD_{600}/0.1 ml and incubated at room temperature for 20 minutes before initiating the experiment. Bafilomycin A_1 (5 μM) was added from a 100 μM stock solution (dissolved in DMSO) 10 minutes prior to the measurements (Abe and Horikoshi, 1995). After detecting the baseline light emission, 100 mM Ca^{2+} was administered into the chamber to generate a Ca^{2+} shock. A Berthold Lumat 9050 luminometer was used to collect aequorin light emission (L) data at 200 millisecond intervals. The data were downloaded directly to a computer and transferred to Microsoft Excel 5.0 for analysis. After measuring maximal light emission from crude cell extracts upon Ca^{2+} addition (L_{max}), L/L_{max} values were plotted on a standard curve to estimate the free cytosolic Ca^{2+} concentrations, as previously described (Miseta et al., 1999a).

Light microscopy

Cells expressing the Pmc1p-GFP fusion protein and/or stained with FM 4-64 were collected, re-suspended in fresh YPD medium, or YPD medium supplemented with Ca^{2+} or EGTA, to 10 OD_{600} units/ml and 10 μl of the culture was mounted on slides coated with concanavalin A, covered with a coverslip and viewed immediately. FM 4-64 staining was carried out as published (Vida and Emr, 1995) with minor modifications. Briefly, yeast cells were grown in the indicated media to mid-log phase, 2 OD_{600} units of cells were collected and re-suspended in 100 μl of YPD (Ca^{2+} was omitted at this step because it decreased total fluorescence). A 1 μl aliquot of an FM 4-64 stock (4 mM in DMSO) was added to the cells, and they were stained for 10-15 minutes at 30°C. Cells were collected and re-suspended in 200 μl fresh media supplemented with Ca^{2+} or EGTA and incubated at 30°C for 40-50 minutes. Light microscopy was conducted using a Leitz Orthoplan microscope with epifluorescence optics and Hoffman Modulation Contrast optics. The images were acquired with a Photometrics CH250 liquid-cooled CCD high-resolution monochromatic camera (Roper Scientific; Tucson, AZ) and analyzed by IPLab Spectrum software from Scanalytics (Fairfax, VA).

Results

PMC1 expression is required for growth of the *pmr1Δ* mutant in an environment containing a reduced level of Ca^{2+} and other divalent cations

We first examined whether the loss of Pmc1p function altered the ability of the *pmr1Δ* strain to grow in the presence of chelating agents that reduced the level of Ca^{2+} and other divalent cations. We found that the *pmr1Δ* strain could grow on YPD plates containing the Ca^{2+} chelator 1,2 bis(2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid (BAPTA; 1 mM), whereas the *pmr1Δ/pmc1Δ* strain could not (Fig. 1A,B). Similar results were obtained with YPD plates containing 2 mM EGTA (data not shown). These results were consistent with the previous conclusion that Pmc1p plays a role in Ca^{2+} uptake into secretory compartments (Bonilla et al., 2002; Durr et al., 1998; Locke et al., 2000). However, it was surprising that Pmc1p influenced cell growth when the availability of Ca^{2+} and other divalent cations was reduced, since previous studies have shown that *PMC1* expression and activity is normally induced by calcineurin when cellular Ca^{2+} stress increases (Cunningham and Fink, 1994b; Marchi et al., 1999). To determine whether calcineurin activation is required for growth of the *pmr1Δ* strain under these conditions, we challenged these strains with cyclosporin A (CsA) on YPD plates containing 1 mM BAPTA (Fig. 1C). We found that neither the *pmr1Δ* nor the *pmr1Δ/pmc1Δ* strains were able to grow in the presence of both CsA and BAPTA, demonstrating that calcineurin activity is necessary for growth of the *pmr1Δ* mutant when the environmental concentration of divalent cations is decreased. CsA inhibition was also observed when these strains were grown on YPD plates containing 2 mM EGTA, but not on YPD plates containing CsA that lacked one of these chelating agents (data not shown). When taken together, these results indicate that one or more genes regulated by the calcineurin signaling pathway, including Pmc1p, plays an important role in Ca^{2+} homeostasis in the *pmr1Δ* strain when the concentration of divalent cations in the environmental is reduced.

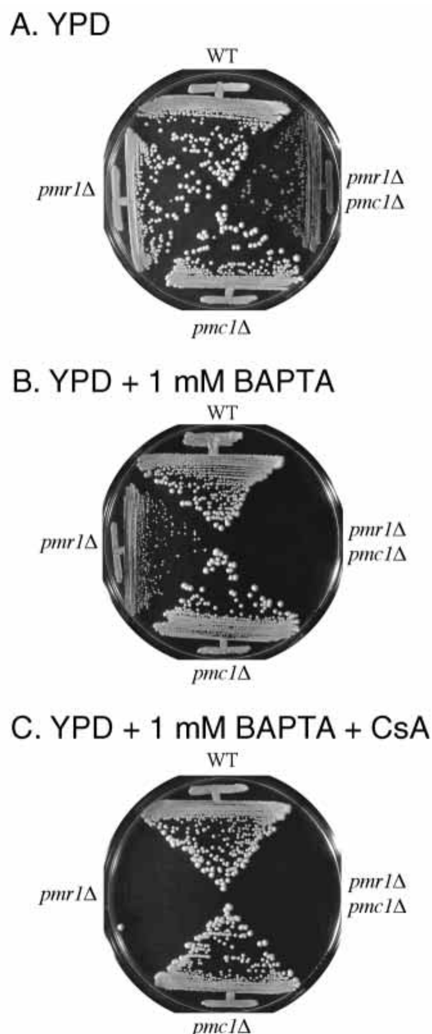


Fig. 1. Pmc1p and calcineurin activity are required for growth of the *pmr1Δ* mutant in a medium containing a reduced level of divalent cations. The indicated strains were streaked on YPD plates containing the following supplements: (A) YPD alone, (B) YPD plus 1mM BAPTA or (C) YPD plus 1mM BAPTA and 10 μg/ml CsA.

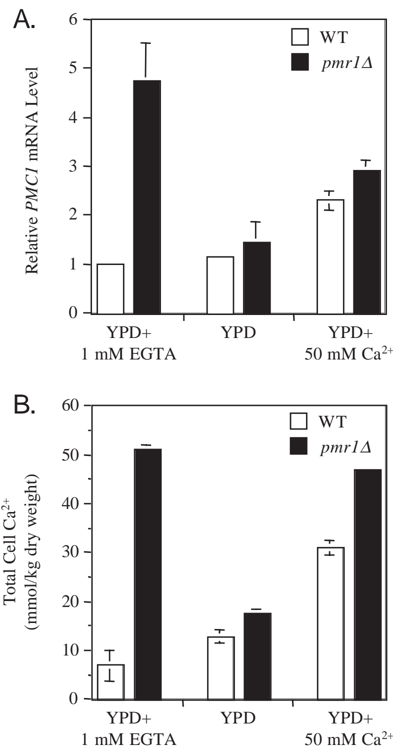


Fig. 2. Ca^{2+} accumulation and *PMC1* transcription are induced when the *pmr1Δ* mutant is grown in a medium containing a reduced level of divalent cations. (A) Relative *PMC1* mRNA levels in strains grown in the presence of different levels of divalent cations. (B) Total cell Ca^{2+} levels in strains grown in the presence of different levels of divalent cations.

The reduced availability of divalent cations leads to increased *PMC1* mRNA levels and increased total cellular Ca^{2+} in the *pmr1Δ* strain

Previous studies have shown that *PMC1* expression increases through a calcineurin-dependent mechanism as the level of environmental Ca^{2+} increases (Cunningham and Fink, 1994b; Marchi et al., 1999). However, the results described above suggest that the normal pattern of *PMC1* expression is altered in the *pmr1Δ* strain. To understand these findings better, we next examined the level of *PMC1* mRNA in the WT and *pmr1Δ* strains using northern blot analysis (Fig. 2A). As expected, we found that the level of *PMC1* mRNA in the WT strain increased with increasing environmental Ca^{2+}. The *PMC1* mRNA level in the *pmr1Δ* strain was only slightly higher than the WT strain when grown in either YPD (which contains 0.3 mM Ca^{2+}) or YPD supplemented with 50 mM $CaCl_2$. By contrast, the steady-state level of *PMC1* mRNA in the *pmr1Δ* strain was ~5-fold higher than normal when these strains were grown in YPD medium containing 1 mM EGTA. In fact, the *PMC1* mRNA level in the *pmr1Δ* strain was higher in cells grown in the presence of a reduced level of divalent cations than under any other condition tested. These results confirm that the pattern of *PMC1* expression is significantly altered in the *pmr1Δ* strain.

Since the expression of the *PMC1* gene is controlled by calcineurin activity (Cunningham and Fink, 1994b; Marchi et al., 1999), the results above suggest that the *pmr1Δ* strain might experience Ca^{2+} stress even when grown in the presence of low environmental Ca^{2+}. To test this possibility, we compared the total cellular Ca^{2+} levels in WT and *pmr1Δ* strains when grown under different environmental Ca^{2+} conditions. Consistent with previous studies (Halachmi and Eilam, 1996; Sorin et al., 1997), we found that the level of cellular Ca^{2+} in the *pmr1Δ* strain was 1.4-fold higher than the WT strain when grown in YPD and 1.5-fold higher than WT in YPD containing 50 mM $CaCl_2$. By contrast, we found that the *pmr1Δ* strain contained ~7.5-fold more total cell Ca^{2+} than the WT strain when grown in YPD medium supplemented with 1 mM EGTA (Fig. 2B). This level of total cellular Ca^{2+} was actually higher than the level measured when this strain was grown in YPD supplemented with 50 mM $CaCl_2$. These results confirm that the *pmr1Δ* strain exhibits excessive Ca^{2+} accumulation when grown in media containing a reduced level of divalent cations, which results in the transcriptional activation of genes controlled by the calmodulin/calcineurin signaling pathway.

Ca^{2+} uptake and accumulation are further increased in the *pmr1Δ/pmc1Δ* strain

The results of previous studies have indicated that Pmc1p

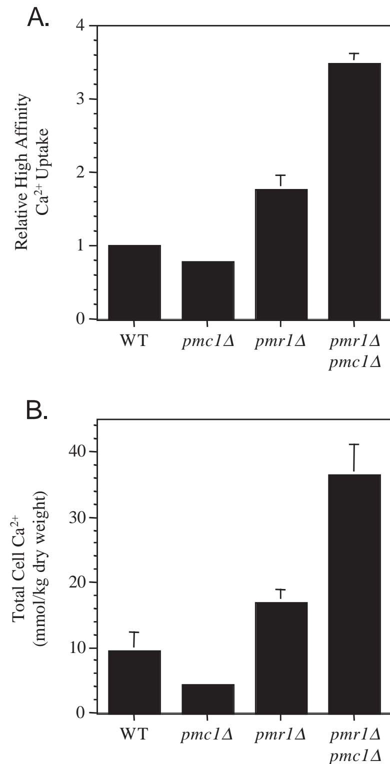


Fig. 3. Ca^{2+} uptake and accumulation are increased in the *pmr1Δ/pmc1Δ* strain relative to the *pmr1Δ* strain in YPD medium. (A) Relative high-affinity $^{45}Ca^{2+}$ uptake during a 30 minute time period. (B) Total cell Ca^{2+} levels measured by flame photometry.

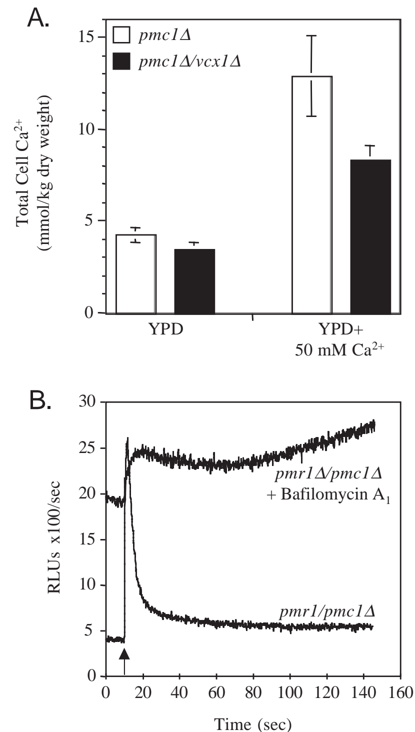


Fig. 4. *VCX1* plays an important role in Ca^{2+} homeostasis in the *pmc1Δ* and *pmr1Δ/pmc1Δ* strains. (A) Total cellular Ca^{2+} levels measured with flame photometry. (B) Cytosolic free Ca^{2+} levels measured with an aequorin Ca^{2+} reporter system. The arrow represents the addition of 100 mM $CaCl_2$ (see Materials and Methods for further details).

contributes to the filling of ER Ca^{2+} stores in a *pmr1Δ* strain (Bonilla et al., 2002; Durr et al., 1998). Given our finding that total cellular Ca^{2+} is significantly higher in a *pmr1Δ* strain when the level of divalent cations in the growth medium is reduced, we next examined Ca^{2+} uptake by the *pmr1Δ/pmc1Δ* strain when grown in YPD medium. Consistent with previous reports, we found that the rate of Ca^{2+} uptake in the *pmr1Δ* strain was 1.8-fold higher than the WT strain (Halachmi and Eilam, 1996; Sorin et al., 1997), whereas the *pmc1Δ* strain had a Ca^{2+} uptake rate that was ~20% lower than normal (Fig. 3A). By contrast, Ca^{2+} uptake in the *pmr1Δ/pmc1Δ* mutant was 3.5-fold higher than the WT strain (and almost 2-fold higher than the *pmr1Δ* strain).

We next examined total cellular Ca^{2+} levels following the growth of these strains in YPD medium. We found that the total cellular Ca^{2+} level in the *pmc1Δ* strain was roughly 2-fold lower than the WT strain, as previously reported (Cunningham and Fink, 1994b). By contrast, the *pmr1Δ/pmc1Δ* strain contained 3.8-fold more Ca^{2+} than the WT strain, and 2.2-fold more total cellular Ca^{2+} than the *pmr1Δ* strain (Fig. 3B). These results demonstrate that the *pmc1Δ* mutation further exacerbates the Ca^{2+} hyper-accumulation phenotype of the *pmr1Δ* strain and suggest that the growth defect observed when the *pmr1Δ/pmc1Δ* strain is grown in a low Ca^{2+} environment is caused by an excessive cellular Ca^{2+} load in combination with a reduced ability to sequester this excess Ca^{2+} adequately into intracellular compartments.

The Ca^{2+}/H^+ Exchanger Vcx1p maintains Ca^{2+} homeostasis in the *pmr1Δ/pmc1Δ* mutant

It was previously shown that Vcx1p activity is downregulated upon calcineurin activation, suggesting that this protein does not play a significant role in Ca^{2+} sequestration under conditions of high Ca^{2+} stress (Cunningham and Fink, 1996; Pozos et al., 1996). Consistent with this conclusion, we previously demonstrated that the presence of a *vcx1Δ* mutation did not have any effect on the level of total cellular Ca^{2+} when the growth medium was supplemented with more than 5 mM $CaCl_2$ (Miseta et al., 1999a). However, some residual Vcx1p activity remains under such repressing conditions, since a *pmc1Δ/vcx1Δ* strain is more sensitive to high extracellular Ca^{2+} than a *pmc1Δ* mutant (Cunningham and Fink, 1994a; Miseta et al., 1999a; Pozos et al., 1996). To gain further insight into the role of Vcx1p in the maintenance of Ca^{2+} homeostasis under conditions of Ca^{2+} stress, we measured total cellular Ca^{2+} levels in the *pmc1Δ* and *pmc1Δ/vcx1Δ* strains. We found that the total cellular Ca^{2+} level in the *vcx1Δ/pmc1Δ* strain was ~20% lower than the *pmc1Δ* mutant when these strains were grown in YPD medium. When grown in YPD supplemented with 50 mM $CaCl_2$, the total cellular Ca^{2+} level increased in both strains, but the level measured in the *vcx1Δ/pmc1Δ* strain was ~36% lower than the *pmc1Δ* strain (Fig. 4A). These results directly implicate Vcx1p in Ca^{2+} sequestration in the *pmc1Δ* mutant under conditions of high Ca^{2+} stress, and suggest that a functionally significant level of Vcx1p activity is maintained

in this strain when grown in the presence of high extracellular Ca^{2+}.

The Vcx1p Ca^{2+}/H^+ exchanger utilizes the proton gradient across the vacuolar membrane to help maintain cytosolic Ca^{2+} levels within a narrow physiological range. The vacuolar proton gradient is maintained by the vacuolar H^+-ATPase (Forster and Kane, 2000), which is sensitive to the inhibitor bafilomycin A_1. We previously used a cytosolic aequorin reporter system to characterize how bafilomycin A_1 influenced the regulation of cytosolic Ca^{2+} levels (Miseta et al., 1999a). We found that Vcx1p plays a key role in rapidly restoring basal cytosolic Ca^{2+} levels following a rise in the cytosolic Ca^{2+} concentration. Since the *pmr1Δ/pmc1Δ* mutant lacks the two major Ca^{2+}-ATPases involved in the maintenance of cellular Ca^{2+} homeostasis, we next used bafilomycin A_1 and the aequorin reporter system to examine the role of Vcx1p in controlling cytosolic Ca^{2+} levels in the *pmr1Δ/pmc1Δ* mutant. We found that bafilomycin A_1 treatment increased the cytosolic Ca^{2+} level in this strain significantly (Fig. 4B). Using a standardization procedure described previously (Miseta et al., 1999a), we found that the cytosolic Ca^{2+} level in the *pmr1Δ/pmc1Δ* mutant increased from ~l60 nM to ~260 nM following bafilomycin A_1 treatment. By contrast, a similar treatment did not alter the basal cytosolic Ca^{2+} levels in WT, *pmr1Δ* and *pmc1Δ* strains (data not shown). These results suggest that Vcx1p plays an important role in the maintenance of the resting cytosolic Ca^{2+} level in the *pmr1Δ/pmc1Δ* strain.

To examine further the role of Vcx1p in Ca^{2+} homeostasis of the *pmr1Δ/pmc1Δ* strain, we next tested the ability of Vcx1p to regulate cytosolic Ca^{2+} levels following the exposure of this strain to a 100 mM $CaCl_2$ shock (Fig. 4B). Immediately following the addition of this Ca^{2+} bolus, the cytosolic Ca^{2+} level rapidly increased to ~300 nM. In the absence of bafilomycin A_1 treatment, we found that the *pmr1Δ/pmc1Δ* mutant could successfully recover from this rapid increase in cytosolic Ca^{2+}, with the resting cytosolic Ca^{2+} level returning to ~180 nM within 30 seconds. By contrast, a brief pre-treatment with bafilomycin A_1 prior to the Ca^{2+} shock completely eliminated the ability of this strain to compensate for this abrupt increase in cytosolic Ca^{2+}. These results demonstrate that Ca^{2+}/H^+ exchange by Vcx1p plays a key role in the maintenance of cellular Ca^{2+} homeostasis in the *pmr1Δ/pmc1Δ* strain.

The *pmr1Δ/pmc1Δ* strain exhibits vacuolar fragmentation

Previous studies have suggested that newly synthesized Pmc1p contributes to the maintenance of Ca^{2+} levels within the secretory pathway during its transit to the vacuole in the *pmr1Δ* mutant (Bonilla et al., 2002; Durr et al., 1998; Locke et al., 2000) (this study). Subcellular fractionation of a *pmr1Δ* strain has shown that Pmc1p is present not only in vacuolar fractions, but also overlaps fractions containing Golgi markers (Marchi et al., 1999). These results suggest that a significant amount of Pmc1p may be retained in the Golgi apparatus in the *pmr1Δ* mutant. To determine whether the Golgi localization of Pmc1p could be visualized directly in yeast cells, we constructed a Pmc1p-GFP fusion protein. Following the integration of this construct into the nuclear genome of *pmc1Δ* and *pmr1Δ/pmc1Δ* strains, we found that the Pmc1p-GFP fusion restored normal Pmc1p function, as indicated by its ability to complement both the Ca^{2+} sensitivity of the *pmc1Δ* mutant and the EGTA sensitivity of the *pmr1Δ/pmc1Δ* mutant (data not shown). Thus, these complemented *pmc1Δ*, and *pmr1Δ/pmc1Δ* strains were functionally equivalent to WT and *pmr1Δ* strains, respectively.

We first asked whether the majority of the Pmc1p-GFP fusion protein co-localized with vacuoles as indicated by FM 4-64, a vital stain that accumulates in the yeast vacuolar membrane (Vida and Emr, 1995). As shown in Fig. 5, we observed complete co-localization of these two markers in the expected vacuolar pattern in WT cells grown in YPD medium. Whereas FM 4-64 and Pmc1p-GFP fluorescence also co-localized completely in the *pmr1Δ* strain, we found that both markers produced an identical pattern of highly fragmented staining. FM 4-64 staining was also used to examine the vacuolar morphology in the *pmc1Δ* and *pmr1Δ/pmc1Δ* strains. The vacuolar morphology was normal in the *pmc1Δ* strain. By contrast, the *pmr1Δ/pmc1Δ* strain exhibited a highly fragmented, frequently tubular, pattern of fluorescence. No Pmc1p-GFP fluorescence could be detected that was distinct from the FM 4-64 staining in either the WT or *pmr1Δ* strain, indicating that any accumulation in the Golgi apparatus (or another subcellular location) was below the resolution of this assay. However, these results demonstrate that significant vacuolar fragmentation occurs in strains containing the *pmr1Δ* mutation when grown in standard YPD medium. On the basis of our finding that the *pmr1Δ* and *pmr1Δ/pmc1Δ* strains both undergo Ca^{2+} stress under these conditions, these results suggest that Ca^{2+} stress might induce vacuolar fragmentation.

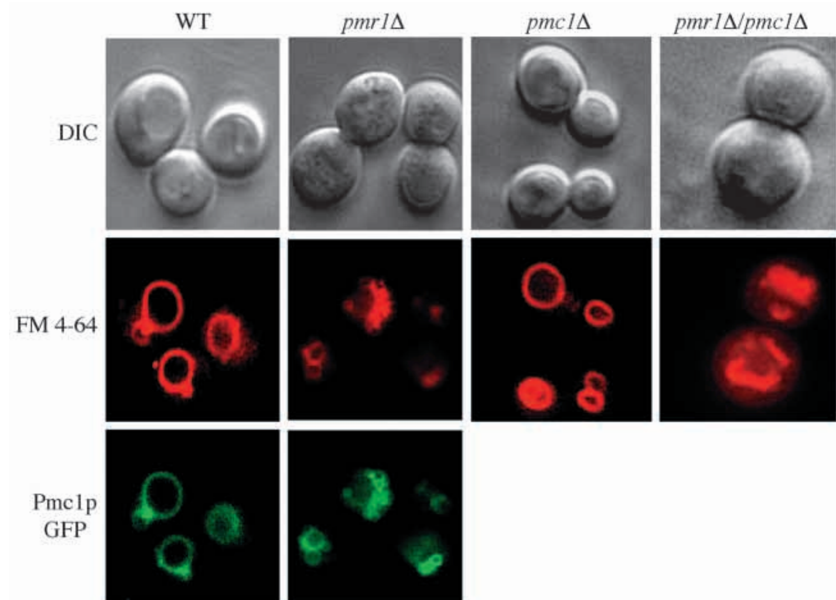
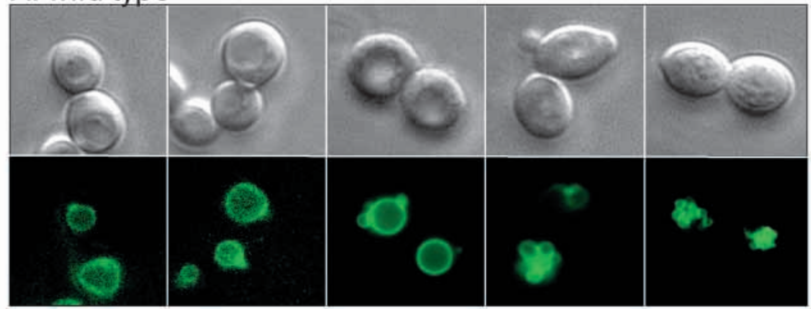


Fig. 5. Vacuoles are fragmented in the *pmr1Δ* and *pmr1Δ/pmc1Δ* strains when grown in YPD medium. Vacuolar morphology was monitored using the vital fluorescent dye FM 4-64 or the Pmc1p-GFP fusion protein as indicated.

To test this hypothesis, we next used the Pmc1p-GFP fusion protein to monitor vacuolar morphology in the WT and *pmr1Δ* strains grown in media containing different Ca^{2+} concentrations (Fig. 6). We found that WT cells grown in low and intermediate environmental Ca^{2+} concentrations (YPD supplemented with 1 mM EGTA, YPD alone, or YPD supplemented with 5 mM $CaCl_2$) exhibited normal vacuolar morphology (Fig. 6A). However, this strain exhibited an increasing degree of vacuolar fragmentation when grown in media containing 50 or 200 mM $CaCl_2$. We found that 70-80% of WT cells grown YPD supplemented with 200 mM $CaCl_2$ contained four or more vacuoles, while 70-80% of cells grown in standard YPD contained three or fewer vacuoles. Furthermore, as the number of the vacuolar structures increased, their size decreased. This vacuolar fragmentation phenotype was independent of osmotic stress, since WT cells grown in YPD medium supplemented with 300 mM $NaCl_2$ did not exhibit any significant change in vacuolar morphology (data not shown).

As shown above, we found that the *pmr1Δ* strain contained highly fragmented vacuoles when grown on YPD medium (Fig. 6B). This fragmented vacuole phenotype was even more severe when grown in the presence of 1 mM EGTA, consistent with our finding that this strain undergoes excessive Ca^{2+} stress under these conditions. Growth of the *pmr1Δ* strain in YPD supplemented with 5-50 mM $CaCl_2$ resulted in a much less severe alteration in vacuolar morphology. Increasing the extracellular $CaCl_2$ concentration to 200 mM $CaCl_2$ led to a fragmented vacuolar morphology similar to that observed in WT cells under these conditions. Overall, these results indicate that vacuolar fragmentation is observed in both WT and *pmr1Δ* strains in conjunction with an elevated level of cellular Ca^{2+} stress.



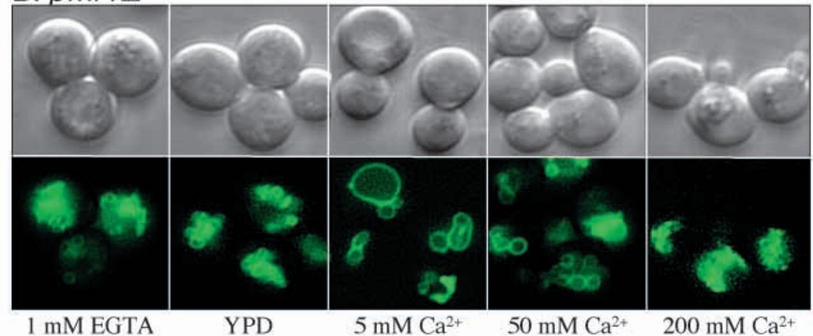


Fig. 6. Vacuolar fragmentation correlates with cellular Ca^{2+} stress in the WT and *pmr1Δ* strains. Vacuolar morphology was monitored by fluorescence of a Pmc1p-GFP fusion. The WT strain (A) and *pmr1Δ* strain (B) were grown under the indicated environmental conditions.

Discussion

Previous studies have shown that a *pmr1Δ* strain lacks the ability to properly maintain normal Ca^{2+} and Mn^{2+} levels in compartments of the secretory pathway. This Ca^{2+} transport defect leads to an increased rate of Ca^{2+} uptake and accumulation by a mechanism that has many characteristics of the mammalian CCE response (Csutora et al., 1999; Lapinskas et al., 1995; Locke et al., 2000). In the current study, we examined how a *pmr1Δ* strain responds to growth in media containing a reduced level of divalent cations. We found that the steady-state level of total cellular Ca^{2+} in the WT strain decreased by twofold when the level of divalent cations in YPD medium was reduced by the addition of 1 mM EGTA. By contrast, we found that the level of total cellular Ca^{2+} increased almost threefold in the *pmr1Δ* strain when the level of divalent cations in YPD medium was reduced. This substantial increase in Ca^{2+} accumulation was sufficient to activate the calcineurin pathway, which increased the expression and activity of intracellular transporters that sequester Ca^{2+} from the cytosol. Consistent with this interpretation, we found that the steady-state level of *PMC1* mRNA increased nearly fivefold in the *pmr1Δ* strain under these conditions.

A previous study found that *PMC1* expression from a multicopy plasmid was capable of suppressing phenotypes associated with the *pmr1Δ* mutation (Durr et al., 1998). This suggested that Pmc1p contributes to the filling of Ca^{2+} stores within the ER or Golgi apparatus during its transit through the secretory pathway. In support of this hypothesis, a *pmr1Δ/pmc1Δ* strain was also shown to exhibit a more severe unfolded protein response (UPR) than a *pmr1Δ* strain, suggesting that the loss of Pmc1p function causes a further depletion of ER Ca^{2+} that inhibits protein folding (Bonilla et al., 2002). It was also shown that the depletion of Golgi Ca^{2+} stores induces cellular Ca^{2+} uptake by a high-affinity Ca^{2+} uptake transporter encoded by the *CCH1* and *MID1* genes. These results led to the hypothesis that yeast cells possess a mechanism that couples the level of Ca^{2+} in the Golgi apparatus to Ca^{2+} uptake that is analogous to the CCE response in mammals (Locke et al., 2000). This model predicts that a *pmr1Δ* strain grows poorly in an environment containing a reduced level of divalent cations because a limiting level of extracellular Ca^{2+} (and/or Mn^{2+}) further reduces its ability to transport these cations efficiently into the cell so that it can replenish the depleted pool of these ions in the Golgi apparatus.

Our finding that Ca^{2+} accumulation is significantly increased in the *pmr1Δ* strain when the availability of divalent cations is reduced cannot be explained solely by a CCE-like mechanism (Csutora et al., 1999; Locke et al., 2000), since it is highly unlikely that the Ca^{2+} level in the Golgi apparatus will be further depleted under conditions where the level of total

cellular Ca^{2+} is threefold higher. We also found that the *pmr1Δ* strain exhibited less vacuolar fragmentation in the presence of 5 mM or 50 mM $CaCl_2$ than either higher or lower concentrations (see Fig. 6), suggesting that a moderate increase in extracellular Ca^{2+} can reduce the rate of cellular uptake (and the resulting level of Ca^{2+} stress). When taken together, these results suggest that a mechanism exists that can couple the rate of cellular Ca^{2+} uptake to the extracellular Ca^{2+} concentration. The results of a previous study also support the existence of an extracellular Ca^{2+}-sensing mechanism. We have shown that the loss of the major isoform of phosphoglucomutase (encoded by the *PGM2* gene) causes a large increase in Ca^{2+} uptake and accumulation when a *pgm2Δ* strain is grown in YP medium containing galactose as carbon source (Fu et al., 2000). Furthermore, we found that a *pgm2Δ/pmr1Δ* strain is unable to grow in YP galactose medium, presumably because the combination of these mutations leads to excessive Ca^{2+} uptake and accumulation that results in an inhibition of cell growth. However, we found that growth of the *pgm2Δ/pmr1Δ* strain could be restored on YP galactose medium when 100 mM $CaCl_2$ was added to the growth medium (Fu et al., 2000). In light of our current results, we propose that this increase in the concentration of $CaCl_2$ in the growth medium can restore growth of the *pgm2Δ/pmr1Δ* strain by reducing Ca^{2+} uptake and accumulation through the action of an extracellular Ca^{2+}-sensing mechanism.

While several mechanisms could be used to monitor the level of extracellular Ca^{2+}, the most straightforward method would utilize a Ca^{2+} sensor on the cell surface (Fig. 7). This cell-surface Ca^{2+} sensor could be functionally equivalent to the extracellular Ca^{2+}-sensing receptor of mammalian cells, which can respond to extremely small changes in the free Ca^{2+} concentration in the blood (Brown et al., 1995; Hebert et al., 1997). The results of the current study provide strong evidence that an extracellular Ca^{2+}-sensing mechanism can also play an important role in coupling the level of environmental Ca^{2+} to cellular Ca^{2+} uptake, homeostasis and signaling in yeast. To our knowledge, such a mechanism has not been proposed previously for yeast cells. This might be due to the fact that this mechanism works in conjunction with other redundant processes that together tightly control cellular Ca^{2+} homeostasis. Such overlapping mechanisms could explain why yeast mutants that maintain abnormally high levels of Ca^{2+} uptake and accumulation (such as the *pmr1Δ* and *pgm2Δ* strains) were necessary to obtain evidence of this novel control mechanism. These mutant strains have shed new light on the mechanisms that couple cellular Ca^{2+} uptake and accumulation. For example, the inability of the *pmr1Δ* strain to grow in the presence of a reduced level of divalent cations allowed us to show that excessive Ca^{2+} accumulation is responsible for this growth defect. This problem is further exacerbated in the *pmr1Δ/pmc1Δ* strain in two ways. First, the loss of Pmc1p further aggravates the reduced ability to properly fill the ER store depletion caused by the *pmr1Δ* mutation. Second, the *pmc1Δ* mutation diminishes the ability of the cell to sequester excess Ca^{2+} in the vacuole. As a result, the cytosolic Ca^{2+} load becomes more severe in the *pmr1Δ/pmc1Δ* strain, whereas its ability to sequester excess Ca^{2+} adequately into the vacuole is decreased. Together, these consequences result in an inability to grow in a Ca^{2+}-depleted environment.

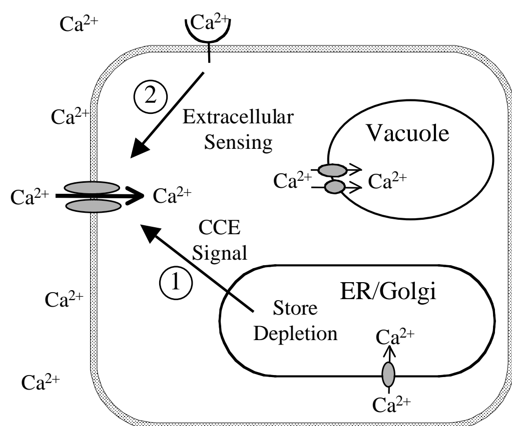


Fig. 7. Model showing how cellular Ca^{2+} uptake in yeast is coordinately regulated by two distinct mechanisms. The first mechanism is a CCE-like response that couples cellular Ca^{2+} uptake to Ca^{2+} store depletion in the ER/Golgi apparatus. The second mechanism couples cellular Ca^{2+} uptake to the level of Ca^{2+} in the extracellular environment.

In order to understand better the functional interplay between Ca^{2+} transporters in yeast, we also examined the role of Vcx1p in maintaining cytosolic Ca^{2+} homeostasis in the *pmr1Δ/pmc1Δ* strain. We found that Vcx1p plays an important role in Ca^{2+} homeostasis over a much broader range of extracellular Ca^{2+} concentrations than previously appreciated (Pozos et al., 1996). Genetic studies have suggested that Vcx1p might play a less important role in Ca^{2+} homeostasis than the Ca^{2+}-ATPases Pmc1p and Pmr1p. Consistent with this conclusion, we have observed that a *pmr1Δ/vcx1Δ* strain is no more sensitive to chelating agents than a *pmr1Δ* strain. This result indicated that Pmc1p alone is sufficient to sequester the high cellular Ca^{2+} that accumulates under these conditions, and suggested that Vcx1p plays only a secondary role in Ca^{2+} homeostasis (R. Kellermayer and D. Bedwell, unpublished). However, in the current study, we show that a *pmr1Δ/pmc1Δ* mutant can successfully cope with a considerable level of Ca^{2+} stress, whereas cellular Ca^{2+} homeostasis is completely disrupted when its vacuolar proton gradient is disrupted by bafilomycin A_1. Since it has been shown that vacuolar vesicles derived from a strain lacking Vcx1p do not possess any Ca^{2+}/H^+ exchange activity (Pozos et al., 1996), these findings strongly indicate that Vcx1p plays the predominant role in maintaining Ca^{2+} homeostasis in the *pmr1Δ/pmc1Δ* mutant. In addition, the previous observation that the combination of the *pmr1Δ*, *pmc1Δ* and *vcx1Δ* mutations together causes synthetic lethality reinforces the importance of Vcx1p in Ca^{2+} regulation in the absence of these two Ca^{2+}-ATPases (Cunningham and Fink, 1996; Miseta et al., 1999a). These results suggest that the Pmr1p, Pmc1p and Vcx1p Ca^{2+} transporters are all capable of independently maintaining cellular Ca^{2+} homeostasis in many yeast strains. Apparently, subtle differences in the extent of Vcx1p inhibition by calcineurin are responsible for conflicting reports regarding the viability of *pmr1Δ/pmc1Δ* strains in different genetic backgrounds (Cunningham and Fink, 1996; Locke et al., 2000) (this report).

We also used a Pmc1p-GFP fusion protein to show that vacuolar fragmentation coincides with Ca^{2+} stress. Our finding that vacuolar fragmentation occurs under diverse conditions

that lead to cellular Ca^{2+} stress in both WT and mutant strains strongly suggests a direct relationship between these two events. The data presented does not allow us to determine whether it is high Ca^{2+} in the cytosol or vacuole that leads to this fragmentation phenomenon. However, in other experiments we found that a *pmc1Δ/vcx1Δ* strain exhibits fragmented vacuoles in media containing 50 mM $CaCl_2$ in a manner similar to the WT strain (R. Kellermayer and D. Bedwell, unpublished). Since this mutant accumulates much less Ca^{2+} in the vacuole than the WT strain (Cunningham and Fink, 1996; Miseta et al., 1999a; Pozos et al., 1996), these results strongly suggest that an elevated cytosolic Ca^{2+} causes vacuolar fragmentation in *S. cerevisiae*.

A recent study examined a large collection of knockout strains from the *Saccharomyces* Genome Deletion Project for defects in homotypic vacuolar fusion (Seeley et al., 2002). Surprisingly, 714 out of 4828 deletion strains examined (~15%) exhibited alterations in vacuole morphology. After excluding a large number of genes thought to influence vacuole morphology by indirect means, 137 genes (~3%) were chosen as candidate *VAM* genes that were thought to play a direct role in vacuolar morphology. Among these were a variety of genes that encoded proteins previously related to homotypic vacuolar fusion, including fusion catalysts, enzymes of lipid metabolism, SNARES, GTPases and their effectors, protein kinases, phosphatases, and cytoskeletal proteins. Another group of genes encoded proteins involved in cation transport (including the *PMR1* gene). Because vacuolar fragmentation was associated with the deletion of these genes, it was reasoned that the loss of these gene products caused defects in vacuolar fusion, resulting in the vacuolar fragmentation phenotype. However, on the basis of the strong correlation between cellular Ca^{2+} stress and vacuolar fragmentation we observed in both WT and mutant yeast strains, we propose that an elevation of cytosolic Ca^{2+} might lead to vacuolar fragmentation as a regulatory response to aid in Ca^{2+} sequestration in WT yeast, rather than simply being the result of a defect in vacuolar fusion associated with high cytosolic Ca^{2+}. On a per unit volume basis, multiple small vacuoles provide a greater surface area than fewer, larger vacuoles. This increased surface/volume ratio could aid in accommodating the function of the increased number of Pmc1p transporters in the vacuolar membrane that are induced by calcineurin activation, thus allowing vacuolar Ca^{2+} sequestration to proceed more efficiently.

In both the current study and a prior study of genes that influence homotypic vacuolar fusion (Seeley et al., 2002), it was shown that a *pmr1Δ* strain exhibits a vacuolar fragmentation phenotype. By contrast, another study found that a *pmr1Δ* mutation reversed the vacuolar fragmentation phenotype associated with oxidative stress in a *sod1Δ* strain (Corson et al., 1999). These results led to the conclusion that oxidative stress associated with the *sod1Δ* mutation altered cellular iron homeostasis, leading to oxidative damage that somehow led to vacuolar fragmentation. It was proposed that the *pmr1Δ* mutation suppressed this vacuolar fragmentation phenotype by raising the concentration of Mn^{2+} in the cytosol, which acted to scavenge free radicals and reduce oxidative stress. Thus, *pmr1Δ* mutations have been associated with both the induction and reversal of vacuolar fragmentation. These markedly different effects suggest the existence of a complex regulatory mechanism that allows the cytosolic levels of different divalent cations to influence vacuolar morphology in distinct ways. Further studies are needed to determine how this complex physiological adaptation is carried out.

The authors thank Lianwu Fu for fruitful discussions, Albert Tousson for providing assistance in digital imaging, and Kyle Cunningham, Kelly Tatchell and Aaron Straight for generously providing strains and other reagents. This work was supported by American Heart Association (Southeast Affiliate) grant 0255121B (to D.M.B.) and Hungarian National Foundation grant OTKA-T038144 (to A.M.).

References

Abe, F. and Horikoshi, K. (1995). Hydrostatic pressure promotes the acidification of vacuoles in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **130**, 307-312.

Antebi, A. and Fink, G. R. (1992). The yeast Ca^{2+}-ATPase homologue, PMR1, is required for normal Golgi function and localizes in a novel Golgi-like distribution. *Mol. Biol. Cell* **3**, 633-654.

Batiza, A. F., Schulz, T. and Masson, P. H. (1996). Yeast respond to hypotonic shock with a calcium pulse. *J. Biol. Chem.* **271**, 23357-23362.

Bonetti, B., Fu, L., Moon, J. and Bedwell, D. M. (1995). The efficiency of translation termination is determined by a synergistic interplay between upstream and downstream sequences in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **251**, 334-345.

Bonilla, M., Nastase, K. K. and Cunningham, K. W. (2002). Essential role of calcineurin in response to endoplasmic reticulum stress. *EMBO J.* **21**, 2343-2353.

Brown, E. M., Vassilev, P. M. and Hebert, S. C. (1995). Calcium ions as extracellular messengers. *Cell* **83**, 679-682.

Burke, D., Dawson, D. C. and Stearns, T. (2000). *Methods in Yeast Genetics: a Cold Spring Harbor Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Corson, L. B., Folmer, J., Strain, J. J., Culotta, V. C. and Cleveland, D. W. (1999). Oxidative stress and iron are implicated in fragmenting vacuoles of *Saccharomyces cerevisiae* lacking Cu, Zn-superoxide dismutase. *J. Biol. Chem.* **274**, 27590-27596.

Cronin, S. R., Khoury, A., Ferry, D. K. and Hampton, R. Y. (2000). Regulation of HMG-CoA reductase degradation requires the P-type ATPase Cod1p/Spf1p. *J. Cell Biol.* **148**, 915-924.

Cronin, S. R., Rao, R. and Hampton, R. Y. (2002). Cod1p/Spf1p is a P-type ATPase involved in ER function and Ca^{2+} homeostasis. *J. Cell Biol.* **157**, 1017-1028.

Csutora, P., Su, Z., Kim, H. Y., Bugrim, A., Cunningham, K. W., Nuccitelli, R., Keizer, J. E., Hanley, M. R., Blalock, J. E. and Marchase, R. B. (1999). Calcium influx factor is synthesized by yeast and mammalian cells depleted of organellar calcium stores. *Proc. Natl. Acad. Sci. USA* **96**, 121-126.

Cunningham, K. W. and Fink, G. R. (1994a). Ca^{2+} transport in *Saccharomyces cerevisiae*. *J. Exp. Biol.* **196**, 157-166.

Cunningham, K. W. and Fink, G. R. (1994b). Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking *PMC1*, a homolog of plasma membrane Ca^{2+} ATPases. *J. Cell Biol.* **124**, 351-363.

Cunningham, K. W. and Fink, G. R. (1996). Calcineurin inhibits VCX1-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **16**, 2226-2237.

Denis, V. and Cyert, M. S. (2002). Internal Ca^{2+} release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. *J. Cell Biol.* **156**, 29-34.

Dunn, T., Gable, K. and Beeler, T. (1994). Regulation of cellular Ca^{2+} by yeast vacuoles. *J. Biol. Chem.* **269**, 7273-7278.

Durr, G., Strayle, J., Plemper, R., Elbs, S., Klee, S. K., Catty, P., Wolf, D. H. and Rudolph, H. K. (1998). The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca^{2+} and Mn^{2+} required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. *Mol. Biol. Cell* **9**, 1149-1162.

Eilam, Y. and Othman, M. (1990). Activation of Ca^{2+} influx by metabolic substrates in *Saccharomyces cerevisiae*: role of membrane potential and cellular ATP levels. *J. Gen. Microbiol.* **136**, 861-866.

Eilam, Y., Lavi, H. and Grossowicz, N. (1985). Mechanism of stimulation of

Ca^{2+} uptake by miconazole and ethidium bromide in yeasts: role of vacuoles in Ca^{2+} detoxification. *Microbios* **44**, 51-66.

Eilam, Y., Othman, M. and Halachmi, D. (1990). Transient increase in Ca^{2+} influx in *Saccharomyces cerevisiae* in response to glucose: effects of intracellular acidification and cAMP levels. *J. Gen. Microbiol.* **136**, 2537-2543.

Emr, S. D., Vassarotti, A., Garrett, J., Geller, B. L., Takeda, M. and Douglas, M. G. (1986). The amino terminus of the yeast F_1-ATPase beta-subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* **102**, 523-533.

Forster, C. and Kane, P. M. (2000). Cytosolic Ca^{2+} homeostasis is a constitutive function of the V-ATPase in *Saccharomyces cerevisiae. J. Biol. Chem.* **275**, 38245-38253.

Fu, L., Miseta, A., Hunton, D., Marchase, R. B. and Bedwell, D. M. (2000). Loss of the major isoform of phosphoglucomutase results in altered calcium homeostasis in *Saccharomyces cerevisiae. J. Biol. Chem.* **275**, 5431-5440.

Halachmi, D. and Eilam, Y. (1996). Elevated cytosolic free Ca^{2+} concentrations and massive Ca^{2+} accumulation within vacuoles, in yeast mutant lacking PMR1, a homolog of Ca^{2+}-ATPase. *FEBS Lett.* **392**, 194-200.

Hartley, A. D., Bogaerts, S. and Garrett, S. (1996). cAMP inhibits bud growth in a yeast strain compromised for Ca^{2+} influx into the Golgi. *Mol. Gen. Genet*. **251**, 556-564.

Hebert, S. C., Brown, E. M. and Harris, H. W. (1997). Role of the Ca^{2+}-sensing receptor in divalent mineral ion homeostasis. *J. Exp. Biol*. **200**, 295-302.

Iida, H., Sakaguchi, S., Yagawa, Y. and Anraku, Y. (1990a). Cell cycle control by Ca^{2+} in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 21216-21222.

Iida, H., Yagawa, Y. and Anraku, Y. (1990b). Essential role for induced Ca^{2+} influx followed by $[Ca^{2+}]i$ rise in maintaining viability of yeast cells late in the mating pheromone response pathway. A study of $[Ca^{2+}]i$ in single *Saccharomyces cerevisiae* cells with imaging of fura-2. *J. Biol. Chem.* **265**, 13391-13399.

Kaibuchi, K., Miyajima, A., Arai, K. and Matsumoto, K. (1986). Possible involvement of RAS-encoded proteins in glucose-induced inositolphospholipid turnover in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **83**, 8172-8176.

Lapinskas, P. J., Cunningham, K. W., Liu, X. F., Fink, G. R. and Culotta, V. C. (1995). Mutations in *PMR1* suppress oxidative damage in yeast cells lacking superoxide dismutase. *Mol. Cell. Biol.* **15**, 1382-1388.

Locke, E. G., Bonilla, M., Liang, L., Takita, Y. and Cunningham, K. W. (2000). A homolog of voltage-gated Ca^{2+} channels stimulated by depletion of secretory Ca^{2+} in yeast. *Mol. Cell. Biol.* **20**, 6686-6694.

Marchi, V., Sorin, A., Wei, Y. and Rao, R. (1999). Induction of vacuolar Ca^{2+}-ATPase and H^+/Ca^{2+} exchange activity in yeast mutants lacking Pmr1, the Golgi Ca^{2+}-ATPase. *FEBS Lett.* **454**, 181-186.

Matheos, D. P., Kingsbury, T. J., Ahsan, U. S. and Cunningham, K. W. (1997). Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*. *Genes Dev.* **11**, 3445-3458.

Miller, J. H. (1992). *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miseta, A., Fu, L., Kellermayer, R., Buckley, J. and Bedwell, D. M. (1999a). The Golgi apparatus plays a significant role in the maintenance of Ca^{2+} homeostasis in the *vps33Δ* vacuolar biogenesis mutant of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **274**, 5939-5947.

Miseta, A., Kellermayer, R., Aiello, D. P., Fu, L. and Bedwell, D. M. (1999b). The vacuolar Ca^{2+}/H^+ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca^{2+} levels in *S. cerevisiae. FEBS Lett.* **451**, 132-136.

Mori, I. C., Iida, H., Tsuji, F. I., Isobe, M., Uozumi, N. and Muto, S. (1998). Salicylic acid induces a cytosolic Ca^{2+} elevation in yeast. *Biosci. Biotechnol. Biochem.* **62**, 986-989.

Nakajima-Shimada, J., Iida, H., Tsuji, F. I. and Anraku, Y. (1991). Monitoring of intracellular calcium in *Saccharomyces cerevisiae* with an apoaequorin cDNA expression system. *Proc. Natl. Acad. Sci. USA* **88**, 6878-6882.

Pozos, T. C., Sekler, I. and Cyert, M. S. (1996). The product of *HUM1*, a novel yeast gene, is required for vacuolar Ca^{2+}/H^+ exchange and is related to mammalian Na^+/Ca^{2+} exchangers. *Mol. Cell. Biol.* **16**, 3730-3741.

Putney, J. W., Jr (1992). Inositol phosphates and calcium entry. *Adv. Second Messenger Phosphoprotein Res.* **26**, 143-160.

Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Dorman, T. E., LeVitre, J., Davidow, L. S., Mao, J. I. and Moir, D. T. (1989). The yeast secretory pathway is perturbed by mutations in *PMR1*, a member of a Ca^{2+} ATPase family. *Cell* **58**, 133-145.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Seeley, E. S., Kato, M., Margolis, N., Wickner, W. and Eitzen, G. (2002). Genomic analysis of homotypic vacuole fusion. *Mol. Biol. Cell* **13**, 782-794.

Sorin, A., Rosas, G. and Rao, R. (1997). PMR1, a Ca^{2+}-ATPase in yeast Golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. *J. Biol. Chem.* **272**, 9895-9901.

Stathopoulos, A. M. and Cyert, M. S. (1997). Calcineurin acts through the *CRZ1/TCN1*-encoded transcription factor to regulate gene expression in yeast. *Genes Dev.* **11**, 3432-3444.

Stathopoulos-Gerontides, A., Guo, J. J. and Cyert, M. S. (1999). Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. *Genes Dev.* **13**, 798-803.

Strayle, J., Pozzan, T. and Rudolph, H. K. (1999). Steady-state free Ca^{2+} in the yeast endoplasmic reticulum reaches only 10 μM and is mainly controlled by the secretory pathway pump pmr1. *EMBO J.* **18**, 4733-4743.

Suzuki, C. and Shimma, Y. I. (1999). P-type ATPase *spf1* mutants show a novel resistance mechanism for the killer toxin SMKT. *Mol. Microbiol.* **32**, 813-823.

Tanida, I., Hasegawa, A., Iida, H., Ohya, Y. and Anraku, Y. (1995). Cooperation of calcineurin and vacuolar H^+-ATPase in intracellular Ca^{2+} homeostasis of yeast cells. *J. Biol. Chem.* **270**, 10113-10119.

Tokes-Fuzesi, M., Bedwell, D. M., Repa, I., Sipos, K., Sumegi, B., Rab, A. and Miseta, A. (2002). Hexose phosphorylation and the putative calcium channel component Mid1p are required for the hexose-induced transient elevation of cytosolic calcium response in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **44**, 1299-1308.

Vida, T. A. and Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* **128**, 779-792.

Withee, J. L., Mulholland, J., Jeng, R. and Cyert, M. S. (1997). An essential role of the yeast pheromone-induced Ca^{2+} signal is to activate calcineurin. *Mol. Biol. Cell* **8**, 263-277.

Yoshimoto, H., Saltsman, K., Gasch, A. P., Li, H. X., Ogawa, N., Botstein, D., Brown, P. O. and Cyert, M. S. (2002). Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae. J. Biol. Chem.* **10**, 31079-31088.