Regulation of Cellular Ca\(^{2+}\) by Yeast Vacuoles*

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Teresa Dunn, Kenneth Gable, and Troy Beeler

From the Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

The role of vacuolar Ca\(^{2+}\) transport systems in regulating cellular Ca\(^{2+}\) was investigated by measuring the vacuolar Ca\(^{2+}\) transport rate, the free energy available to drive vacuolar Ca\(^{2+}\) transport, the ability of the vacuole to buffer luminal Ca\(^{2+}\), and the vacuolar Ca\(^{2+}\) efflux rate. The magnitude of the Ca\(^{2+}\) gradient generated by the vacuolar H\(^{+}\) gradient best supports a 1 Ca\(^{2+}\):2 H\(^{+}\) coupling ratio for the vacuolar Ca\(^{2+}\)/H\(^{+}\) exchanger. This coupling ratio along with a cytosolic Ca\(^{2+}\) concentration of 125 nm would give a vacuolar free Ca\(^{2+}\) concentration of ~50 μM. The total vacuolar Ca\(^{2+}\) concentration is ~2 mM due to Ca\(^{2+}\) binding to vacuolar polyphosphate. The Ca\(^{2+}\) efflux rate from the vacuole is less than the growth rate indicating that the steady-state Ca\(^{2+}\) loading level of the vacuole is dependent mainly on the Ca\(^{2+}\) transport rate and the rate that vacuolar Ca\(^{2+}\) is diluted by growth. Based on the kinetic parameters of vacuolar Ca\(^{2+}\) accumulation in vitro, the maximum rate of Ca\(^{2+}\) accumulation in vivo is expected to be ~0.2 nmol of Ca\(^{2+}\) min\(^{-1}\) mg protein\(^{-1}\), a rate that is similar to the cellular Ca\(^{2+}\) accumulation rate. The cytosolic Ca\(^{2+}\) concentration increases from 0.1 μM to 1–2 μM as the extracellular Ca\(^{2+}\) concentration is raised from 0.3 μM to 50 μM. The rise in cytosolic Ca\(^{2+}\) concentration increases cellular Ca\(^{2+}\) from 10 to 300 nmol Ca\(^{2+}\)/mg by increasing the rate of vacuolar Ca\(^{2+}\) accumulation but does not significantly alter the cellular growth rate.

Although Ca\(^{2+}\) is believed to be an important regulatory messenger (1, 2) in Saccharomyces cerevisiae, little is known about the Ca\(^{2+}\) pumps and Ca\(^{2+}\) channels which regulate the cytosolic Ca\(^{2+}\) concentration. The best-characterized Ca\(^{2+}\) transport system in S. cerevisiae is the vacuolar Ca\(^{2+}\)/H\(^{+}\) exchanger (3–6). The importance of vacuolar Ca\(^{2+}\) transport in regulating cellular Ca\(^{2+}\) is suggested by the observation that mutants defective in the vacuolar H\(^{+}\)-ATPase (7–10) have reduced growth rates in 100 mM Ca\(^{2+}\), whereas 100 mM Ca\(^{2+}\) has little influence on the wild-type growth rate. Since cytosolic Ca\(^{2+}\) concentration is elevated in some of the mutants that have vacuolar defects (8, 12), it could be argued that vacuolar Ca\(^{2+}\) transport regulates cytosolic Ca\(^{2+}\). However, it is also possible that the elevated cytosolic Ca\(^{2+}\) is an indirect consequence of the vacuolar defect.

The goal of the study presented here is to evaluate the potential role of the vacuole in regulating the cellular Ca\(^{2+}\) concentration under physiological conditions in wild-type cells grown in media containing both high and low Ca\(^{2+}\) concentrations.

EXPERIMENTAL PROCEDURES

Materials—Arsenazo III was obtained from Aldrich. Zymolyase 100T was supplied by Seikagaku Kogyo, Rockville, MD. 4Ca\(^{2+}\) was purchased from DuPont-New England Nuclear. All other chemicals were purchased from Sigma.

Yeast Strains/Growth—The yeast strain used in this work was Cuh3 (S288C, MATb his4-619 ura3-52). Cells were grown according to standard procedures (13). Standard yeast extract bacto-peptone dextran (YPD) and synthetic media were prepared according to Sherman (13). Low phosphate YPD media was made according to the method described by Rubin (14).

Measurement of Ca\(^{2+}\) Accumulation by Whole Cells—Two methods were used to measure Ca\(^{2+}\) accumulation by cells. In one method, cells were incubated in media containing tracer 4Ca\(^{2+}\) (0.1–1 μCi/ml) for different periods of time, and aliquots were removed, washed three times, and centrifuged at 20°C in YPD medium at 4 °C, and filtered through Beckman Ready Organic™ scintillation mixture, and the amount of Ca\(^{2+}\) in the cell was determined by scintillation counting.

In the other method, cells were incubated in media containing 1–100 mM Ca\(^{2+}\) for different periods of time and then harvested and washed by centrifugation (4°C). The cells were resuspended in 0.1 μM KCl, 10 mM PIPES, pH 7.0, 0.1 mM arsenazo III at a density 10^7/ml (36°C). Enough digitonin (1 mg/ml) to permeabilize all the cell membranes was added, and the amount of Ca\(^{2+}\) released from the cell was determined spectrophotometrically using an SLM-Aminco DW2; dual wavelength spectrophotometer by measuring the increased absorbance caused by the formation of the arsenazo III-Ca\(^{2+}\) complex at 660 nm using 885 nm as a reference wavelength. When both methods (filtration and spectrophotometric) were compared under the same experimental conditions, the results were essentially the same.

Isolation of Vacuole Membrane Vesicles—Vacuole membrane vesicles (vesiculated vacuoles) were prepared by the method developed by Anraku and co-workers (3) and stored at ~70 °C in 0.1 μM potassium glutamate, 10 mM PIPES, pH 7.0, and 10% glycerol.

Permeabilization of Yeast Cells—Ca\(^{2+}\) accumulation by intact vacuoles was measured using either osmotically shocked partially regenerated spheroplasts (15) or cells permeabilized by treatment with the detergent digitonin. Treatment of cells with 0.2 mg digitonin/mg protein for 10 min at 26°C selectively permeabilizes the plasma membrane.

Measurement of Polyphosphate—Polyphosphate was extracted from the cells by the method described by Clark et al. (16) and measured by the procedure described by Ames (17).

RESULTS

Effect of Ca\(^{2+}\) on the Growth Rate and Ca\(^{2+}\)-Loading Level of S. cerevisiae cells—The role of the vacuole in regulating cellular Ca\(^{2+}\) was investigated. Varying the Ca\(^{2+}\) concentration in the growth medium from 1 μM to 100 mM has little effect on the growth rate of S. cerevisiae cells (18–23). The tolerance of S. cerevisiae to high Ca\(^{2+}\) concentrations is not due to the inability of Ca\(^{2+}\) to enter the cell since the cellular Ca\(^{2+}\) increases as the extracellular Ca\(^{2+}\) concentration is raised (Fig. 1, inset).

The abbreviations used are: PIPES, 1,4-piperazinebis(ethanesulfonic acid); MES, 4-morpholineethanesulfonic acid.

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† To whom correspondence should be addressed: Dept. of Biochemistry, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814. Tel.: 301-295-3592; Fax: 301-295-3512.
In normal YPD medium (~0.3 mM Ca\(^{2+}\)), the cellular Ca\(^{2+}\) is 10–20 nmol/mg protein. Addition of 50 mM Ca\(^{2+}\) to the YPD growth medium causes a relatively slow increase in the cellular Ca\(^{2+}\) to ~225 nmol/mg protein after 5 h (Fig. 1) without changing the growth rate.

**Ca\(^{2+}\) Accumulation by Permeabilized Cells and Vacuolar Membrane Vesicles**—The following experiments were designed to determine the relationship between the cytosolic Ca\(^{2+}\) concentration and vacuolar Ca\(^{2+}\) accumulation. The kinetic and thermodynamic parameters for vacuolar Ca\(^{2+}\) transport were investigated.

ATP initiates Ca\(^{2+}\) accumulation by isolated vacuole membrane vesicles (4) as well as by permeabilized partially regenerated spheroplasts (2). Since nigerin and other proton ionophores inhibit ATP-dependent Ca\(^{2+}\) accumulation (4), it was proposed by Ohashi and Anraku that ATP hydrolysis by the vacuolar H\(^{+}\)-ATPase establishes a proton gradient that drives Ca\(^{2+}\) transport. The Ca\(^{2+}\) (Km) of the Ca\(^{2+}\) transporter in both the isolated vacuole membrane vesicles (data not shown) and permeabilized cells is 25 \(\mu\)M at pH 7.0 and 1 mm Mg\(^{2+}\) (Fig. 2B). Permeabilized cells have a maximum Ca\(^{2+}\) accumulation rate (Vmax) of 35 nmol of Ca\(^{2+}\) min\(^{-1}\) (mg cell protein\(^{-1}\)) (Fig. 2B). Therefore, the vacuolar Ca\(^{2+}\) accumulation rate in the presence of 125 I\(^{-}\)Ca\(^{2+}\) is expected to be ~0.2 nmol Ca\(^{2+}\) min\(^{-1}\) (mg cell protein\(^{-1}\)) which is comparable to the rate of whole cell Ca\(^{2+}\) accumulation in YPD medium (0.1–0.2 nmol Ca\(^{2+}\) min\(^{-1}\) (mg cell protein\(^{-1}\))). Thus, the overall rate of cellular Ca\(^{2+}\) accumulation could depend on the rate of vacuolar Ca\(^{2+}\) accumulation and not on the rate of Ca\(^{2+}\) influx across the plasma membrane.

**Effect of the Proton Gradient on Ca\(^{2+}\) Accumulation by Vacuole Membrane Vesicles**—To determine if ATP is required directly by the Ca\(^{2+}\) transport system, H\(^{+}\) gradients across the yeast vacuolar membrane were formed in the absence of ATP by diluting K\(^{+}\)-equilibrated vacuolar membrane vesicles into solutions containing differing ratios of Tris\(^{+}\) and K\(^{+}\) and the ionophore, nigerin. Nigerin mediates the exchange of one proton for one K\(^{+}\) causing the formation of a H\(^{+}\) gradient which is expected to equal the K\(^{+}\) gradient. Ca\(^{2+}\) accumulation was measured spectrophotometrically using the Ca\(^{2+}\) indicator arszenazo I\(^{111}\) (50 L\(^{-}\)IIM) (and therefore on the H\(^{+}\) gradient). No Ca\(^{2+}\) accumulation was observed in the absence of nigerin or when nigerin is replaced with the K\(^{+}\) ionophore, valinomycin, demonstrating that neither a K\(^{+}\) gradient nor K\(^{+}\) diffusion potential (inside-negative) drives Ca\(^{2+}\) transport. An apparent H\(^{+}\) K\(_{m}\) of 2 \(\mu\)M is determined from the relationship between the K\(^{+}\) gradient and the Ca\(^{2+}\) transport rate (Fig. 3B).

**The Ca\(^{2+}\)/H\(^{+}\) Exchanger Coupling Ratio**—To determine the stoichiometry of the Ca\(^{2+}\)/H\(^{+}\)-exchanger, the free intravesicular Ca\(^{2+}\) concentration established by the proton gradient generated by the vacuolar H\(^{+}\)-ATPase was determined. Vesicles were equilibrated with different concentrations of Ca\(^{2+}\) and \(50\text{Ca}^{2+}\), and the amount of Ca\(^{2+}\) trapped within the vesicles was measured by filtration (Fig. 4A). The trapped vesicular Ca\(^{2+}\) can be divided into a component that is directly proportional to the Ca\(^{2+}\) concentration and another that saturates. The component that increases linearly with the Ca\(^{2+}\) concentration represents free Ca\(^{2+}\). The trapped volume of vacuole membrane vesicles is calculated to be 2.4 \(\mu\)l/mg protein, a value similar to that of other biomembrane vesicles. The saturable component probably represents Ca\(^{2+}\) binding to residual polyphosphate in the vesicle lumen (24).
grown in YPD medium. Ca2+ binding to polyphosphate was determined. Next, Ca2+ binding to cellular polyphosphate was compared with that of synthetic polyphosphate. Finally, the Ca2+ sequestration was investigated. The strain used in this study accumulates ~0.1 mg polyphosphate/mg protein when grown in YPD medium. Ca2+ binding to polyphosphate was investigated using several experimental approaches. First, properties of Ca2+ binding to synthetic polyphosphate were determined. Next, Ca2+ binding to cellular polyphosphate was compared with that of synthetic polyphosphate. Finally, the effect of the vacuolar polyphosphate content on Ca2+ accumulation by whole cells and by cells with the plasma membrane permeabilized was determined.

The affinity and capacity of polyphosphate to bind Ca2+ was determined spectrophotometrically using the Ca2+-indicator arsenazo III. The free Ca2+ concentration in the presence of 80 μg/ml polyphosphate was measured as the total Ca2+ concentration varied (Fig. 5).

Synthetic polyphosphates containing averages of 5, 18, and 31 phosphate residues were used. At pH 6.0 (close to the vacuolar pH in vivo (26,27)), the Ca2+ dissociation constant is 6–8 μM (Fig. 5). The 31-residue polyphosphate binds 1 Ca2+/2.5 phosphate residues with high affinity suggesting that, except for the 3 phosphate residues on each end, the high affinity Ca2+-binding site is formed by 2 phosphate residues. The binding of Ca2+ does not change significantly when the pH is decreased to 5.7 or increased to 6.8 (data not shown); however, Mg2+ is a strong competitor for Ca2+ binding (Fig. 5). In the presence of 1 mM Mg2+, the dissociation constant is increased to ~1 mM. The data indicate that Mg2+ and Ca2+ bind to polyphosphate with similar affinities.

Treatment of yeast cells with high detergent concentrations exposes intracellular high affinity Ca2+-binding sites (Fig. 6). The detergent-exposed latent Ca2+ binding activity of S. cerevisiae can be primarily attributed to polyphosphate for the following reasons. 1) Latent Ca2+ binding activity is directly proportional to the polyphosphate content of the cells which varies with the phosphate concentration (0.01–1 mM) of the growth media. 2) The Ca2+/phosphate residue stoichiometry (0.4 Ca2+/phosphate residue in 50 μM Ca2+) of the latent Ca2+ binding is
Vacuolar Ca²⁺ Transport

The role of polyphosphate in sequestering cellular Ca²⁺ is further demonstrated by measuring the effect of cellular polyphosphate levels on the in vivo Ca²⁺ content (Fig. 7B). The amount of cellular Ca²⁺ increases with increasing cellular polyphosphate. The ratio of cellular Ca²⁺ to polyphosphate residues remains constant at about 0.003, once again demonstrating that the in vivo vacuolar Ca²⁺ level for cells grown in 0.3 mM Ca²⁺ is ~100 times lower than the level of polyphosphate Ca²⁺-binding sites. The Ca²⁺ content of cells growing in 50 mM Ca²⁺ is also proportional to cellular polyphosphate content; however, the ratio of Ca²⁺ to polyphosphate residues increases to ~0.2 (data not shown).

Vacuolar Ca²⁺ Efflux—The above data indicate that the rate-limiting step of Ca²⁺ accumulation by S. cerevisiae cells is vacuolar transport and that polyphosphate acts as a Ca²⁺ sink within the vacuole lumen. The next question that was addressed was whether Ca²⁺ sequestration in the vesicles was...
from the vacuole lumen while the reason that the intrinsic exchanger fails to release Ca\(^{2+}\) may have a low affinity for protons (low pK) on the cytosolic side blocking the exchange of cytosolic protons with luminal Ca\(^{2+}\). reversible. The rate of Ca\(^{2+}\) efflux from yeast vacuoles was measured both in vitro and in vivo. Permeabilized cells equilibrated with 25 \(\mu\)M Ca\(^{2+}\) sequester -6 nmol Ca\(^{2+}\)/mg protein (about 0.14 nmol/mg would be free, most of the rest is bound to polyphosphate) (Fig. 8). This observation further supports the conclusion that the free vacuole Ca\(^{2+}\) concentration in vivo is \(-30 \mu\)M since cells accumulate 10-20 nmol Ca\(^{2+}\)/mg protein. The rate of Ca\(^{2+}\) efflux from the vacuole of permeabilized cells was measured following dilution into an EGTA solution. The Ca\(^{2+}\) efflux rate was only 0.016 nmol Ca\(^{2+}\)/min mg (mg protein)\(^{-1}\); therefore, the halftime for Ca\(^{2+}\) release from the vacuole exceeds the doubling time for cells under normal growth conditions. 45Ca\(^{2+}\) release was observed when unlabeled Ca\(^{2+}\) was added to the dilution medium, but this is due to 45Ca\(^{2+}\)-Ca\(^{2+}\) exchange mediated by the Ca\(^{2+}\)/H\(^{+}\) exchanger since in parallel experiments it was observed that extravacuolar 45Ca\(^{2+}\) was accumulated by vacuoles in exchange with luminal unlabeled Ca\(^{2+}\).

The Ca\(^{2+}\) ionophore, A23187, was able to induce the release of all sequestered Ca\(^{2+}\) in the absence of extravacuolar Ca\(^{2+}\). Like the intrinsic Ca\(^{2+}\)/H\(^{+}\) exchanger, A23187 mediates the transmembrane exchange of one Ca\(^{2+}\) for two protons. Perhaps the reason that the intrinsic exchanger fails to release Ca\(^{2+}\) from the vacuole lumen while A23187 does is that A23187 has a relatively high affinity for protons (high pK) so that it transports protons in both directions, while the intrinsic exchanger may have a low affinity for protons (low pK) on the cytosolic pool and the high Ca\(^{2+}\) binding capacity of the vacuolar pool, and the amount of Ca\(^{2+}\) in this stable pool increases with increasing extracellular Ca\(^{2+}\) concentration. This experiment indicates that the in vivo vacuolar Ca\(^{2+}\) efflux rate is also very low.

**DISCUSSION**

**Model of Ca\(^{2+}\) Metabolism by S. cerevisiae**—A model for Ca\(^{2+}\) metabolism by S. cerevisiae cells is presented in the inset of Fig. 9. Steady-state Ca\(^{2+}\) gradients across the plasma membrane are rapidly established (within seconds) following changes in the extracellular Ca\(^{2+}\) concentration. The mechanisms by which Ca\(^{2+}\) enters the cell and is actively pumped out are not known, but a 25,000-fold Ca\(^{2+}\) gradient across the plasma membrane can apparently be maintained. The rate of Ca\(^{2+}\) accumulation by the vacuole (the major Ca\(^{2+}\)-sequestering organelle) is dependent on the rate of Ca\(^{2+}\) transport mediated by the Ca\(^{2+}\)/2H\(^{+}\) exchanger and the cell growth rate. The cellular Ca\(^{2+}\) uptake rate is predicted to increase linearly with the cytosolic Ca\(^{2+}\) concentration because of the relatively high Km (25 \(\mu\)M) and the high Ca\(^{2+}\) binding capacity of the vacuolar lumen (\(-400 \text{ nmol Ca}^{2+}/\text{mg protein}\)). Because most of the cellular Ca\(^{2+}\) is found in the vacuole, the rate of cellular Ca\(^{2+}\) accumulation is dependent on the rate of vacuolar Ca\(^{2+}\) accumulation. Because the vacuolar Ca\(^{2+}\) efflux rate is less than the growth rate, the steady-state vacuolar Ca\(^{2+}\) level is reached when the rate of vacuolar Ca\(^{2+}\) transport is equal to the rate of vacuolar Ca\(^{2+}\) dilution by growth. The rate of vacuolar Ca\(^{2+}\) dilution through growth would be k\(_{\text{growth}}\) x [Ca\(_{\text{vacuolar}}\), where k\(_{\text{growth}}\) is the rate constant for cell growth and [Ca\(_{\text{vacuolar}}\) is the
total vacuolar Ca\(^{2+}\) content. If vacuolar Ca\(^{2+}\) transport follows Michaelis-Menten kinetics, \(v = V_{\text{max}}[\text{Ca}^{2+}]_{\text{cytosol}}/[\text{Ca}^{2+}]_{\text{cytosol}} + K_M\), the steady-state vacuolar Ca\(^{2+}\) content would be \(V_{\text{max}}[\text{Ca}^{2+}]_{\text{cytosol}}/[\text{Ca}^{2+}]_{\text{cytosol}} + K_M\). Based on the kinetic parameters (\(V_{\text{max}} = 35 \text{ nmol Ca}^{2+} \text{ min}^{-1} \text{ (mg protein)}^{-1}; K_M = 25 \mu\text{M}\)) determined from permeabilized cells, and a growth rate of \(6 \times 10^{-3} \text{ min}^{-1}\), the total vacuolar Ca\(^{2+}\) content is predicted to be directly proportional to the cytosolic Ca\(^{2+}\) concentration to 1-2 nmol Ca\(^{2+}\) (mg protein) (Fig. 9A). According to the proposed model, the cytosolic Ca\(^{2+}\) concentrations that would give the observed cellular Ca\(^{2+}\) levels of 10-300 nmol Ca\(^{2+}\)/(mg protein) would be 0.1-1.4 \muM Ca\(^{2+}\), meaning the cytosolic Ca\(^{2+}\) would have to increase from 0.1 to 1.4 \muM as the extracellular Ca\(^{2+}\) increases from 0.3 to 100 \muM (Fig. 9B).

Addition of 100 mM Ca\(^{2+}\) to the growth medium has no significant effect on the growth rate suggesting that increasing the cytosolic Ca\(^{2+}\) concentration to 1-2 \muM does not alter the cellular physiology of exponentially growing yeast cells. In this regard, it is interesting that at these cytosolic Ca\(^{2+}\) concentrations, calmodulin would be expected to be activated by Ca\(^{2+}\) and yet there is no influence on cell growth. These results complement the findings of Geiser et al. (28) which demonstrate that, while calmodulin is required for growth, Ca\(^{2+}\) binding to calmodulin is apparently not required. Taken together these results indicate that Ca\(^{2+}\) binding to calmodulin does not affect vegetative cell growth.

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