The vacuolar Ca^{2+}/H^+ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca^{2+} levels in *S. cerevisiae*

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Abstract **It is well established that the vacuole plays an important role in the cellular adaptation to growth in the presence of elevated extracellular Ca^{2+} concentrations in *Saccharomyces cerevisiae*. The Ca^{2+} ATPase Pmc1p and the Ca^{2+}/H^+ exchanger Vcx1p/Hum1p have been shown to facilitate Ca^{2+} sequestration into the vacuole. However, the distinct physiological roles of these two vacuolar Ca^{2+} transporters remain uncertain. Here we show that Vcx1p can rapidly sequester a sudden pulse of cytosolic Ca^{2+} into the vacuole, while Pmc1p carries out this function much less efficiently. This finding is consistent with the postulated role of Vcx1p as a high capacity, low affinity Ca^{2+} transporter and suggests that Vcx1p may act to attenuate the propagation of Ca^{2+} signals in this organism.**

Key words: Ca^{2+}/H^+ exchanger; Ca^{2+} ATPase; Vacuole; Aequorin; Yeast

1. Introduction

Cytosolic Ca^{2+} levels are normally maintained in the 50–200 nM concentration range in the yeast *Saccharomyces cerevisiae* [1–5]. However, the total cellular Ca^{2+} concentration is more than 10 000-fold higher (2–3 mM). It is estimated that at least 90% of the total cellular Ca^{2+} is sequestered within the vacuole [1]. This Ca^{2+} is complexed with vacuolar polyphosphate in a relatively stable form referred to as the non-exchangeable Ca^{2+} pool. This name is derived from the fact that vacuolar Ca^{2+} exchanges with the environment slower than Ca^{2+} located in either the cytosol or other intracellular compartments [6,7].

Several mutations that reduce vacuolar Ca^{2+} sequestration have been shown to prevent cell growth in the presence of high environmental Ca^{2+} (= 100 mM) [6]. We recently found that the severe class C vacuolar biogenesis mutant *vps33*Δ is sensitive to both high (100 mM) and low (0.001 mM) extracellular Ca^{2+} concentrations [5]. The *vps33*Δ strain also has elevated cytosolic Ca^{2+}, and is severely compromised in its ability to reduce cytosolic Ca^{2+} levels upon challenge with elevated environmental Ca^{2+}. In addition, other studies have shown that vacuolar acidification is a prerequisite for vacuolar Ca^{2+} sequestration [8,9]. These observations are consistent with the hypothesis that the vacuole plays an important role in the maintenance of Ca^{2+} homeostasis in yeast.

Two proteins known to mediate vacuolar Ca^{2+} sequestration are the Ca^{2+} ATPase Pmc1p and the Ca^{2+}/H^+ exchanger Vcx1p/Hum1p [9–11]. Deletion of the *PMC1* gene causes increased sensitivity to elevated levels of environmental Ca^{2+}, whereas deletion of the *VCX1* gene does not significantly alter Ca^{2+} sensitivity. However, the deletion of both genes leads to a greater sensitivity to environmental Ca^{2+} than the loss of either gene alone [9,11]. This indicates that the function of these two Ca^{2+} transporters overlap to some extent. Both the *PMC1* and *VCX1* genes are regulated by the cytosolic Ca^{2+} level through the calmodulin-calcineurin signaling pathway. However, increased cytosolic Ca^{2+} was shown to increase Ca^{2+} uptake by Pmc1p and to inhibit Ca^{2+} transport by Vcx1p [10,11]. Thus, the regulation of these transporters in response to Ca^{2+} stress is fundamentally different.

In addition to the lack of a Ca^{2+}-sensitive growth phenotype, the fraction of total cellular Ca^{2+} associated with the non-exchangeable Ca^{2+} pool in a *vcx1*Δ strain is also not significantly altered [5,11,12]. Hence, it might be concluded that Vcx1p is a relatively insignificant player in the maintenance of overall Ca^{2+} homeostasis. However, *VCX1* has been shown to be responsible for a significant level of Ca^{2+} uptake in short-term, cell-free [^{45}Ca] uptake assays [9,11]. To determine the role played by Vcx1p in the response to increased cytosolic Ca^{2+} in living cells, we asked how the *pmc1*Δ and *vcx1*Δ mutations influenced the ability of cells to response to an environmental Ca^{2+} shock. Our results indicate that the loss of Pmc1p has little or no effect on the ability to restore basal cytosolic Ca^{2+} levels following a Ca^{2+} shock. In contrast, the loss of Vcx1p results in a significant defect in short-term adaptation to a sudden exposure to high environmental Ca^{2+}. This finding is consistent with its postulated role as a high capacity, low affinity Ca^{2+} transporter [13,14], and suggests that Vcx1p may function to attenuate the propagation of Ca^{2+} signals under normal conditions.

2. Materials and methods

2.1. Strains used

The parental strain SEY6210 is *MAT*α *ura3-52, leu2-3 112, his3-*Δ*200, trp1-*Δ*901, lys2-801, suc2-*Δ*9*. The *pmc1*Δ strain YDB224 was derived from SEY6210 by deleting the *PMC1* gene (*pmc1*Δ::TRP1). Similarly, the *vcx1*Δ strain YDB225 was derived from SEY6210 by deleting the *VCX1* gene (*vcx1*Δ::URA3) [5]. To carry out cytosolic Ca^{2+} measurements, these strains were transformed with the plasmid *pEVP11*, which expresses the apoaequorin gene [4,5]. This plasmid was a gift from Patrick Masson (University of Wisconsin).

2.2. Culture media

Yeast strains were grown on YP medium containing 2% D-glucose (YPD), or synthetic medium containing 2% D-glucose (SD) and other required nutrients [15]. All growth media were buffered with 40 mM MES-Tris, pH 5.5.

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2.3. Measurement of cytosolic Ca^{2+} concentration

Strains expressing apoaequorin were grown in SD medium and harvested at 1 OD_{600}/ml. Ten OD units of cells were resuspended in 0.2 ml of aequorin test medium (SD+2 mM EGTA+40 mM MES-Tris, pH 6.5). The final Ca^{2+} concentration of this medium was calculated to be ~6 μM using the MaxChelator program. To activate the cytosolic apoaequorin in the cells to aequorin, 10 μl of 0.59 mM coelenterazine (dissolved in methanol) was added and the cells incubated for 20 min at room temperature (RT). The cells were harvested by brief centrifugation in a microcentrifuge and washed with 0.5 ml test medium. Finally, the cells were resuspended in 1 ml test medium and allowed to equilibrate for 20 min before initiating the experiment. The calculation of cytosolic Ca^{2+} concentrations were carried out using a standard curve as previously described [5,16]. Where indicated, bafilomycin A_1 (dissolved in DMSO) was added to a final concentration of 5 μM and incubated with cells for 10 min before measuring cytosolic Ca^{2+} concentrations were determined. Control cells in these experiments were mock treated for a similar period of time with DMSO alone.

3. Results

3.1. Responses of wild-type, vcx1Δ and pmc1Δ strains to elevated environmental Ca^{2+}

We initially examined the ability of the wild-type, *pmc1*Δ, and *vcx1*Δ strains to grow on YPD plates supplemented with increasing concentrations of $CaCl_2$. No growth inhibition was observed for any of these strains on plates supplemented with 50 mM $CaCl_2$ (Fig. 1). However, when 400 mM $CaCl_2$ was added to the plates the growth of both the wild-type and *vcx1*Δ strains was reduced, while the *pmc1*Δ strain was unable to form visible colonies. To better understand how high Ca^{2+} levels inhibited the growth of the *pmc1*Δ strain, we monitored the response of these strains to a Ca^{2+} challenge when grown

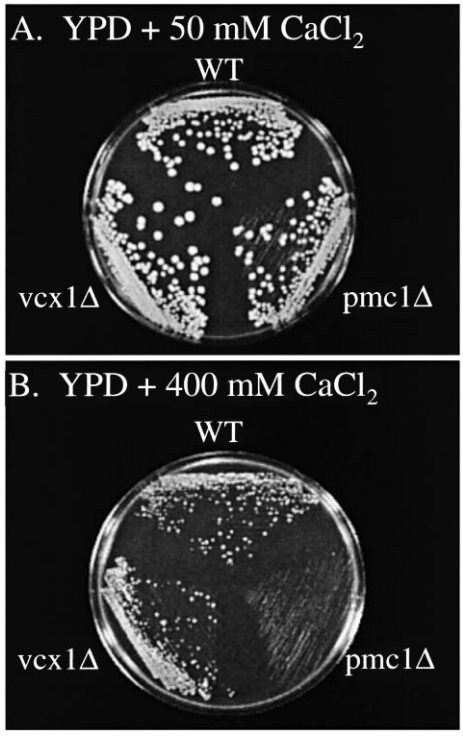

Fig. 1. Sensitivity of vacuolar transporter mutants to elevated environmental Ca^{2+}. The indicated strains were streaked onto YPD plates containing A: 0.3 mM $CaCl_2$, B: 50 mM $CaCl_2$, or B: 400 mM $CaCl_2$. The plates were then incubated at 30°C for 48 h.

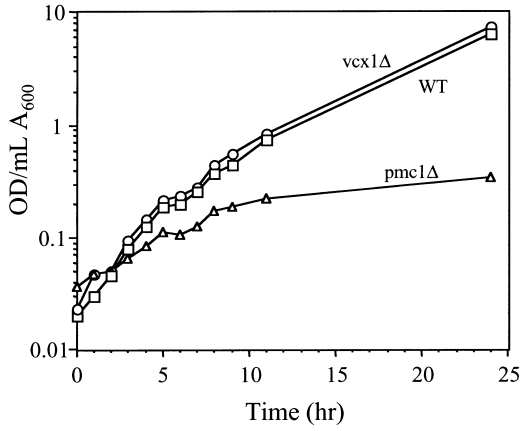

Fig. 2. Growth of vacuolar transporter mutants following the addition of high environmental Ca^{2+}. Strains were inoculated from a mid-log YPD culture and grown in YPD medium at 30°C to a cell density of 0.1–0.2 OD_{600} units/ml. At $t = 5$ h, 400 mM $CaCl_2$ was then added and growth was continued overnight.

in liquid YPD medium. Wild-type, *vcx1*Δ and *pmc1*Δ strains were initially grown in normal YPD medium. When the cell density reached 0.1–0.2 A_{600} units/ml, 400 mM $CaCl_2$ was added to the culture medium to induce a rapid Ca^{2+} shock (Fig. 2). We found that this sudden exposure to Ca^{2+} resulted in an initial growth lag in all three strains that lasted for 1–2 h. Following this lag period, all three strains (including the *pmc1*Δ strain) initially resumed growth. The wild-type and *vcx1*Δ strains continued to grow overnight and reached similar cell densities (6–7 OD_{600} units/ml). In contrast, the cell density of the *pmc1*Δ strain increased from 0.11 OD_{600} units/ml (the cell density when excess $CaCl_2$ was added) to a final density of 0.34 OD_{600} units/ml. These results indicate that the *pmc1*Δ strain can grow for 1–2 generations in the presence of high environmental Ca^{2+} before inhibition occurs.

3.2. Cytosolic Ca^{2+} levels in wild-type, vcx1Δ and pmc1Δ strains

To directly monitor cytosolic free Ca^{2+} concentrations, the wild-type, *pmc1*Δ, and *vcx1*Δ strains were each transformed with a plasmid expressing the apoaequorin gene. Cultures were grown in SD medium, harvested, and resuspended in SD medium supplemented with 2 mM EGTA to reduce the extracellular Ca^{2+} concentration to ~6 μM. The cytoplasmic apoaequorin expressed in each strain was then converted to active aequorin by incubation with coelenterazine, and the measurement of Ca^{2+}-dependent light emission was initiated to determine the basal cytosolic Ca^{2+} concentration. We found that the resting cytosolic Ca^{2+} concentration of the wild-type, *pmc1*Δ, and *vcx1*Δ strains were all in the 70–80 nM range when cells were incubated in this low Ca^{2+} medium. To examine the role of the Pmc1p and Vcx1p transporters in the cellular response to a rapid change in environmental Ca^{2+}, 50 mM $CaCl_2$ was injected into the medium (Fig. 3A). All three strains responded to this environmental Ca^{2+} shock with an initial sharp elevation in cytosolic Ca^{2+} levels. However, the peak cytosolic Ca^{2+} level observed in the *vcx1*Δ strain (~400 nM) was significantly higher than was observed in either the wild-type strain (~290 nM) or the *pmc1*Δ strain (~250 nM). Both the wild-type and *pmc1*Δ strains recovered quickly after the Ca^{2+} shock, with each reaching a new

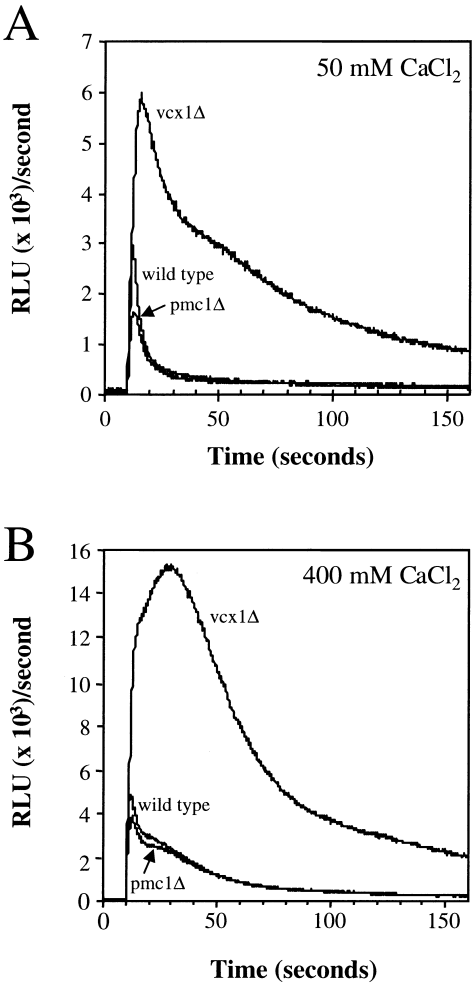

Fig. 3. Aequorin measurements of cytosolic Ca^{2+} levels following exposure to high environmental Ca^{2+}. Ca^{2+}-dependent light emission was recorded for 10 s in low Ca^{2+} medium to determine the basal cytosolic Ca^{2+} concentration. $CaCl_2$ was then added to a final concentration of A: 50 mM or B: 400 mM and changes in cytosolic Ca^{2+} were monitored for an additional 150 s.

steady-state level of 90–100 nM within 30 s of the initial exposure to high extracellular Ca^{2+}. In contrast, the recovery of the *vcx1*Δ strain was much more gradual, with the cytosolic Ca^{2+} concentration reaching only 260 nM after 150 s. These results suggest that the loss of the Vcx1p transporter, but not the Pmc1 transporter, leads to a severe defect in the ability of yeast cells to maintain the normal cytosolic Ca^{2+} level upon exposure to an environmental Ca^{2+} insult.

We next challenged these strains with 400 mM $CaCl_2$, which inhibits growth of the *pmc1*Δ strain after a few generations but does not significantly effect the growth of either the wild-type or the *vcx1*Δ strains (Fig. 3B). Under these conditions, the wild-type strain reached a maximum cytosolic Ca^{2+} level of ~320 nM Ca^{2+}, while the *pmc1*Δ strain reached a peak level of ~300 nM Ca^{2+}. These levels were only slightly higher than those observed after these strains were exposed to 50 mM $CaCl_2$. The peak levels of these strains rapidly decreased to a new cytosolic steady-state Ca^{2+} concentration of 100 nM within 90 s, indicating that the *pmc1*Δ strain was still able to regulate its cytosolic Ca^{2+} level as efficiently as the wild-type strain under these extreme conditions of Ca^{2+} stress. In contrast, the peak cytosolic Ca^{2+} concentration in the *vcx1*Δ strain reached ~510 nM, and decreased to a level of only 260 nM after 150 s. Thus, the peak was again much higher and the recovery phase was more gradual in the *vcx1*Δ strain than for either the wild-type or *pmc1*Δ strains. These results again indicate that the loss of Vcx1p significantly reduces the ability of cells to properly control cytosolic Ca^{2+} levels. In contrast, the loss of Pmc1p does not have a significant effect on the ability of cells to carry out the short-term adaptation of cytosolic Ca^{2+} levels, even when exposed to growth inhibitory levels of environmental Ca^{2+}. The ability of this strain to properly control cytosolic Ca^{2+} levels upon exposure to this extreme level of Ca^{2+} stress is also consistent with its ability to grow for 1–2 generations under these conditions.

It should be noted that although the wild-type and *pmc1*Δ strains showed only modest differences in their response to Ca^{2+} shock, the peak cytosolic Ca^{2+} level was consistently lower in the *pmc1*Δ strain when exposed to varying concentrations of extracellular Ca^{2+} in many independent experiments. We speculate that this difference may indicate that the *pmc1*Δ strain has either an increased rate of Ca^{2+} sequestration from the cytosol (possibly mediated by a compensatory increase in activity of Vcx1p), or a decreased rate of Ca^{2+} uptake across the plasma membrane.

3.3. *Effect of bafilomycin A on the maintenance of cytosolic Ca^{2+} levels*

As outlined above, previous studies found that the *VCX1* (*HUM1*) gene encodes a protein that functions as a vacuolar Ca^{2+}/H^+ exchanger [9,11]. This suggested that a loss of the H^+ gradient across the vacuolar membrane may also reduce the ability of the cell to properly regulate cytosolic Ca^{2+} levels through Vcx1p function. To test this possibility, we utilized the compound bafilomycin A_1, which is a specific inhibitor of the vacuolar H^+ ATPase [17,18]. WT cells were incubated for 10 min in the presence or absence of bafilomycin A_1. A $CaCl_2$ solution was then injected into the cuvette at a final concentration of 100 mM (Fig. 4A). We found that this short treatment of cells with bafilomycin A_1 caused the peak cytosolic Ca^{2+} level in the WT strain to increase from ~260 mM to a level of ~360 nM. Similarly, the level of cytosolic Ca^{2+} following recovery increased from ~80 nM to ~150 nM after exposure to this compound. Both the peak and post-recovery cytosolic Ca^{2+} levels observed in the treated cells were similar to the cytosolic Ca^{2+} levels measured in parallel assays in the *vcx1*Δ strain. These results provide further evidence that the primary control of the cytosolic Ca^{2+} concentration in the WT strain is provided by the Vcx1p Ca^{2+}/H^+ exchanger.

If Vcx1p represents the only Ca^{2+}/H^+ exchanger capable of efficiently sequestering cytosolic Ca^{2+} in this organism, a similar short treatment with bafilomycin A_1 might be predicted to have no further effect on the ability of a *vcx1*Δ strain to recover from a Ca^{2+} shock. To test this possibility, we treated the *vcx1*Δ strain with bafilomycin A_1 and again exposed it to a 100 mM Ca^{2+} shock (Fig. 4B). We found that treatment with this compound resulted in an increase in the peak cytosolic Ca^{2+} level from ~380 nM to ~425 nM, while the cytosolic Ca^{2+} level following recovery also increased from ~150 nM to ~255 nM. These results indicate that bafilomycin A_1 can also compromise the ability of the *vcx1*Δ strain to recover from a Ca^{2+} shock.

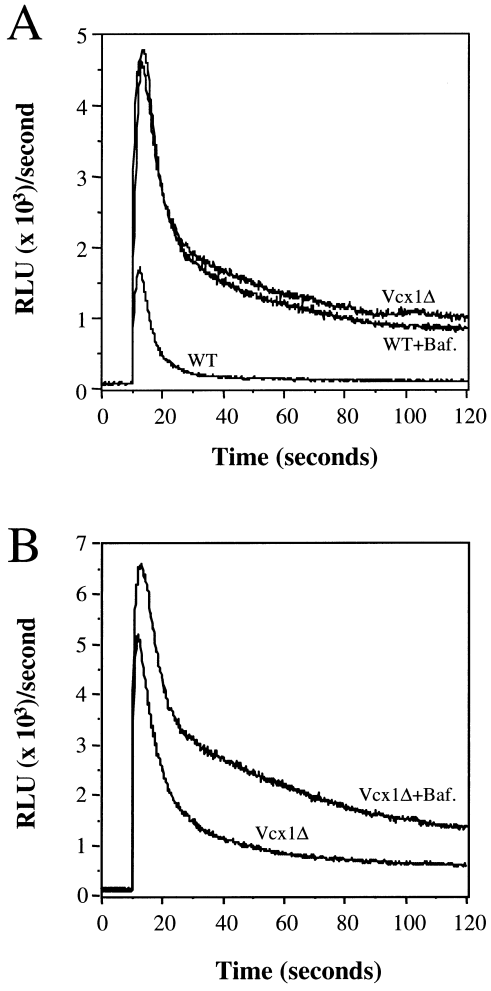

Fig. 4. Effect of bafilomycin A_1 on cytosolic Ca^{2+} levels following exposure to high environmental Ca^{2+}. WT or *vcx1*Δ cells were pretreated for 10 min with either bafilomycin A_1 (in DMSO) or DMSO alone. Ca^{2+}-dependent light emission was then recorded for 10 s in low Ca^{2+} medium to determine the basal cytosolic Ca^{2+} concentration. $CaCl_2$ was then added to a final concentration of 100 mM, and light emission was recorded for an additional 110 s. A: Effect of bafilomycin A_1 on the response of the WT strain to Ca^{2+} shock. B: Effect of bafilomycin A_1 on the response of the *vcx1*Δ strain to Ca^{2+} shock.

4. Discussion

In previous studies, overproduction of Vcx1p was found to suppress the Ca^{2+} sensitivity of a *pmc1* strain [11] and the Mn^{2+} sensitivity of a calcineurin-deficient strain [9]. Both of these studies concluded that the *VCX1* gene encodes a vacuolar Ca^{2+}/H^+ exchanger. However, only a very modest sensitivity to elevated environmental Ca^{2+} was observed in a *vcx1*Δ mutant. In addition, a significant increase in Ca^{2+} sensitivity attributable to a loss of Vcx1p activity was observed only when the *vcx1*Δ mutation was combined with either a *pmc1*Δ or a calcineurin mutation. Consistent with these observations, we did not observe any significant growth difference between a wild-type and *vcx1*Δ strain on YPD plates containing 400 mM $CaCl_2$. These results suggest that Vcx1p plays a role in the maintenance of cellular Ca^{2+} homeostasis that is largely redundant with Pmc1p under low to moderate extracellular Ca^{2+} concentrations. However, they appear to be at odds with the finding that Vcx1p activity represents a significant amount of vacuolar Ca^{2+} uptake in vitro [9,11].

Cunningham and Fink reported that Vcx1p activity was significantly reduced when cells are exposed to high extracellular Ca^{2+} [11]. We have also found that total cellular Ca^{2+} levels are lower than wild-type in a *vcx1*Δ strain at low extracellular Ca^{2+} levels (0.01 mM and 0.3 mM), but are indistinguishable from the wild-type strain when the extracellular Ca^{2+} level was increased to 5 mM or above (unpublished results). Furthermore, the Ca^{2+} sensitivity of a *pmc1*Δ strain can be reversed by cyclosporin A, a compound that inhibits calcineurin activity ([10]; our unpublished results). This pattern of regulation raises the possibility that the calcineurin-mediated inactivation of Vcx1p may take place in order to allow the efficient propagation of strong intracellular Ca^{2+} signals. These signals could then induce the expression and function of other Ca^{2+} transporters (such as Pmc1p and Pmr1p) that may have a higher threshold for induction [12].

To test this hypothesis, we introduced a cytosolic form of apoaequorin into the wild-type, *vcx1*Δ and *pmc1*Δ strains. We found that the basal cytosolic Ca^{2+} level in each of these strains was similar when tested in a medium containing a low concentration of Ca^{2}. However, when these cells were exposed to a Ca^{2+} shock with either 50 mM or 400 mM $CaCl_2$, we observed a significantly greater elevation in the cytosolic Ca^{2+} level in the *vcx1*Δ strain than in either the wild-type or *pmc1*Δ strains. The recovery phase observed following the Ca^{2+} shock was also much more prolonged in the *vcx1*Δ strain. These results demonstrate that the Vcx1p transporter plays a pivotal role in rapidly eliminating excess Ca^{2+} from the cytosol. Furthermore, these findings are consistent with the model that Vcx1p acts to attenuate the induction of cytosolic Ca^{2+} signaling pathways under conditions where it is active (below 5 mM extracellular Ca^{2+}).

It is also noteworthy that the *pmc1*Δ strain, like the wild-type strain, is able to efficiently sequester excess cytosolic Ca^{2+} following a 400 mM Ca^{2+} shock. This finding seems at odds with the fact that this strain cannot grow on YPD plates containing 400 mM $CaCl_2$. However, our results indicate that high Ca^{2+} does not immediately inhibit growth of the *pmc1*Δ strain. Instead, the *pmc1*Δ strain was able to grow for 1–2 generations after Ca^{2+} addition. A previous study found that activation of the calmodulin-calcineurin signaling pathway stimulated Pmc1p function, but inhibited Vcx1p function [11]. Our measurements of cytosolic Ca^{2+} indicate that the inactivation of Vcx1p function does not occur immediately, since the Vcx1p transporter retains the ability to rapidly eliminate cytosolic Ca^{2+} immediately following a Ca^{2+} shock in the *pmc1*Δ strain. The eventual down-regulation of Vcx1p in this strain may ultimately reduce the cell's ability to sequester cytosolic Ca^{2+}, resulting in a gradual cessation of growth.

Based upon these findings, we propose that the Vcx1p and Pmc1p transporters play complementary roles in Ca^{2+} homeostasis. Under conditions where Vcx1p is active, it functions to rapidly sequester cytosolic Ca^{2+} and attenuate the activation of Ca^{2+} signaling pathways. In contrast, Pmc1p appears to play a minimal role in the rapid sequestration of cytosolic Ca^{2+} under conditions where Vcx1p is active. When the level of environmental Ca^{2+} is increased, the calmodulin-calcineurin pathway is activated. This leads to the down-regulation of

Vcx1p function, which allows the maximal induction of Ca^{2+}-mediated signal transduction pathways and a subsequent increase in activity of other Ca^{2+} transporters such as Pmr1p and Pmc1p. These transporters may be optimally suited to sustain growth under conditions of high Ca^{2+} stress.

Finally, we found that bafilomycin A_1 treatment of the *vcx1*Δ strain also resulted in a reduced ability to properly control cytosolic Ca^{2+} levels upon exposure to an environmental Ca^{2+} shock. This result is in contrast to the observation that treatment of WT cells with this compound results in a response to Ca^{2+} stress that is identical to the response seen with the *vcx1*Δ strain. It is possible that the absence of the Vcx1p transporter may result in the induction or activation of a new transporter capable of coupling vacuolar Ca^{2+} uptake to the vacuolar H^+ gradient. Further studies are required to determine the source of this additional Ca^{2+} transport activity.

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