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Activation of Divalent Cation Influx into *S. cerevisiae* Cells by Hypotonic Downshift

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Abstract. Subjecting *Saccharomyces cerevisiae* cells to a hypotonic downshift by transferring cells from YPD medium containing 0.8 M sorbitol to YPD medium without sorbitol induces a transient rapid influx of Ca^{2+} and other divalent cations into the cell. For cells grown in YPD at 37°C, this hypotonic downshift increases Ca^{2+} accumulation 6.7-fold. Hypotonic downshift-induced Ca^{2+} accumulation and steady-state Ca^{2+} accumulation in isotonic YPD medium are differentially affected by dodecylamine and Mg^{2+}. The Ca^{2+}-influx pathway responsible for hypotonic-induced Ca^{2+} influx may account for about 10–35% of Ca^{2+} accumulation by cells growing in YPD. Ca^{2+} influx is not required for cells to survive a hypotonic downshift. Hypotonic downshift greatly reduces the ability of *S. cerevisiae* cells to survive a 5-min exposure to 10 mM Cd^{2+} suggesting that mutants resistant to acute Cd^{2+} exposure may help identify genes required for hypotonic downshift-induced divalent cation influx.

Key words: *Saccharomyces cerevisiae* — Calcium — Mechanosensitive channel — Cadmium — Zinc — Dodecylamine — Osmotic downshift — Hypotonic downshift

Introduction

The mechanisms by which Ca^{2+} enter *S. cerevisiae* cells are not well understood. Two transport systems that mediate divalent cation influx into *S. cerevisiae* cells have been described: the ''general divalent cation transporter'' (Rothstein et al., 1958; Fuhrman & Rothstein, 1968; Borst-Pauwels, 1988) and the mechanosensitive channel (Gustin et al., 1986; Saimi et al., 1988; Gustin, 1991). Both systems are relatively nonspecific and low-affinity. The primary function of the general divalent cation transporter may be to mediate Mg^{2+} influx into the cell, however, other divalent cations are also transported into the cell when present in the growth medium at relatively high concentrations (0.01–10 mM). The order of affinity for general divalent cation transport (from greatest to least) is Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+} and Sr^{2+}. At low concentrations (<1 μM), essential metals are transported into the cell by specific, high-affinity transport systems (Mn^{2+}: Supek et al., 1996; Cu^{2+}: Dancis et al., 1994; Yuan et al., 1995; Zn^{2+}: Zhao & Eide, 1996; Fe^{2+}: Askwith et al., 1994; Stearman et al., 1996).

Kung and coworkers, using a patch clamp, observed a 40 pS mechanosensitive channel that carries Ca^{2+} across the *S. cerevisiae* plasma membrane (Gustin et al., 1986; Saimi et al., 1988; Gustin, 1991). This was the only plasma membrane channel observed to mediate Ca^{2+} influx, although the channel had low ion selectivity. Application of pressure equivalent to that generated by a 3-mOsm gradient across the membrane is sufficient to activate the channel (Saimi et al., 1988). Batiza, Schulz and Masson (1996) measured a hypotonic downshift-induced rise in cytosolic Ca^{2+} in *S. cerevisiae* cells using AEQUORIN as a reporter.

Ca^{2+} influx into cells may be part of a signaling pathway initiated by increased membrane tension arising from changes in the osmolarity of the medium or from cell wall remodeling during growth (Kamada et al., 1995). The purpose of this study was to characterize the properties of Ca^{2+} influx induced by a hypotonic downshift and to address its role in regulating cellular Ca^{2+}.

Materials and Methods

Strains and Media

Saccharomyces cerevisiae cells (strain CuH3 = *Mata ura3-52 his4-619*) were grown according to standard procedures (Sherman, Fink &

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Hicks, 1986) in YPD (1% yeast extract, 2% Bactopeptone, 2% dextrose) medium. YPD + 0.8 M sorbitol was prepared by diluting an autoclaved 2× YPD solution with an autoclaved 1.6 M sorbitol solution.

Measurement of $^{45}Ca^{2+}$ Accumulation by *S. cerevisiae* Cells upon Osmotic Downshift

Cells were resuspended or diluted (final concentration of 2×10^7 cells/ml) into the Ca^{2+}-uptake media (which varied according to the experiment as described in the figure legends) containing 1 or 2 μCi/ml $^{45}Ca^{2+}$. At various times, 1-ml aliquots of the cells were Millipore filtered. The filters were washed four times with 5-ml of ice-cold 20 mM $MgSO_4$, and dried. The cell-associated $^{45}Ca^{2+}$ was determined by scintillation counting.

Measurement of Divalent Cations by the Indicator Arsenazo III

S. cerevisiae cells were grown in YPD + 0.8 M sorbitol at 37°C to an OD_{500nm} of about 1. The cells were washed twice by centrifugation with 0.1 M KCl, 10 mM HEPES (pH 7.0), 0.75 mM $MgSO_4$ containing 0.8 M sorbitol and resuspended in the same solution to an OD_{600nm} of 50. Cells were diluted 50-fold into the KCl solution containing 100 μM arsenazo III and 75 μM of either Ca^{2+}, Sr^{2+}, Zn^{2+}, Mn^{2+}, or Cd^{2+} with or without 0.8 M sorbitol. The arsenazo III:divalent cation difference absorbance was measured using an AMINCO DW500 dual wavelength spectrophotometer with the wavelength pair set to either 685 nm–660 nm (Ca^{2+}), 700 nm–649 nm (Sr^{2+}), 567 nm–613 nm (Zn^{2+}), 578 nm–611 nm (Mn^{2+}), or 576 nm–603 nm (Cd^{2+}). A calibration curve for each divalent cation was produced by titrating arsenazo III with the appropriate divalent cation.

Results

Hypotonic Downshift Induces Calcium Influx into *S. cerevisiae* Cells

During growth in 0.8 M sorbitol, *S. cerevisiae* cells increase their intracellular osmolarity by inducing glycerol synthesis (Varela et al., 1992; Larsonn et al., 1993; Albertyn et al., 1994). Shifting cells grown in YPD containing 0.8-M sorbitol into YPD medium lacking sorbitol (hypotonic downshift) is expected to increase membrane tension caused by water influx into the cells. Subjecting *S. cerevisiae* cells to this hypotonic downshift induces rapid Ca^{2+} influx (6 nmol mg^{-1} min^{-1}) (Fig. 1*A*). The Ca^{2+} concentration of YPD medium is about 0.3 mM (Dunn, Gable & Beeler, 1994). Hypotonic downshift-induced Ca^{2+} influx (measured at 26°C) for cells grown at 26°C is more transient than for cells grown at 37°C (Fig. 1*B vs.* 1*A*). The rate of Ca^{2+} entry depends on the magnitude of the osmotic gradient (Fig. 1*B* insert). Hypotonic downshift-induced Ca^{2+} influx is not observed in the absence of glucose suggesting that this process is either energy-dependent or regulated by glucose. No loss of cell viability is observed upon hypotonic downshift in the presence of 10 mM EGTA, a divalent cation chelator, or 10 mM $CaCl_2$ indicating that neither decreased nor increased Ca^{2+} influx influences the ability of cells to survive the hypotonic downshift in YPD medium.

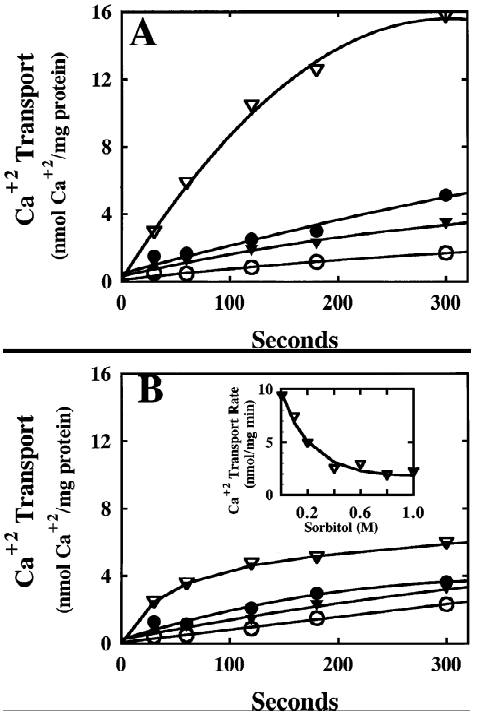

Fig. 1. Effect of osmotic gradient Ca^{2+} uptake by *S. cerevisiae* cells. (*A*) *S. cerevisiae* cells were grown in YPD at 37°C with (▽,▼) and without (○,●) 0.8 M sorbitol to an OD_{600nm} of about 1. The cells were collected by centrifugation and resuspended in YPD at 26°C containing 1 μCi $^{45}Ca^{2+}$ with (○,▼) and without (●,▽) 0.8 M sorbitol. Cell-associated Ca^{2+} was determined at the indicated times as described in Materials and Methods. (*B*) Same as *A*, except cells were grown at 26°C instead of 37°C. Insert: Cells were grown at 26°C in YPD + 0.8 M sorbitol. Ca^{2+} accumulation was measured upon transfer to YPD containing the indicated sorbitol concentration.

Hypertonic upshift initiated by addition of 0.8 M sorbitol to cells in YPD decreases the cellular Ca^{2+} accumulation rate by 45% as compared with cells maintained in YPD (Fig. 1).

Kovac (1985) has demonstrated that the rate of Ca^{2+} accumulation by *S. cerevisiae* cells in YPD is proportional to the Ca^{2+} concentration in the range of 0.04 to 0.8 mM. The Ca^{2+} uptake rate is also proportional to the Ca^{2+} concentration in the range of 1 to 50 mM (Fig. 2). In contrast, hypotonic downshift-induced Ca^{2+} influx saturates at about 10 mM Ca^{2+} (Fig. 2). Since Ca^{2+} accumulation by *S. cerevisiae* cells is dependent on vacuolar sequestration of cytosolic Ca^{2+} into a nonexchangeable pool (Dunn et al., 1994), it is not known whether the saturation of Ca^{2+} accumulation is due to saturation of the plasma membrane or vacuolar transport systems.

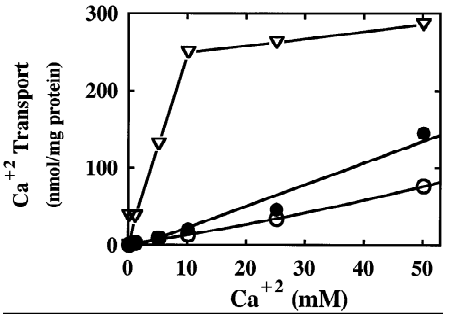

Fig. 2. Effect of Ca^{2+} concentration on the rate of Ca^{2+} influx. *S. cerevisiae* cells were grown in YPD with (▽,○) or without (●) 0.8 M sorbitol at 37°C to an OD_{600nm} of about 1. The cells were collected by centrifugation and resuspended in YPD medium with 2 μCi/ml $^{45}Ca^{2+}$ and the indicated Ca^{2+} concentration with (○) or without (▽,●) 0.8 M sorbitol. The Ca^{2+} concentration of YPD without Ca^{2+} supplementation is about 0.3 mM. After a 1-min incubation, 1-ml aliquots were Millipore filtered. The filters were washed four times with 5 ml of ice-cold 20 mM $MgSO_4$, and dried. The cell-associated $^{45}Ca^{2+}$ was determined by scintillation counting.

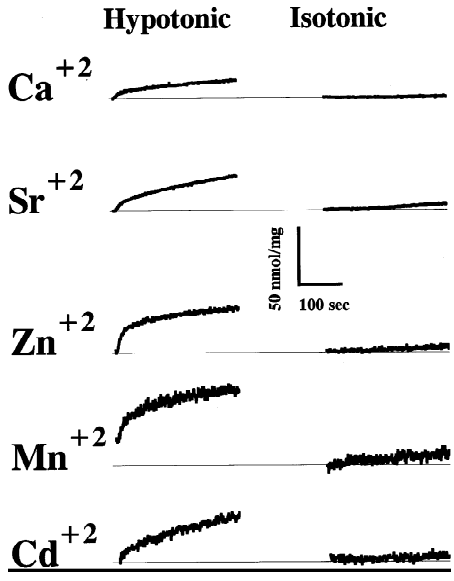

Fig. 3. Effect of an osmotic downshift on influx of Ca^{2+}, Sr^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+}. *S. cerevisiae* cells were grown in YPD + 0.8 M sorbitol at 37°C to an OD_{600nm} of about 1. The cells were washed, concentrated to an OD_{600nm} of 50, and diluted 50-fold into a KCl solution containing arsenazo III and the indicated divalent cation with (right) or without (left) 0.8 M sorbitol. Divalent cation accumulation was measured by monitoring the arsenazo III:divalent cation difference absorbance as described in Materials and Methods.

Hypotonic Downshift-Induced Cation Influx has Low Ion Selectivity; Hypotonic Downshift in the Presence of Cadmium Results in Increased Cadmium Toxicity

Hypotonic downshift-induced divalent cation influx has low selectivity. A transient influx of Sr^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+} as well as Ca^{2+} is induced by hypotonic downshift (Fig. 3). $^{22}Na^{+}$ influx was also stimulated by osmotic downshift in a manner similar to that of Ca^{2+} (*data not shown*).

The large increase in Cd^{2+} influx induced by hypotonic downshift greatly increases Cd^{2+} toxicity. Hypotonic-induced Cd^{2+} influx increased Cd^{2+} toxicity about tenfold (Fig. 4*A*). In the absence of a hypotonic downshift, half of the cells survived a 10-min exposure to 30 mM Cd^{2+}. With a hypotonic downshift, only 2-mM Cd^{2+} was required to kill half the cells. Enhancement of Cd^{2+} toxicity by hypotonic downshift is blocked by 50 mM Ca^{2+} (Fig. 4*A*), and 25 μM dodecylamine (Fig. 4*B*). As shown below, dodecylamine is an inhibitior of hypotonic-induced Ca^{2+} influx. The apparent dependency of Cd^{2+} toxicity on hypotonic downshift-induced Cd^{2+} influx suggests that isolation of mutants that are resistant to acute exposure to high Cd^{2+} concentrations might be useful in identifying genes required for hypotonic-induced divalent cation influx.

Alkylamines Block Hypotonic Downshift-induced Cation Influx

The properties of hypotonic downshift-induced Ca^{2+} influx were compared to those of Ca^{2+} influx by cells maintained in constant osmolarity. Gadolinium (10 μM) applied from the cytoplasmic side blocked the mechanosensitive channel (Gustin et al., 1988). Gadolinium (10–100 μM) had no significant effect on cellular Ca^{2+} accumulation in YPD with or without a hypotonic downshift (*data not shown*) indicating that the mechanosensitive channel may not mediate hypotonic downshift-induced Ca^{2+} influx. However, it is also possible that gadolinium does not block the mechanosensitive channel from the extracellular side, or that the channels are less accessible in intact yeast due to the cell wall. Gadolinium at concentrations greater than 0.1 mM precipitated in YPD. Batiza, Schulz & Masson (1996) recently measured increased cytosolic Ca^{2+} upon hypotonic shock using an AEQUORIN calcium reporter system. In their studies, 10 mM gadolinium was used to block the hypotonic shock-induced Ca^{2+} signal.

A variety of amphipathic cations were tested for their ability to block hypotonic downshift-induced Ca^{2+} accumulation. The most effective blockers were found to be primary alkylamines (Fig. 5*A*). Alkylamines with 10 to 15 carbons block 75% of the hypotonic downshift-induced Ca^{2+} influx and 25% of Ca^{2+} accumulation in YPD without a hypotonic downshift (Fig. 5*B*). Under the assay conditions, the alkylamines did not affect cell viability. These results are consistent with 25 to 35% of the Ca^{2+} transport in the absence of an osmotic

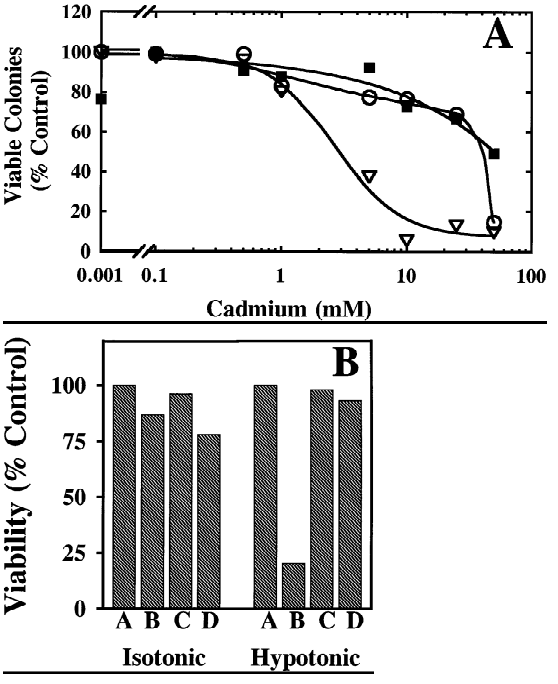

Fig. 4. Effect of osmotic downshift on Cd^{2+} toxicity. Panel *A. S. cerevisiae* cells were grown in YPD containing 0.8 M sorbitol at 37°C to an OD_{600nm} of about 1. The cells were diluted to 10^5/ml in YPD containing the indicated Cd^{2+} concentration with (○) or without (▽,■) 0.8 M sorbitol. In one series, the dilution media also contained 50 mM Ca^{2+} (■). After a 5-min incubation at 37°C, 100 μl aliquots were plated onto YPD + 10 mM $CaCl_2$ agar plates to determine number of viable cells. (*B*) Cells were grown as in panel *A*, collected by centrifugation and resuspended to an OD_{600} of 0.9 in YPD (*A*) or YPD containing (*B*) 10 mM $CdCl_2$, (*C*) 25 μM dodecylamine, or (*D*) 10 mM $CdCl_2$, 25 μM dodecylamine. After a 10-min incubation at 37°C, the cells were diluted 10,000-fold and 100 μl aliquots were plated onto YPD/sorbitol agar plates to determine the number of viable cells.

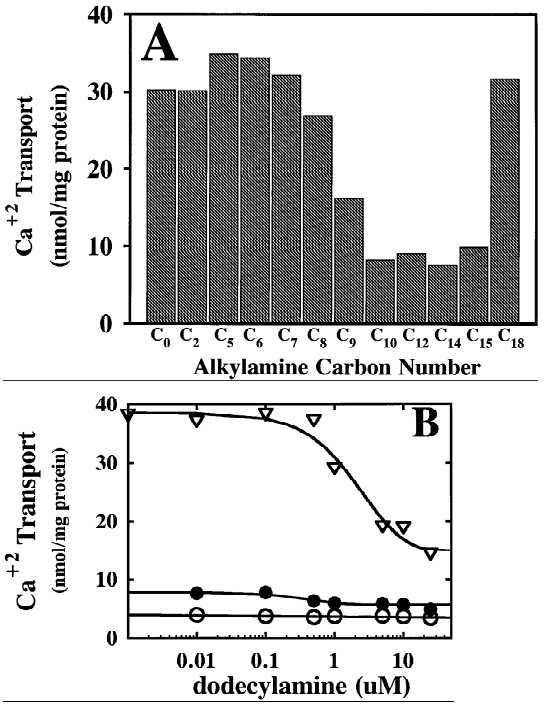

Fig. 5. Inhibition of Ca^{2+} accumulation by alkylamines. (*A*) Inhibition of Ca^{2+} accumulation as a function of alkyl chain length. *S. cerevisiae* cells were grown in YPD containing 0.8 M sorbitol at 37°C to an OD_{600nm} of about 1. The cells were washed twice by centrifugation and resuspended in KCl solution to an OD_{600nm} of 50. To measure Ca^{2+} uptake, cells were diluted 50-fold into KCl solution containing 1 mM Ca^{2+} + $^{45}Ca^{2+}$ (1 μCi/ml) + the indicated alkylamine (25 μM). After a 5-min incubation, 1-ml aliquots were filtered and processed for determination of cellular Ca^{2+} accumulation (Materials and Methods). (*B*) Inhibition of Ca^{2+} accumulation as a function of the dodecylamine concentration. Cells were grown in YPD with (▽,○) or without (●) 0.8 M sorbitol at 37°C to an OD_{600nm} of about 1. The cells were washed twice by centrifugation and resuspended in KCl solution with (○) and without (▽,●) 0.8 M sorbitol, and Ca^{2+} uptake was measured at the indicated dodecylamine concentration as described in *A*.

downshift being mediated by the same transport system that mediates hypotonic downshift-induced Ca^{2+} influx.

Effect of Divalent Cations on Hypotonic Downshift-induced Ca^{2+} Accumulation

Numerous divalent cations have been shown to inhibit Ca^{2+} accumulation by *Saccharomyces cerevisiae* (Borst-Pauwels, 1981). The effect of these divalent cations on Ca^{2+} accumulation by cells maintained at constant osmolality was compared to their effect on hypotonic downshift-induced Ca^{2+} accumulation. All divalent cations tested (Cd^{2+}, Zn^{2+}, Mg^{2+}, Sr^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Ba^{2+}) decreased Ca^{2+} accumulation by cells under isotonic conditions. The effect of divalent cations on hypotonic downshift-induced Ca^{2+} accumulation is more complex (Fig. 6). Sr^{2+} and Ba^{2+} appear to affect both hypotonic downshift-induced Ca^{2+} influx and Ca^{2+} influx in the absence of osmotic change similarly. By contrast Mg^{2+}, Ni^{2+}, and Mn^{2+} have little influence on the hypotonic downshift-induced accumulation.

Surprisingly, Cd^{2+} and Zn^{2+} increase hypotonic downshift-induced Ca^{2+} accumulation 6- and 3-fold respectively while decreasing Ca^{2+} accumulation in isotonic conditions (Fig. 6). The enhancement of Ca^{2+} accumulation could be due to inhibition of Ca^{2+} efflux across the plasma membrane or to increased Ca^{2+} influx. Cd^{2+} influx induced by a hypotonic downshift in the presence of 1-mM Cd^{2+} transiently (about 6 min.) increases Ca^{2+} accumulation by cells even after restoring high osmolarity (Fig. 7). The effect of Cd^{2+} on Ca^{2+} uptake is apparently reversible, and the rate at which normal Ca^{2+} accumulation is restored after the hyper-

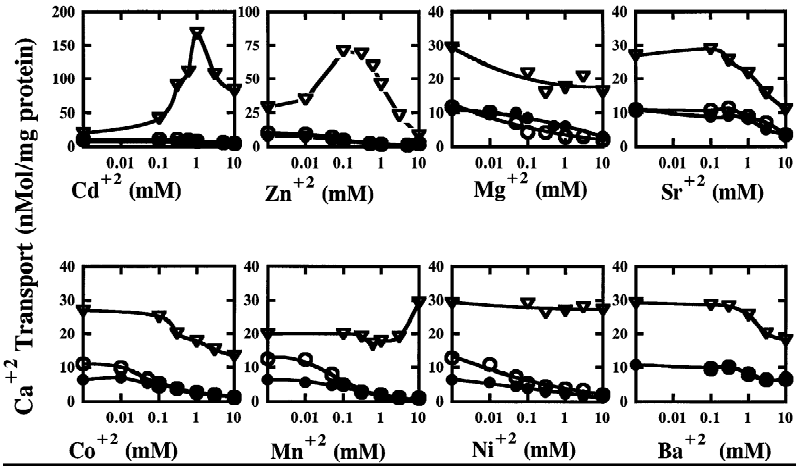

Fig. 6. Effect of divalent cations on Ca^{2+} accumulation. Cells were grown in YPD with (○,▽) or without (●) 0.8 M sorbitol at 37°C to an OD_{600nm} of about 1. Cells were washed and resuspended in KCl solution containing 2% glucose with (○,▽) or without (●) 0.8 M sorbitol to an OD_{600nm} of 50. To measure Ca^{2+} uptake, cells were diluted 50-fold into KCl solution with (○) or without (●,▽) 0.8 M sorbitol containing 1 mM Ca^{2+} + $^{45}Ca^{2+}$ (1 μCi/ml) and the indicated concentration of divalent cation. After 5 min, cell-associated Ca^{2+} was measured as described in Materials and Methods.

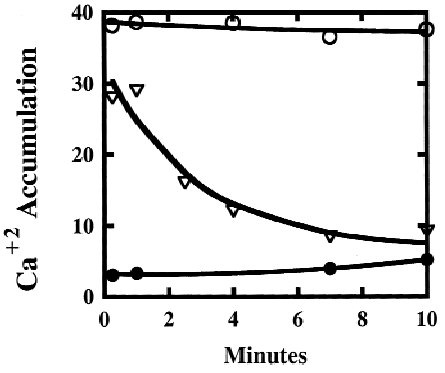

Fig. 7. Increased Ca^{2+} accumulation caused by Cd^{2+} influx during osmotic downshift. Cells were grown in YPD + 0.8 M sorbitol at 37°C, washed and concentrated in KCl solution to 50 OD_{600nm}/ml. Cells were diluted 50-fold into KCl solution + 2% glucose containing either 10 mM $CdCl_2$ (○), 10 mM $CdCl_2$, 0.8 M sorbitol (●), or 1 mM $CdCl_2$ (▽). After a 5-min incubation, the cells were centrifuged in a microfuge and resuspended in 0.1 M KCl, 0.8 M sorbitol, 0.2 M glucose, 10 mM HEPES (pH 7.0). At the indicated time, 1 mM $CaCl_2$ + 1 μCi/ml $^{45}Ca^{2+}$ was added, the samples were incubated for 1 min and cell-associated Ca^{2+} was determined (Materials and Methods).

tonic downshift likely reflects the rate of Cd^{2+} sequestration in the cell.

Discussion

S. cerevisiae cells exhibit normal growth over a wide range of osmolarity by regulating the intracellular osmolarity, transporters in the plasma membrane, and cell wall synthesis (Klis, 1994; Thevelein, 1994). Cells tolerate rapid changes in the extracellular osmolarity. Shifting cells from high to low osmotic medium is expected to increase intracellular pressure due to the influx of water into the cell. Cells are likely to have mechanisms to monitor changes in the membrane tension caused by alterations of extracellular osmolarity. In this study, we characterized the effect of rapid osmotic downshift on the influx of Ca^{2+} and other divalent cations into cells.

Cells growing in isotonic medium may also undergo increases in membrane tension similar to those generated by a hypotonic downshift. In general, *S. cerevisiae* cells have a higher concentration of solutes inside than outside the cell. As a consequence water flows into the cell by osmosis increasing internal pressure. The cell wall prevents swelling and lysis of the cells by this high intracellular pressure. During cell growth, new cell wall and plasma membrane is deposited in specific growth regions (the bud tip in *S. cerevisiae*). Expansion of the cell wall must be regulated to allow increase in cell size without cell lysis. The effect of hypotonic downshift on membrane tension may mimic the localized effects of increased membrane tension caused by cell wall expansion and remodeling. Therefore, the divalent cation influx induced by hypotonic downshift may reflect what is occuring locally at the bud tip.

Hypotonic downshift increases Ca^{2+} accumulation 6.5-fold. This increased permeability is not specific for Ca^{2+}; hypotonic downshift-induced Sr^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+} influx is also observed. The mechanism of increased divalent cation influx is not known. One possibility is that a mechanosensitive channel is activated by increased membrane tension. Indeed patch clamp studies do indicate that the plasma membrane contains a mechanosensitive channel with low ion specificity (Gustin et al., 1988; Saimi et al., 1988; Gustin, 1991). Another possibility is that increased membrane tension induces transient leaks in the plasma membrane. These leaks would be rapidly repaired since no cell death was observed following a hypotonic downshift and within 5 min after the hypotonic downshift, the Ca^{2+} accumulation rate returns to normal levels.

Dodecylamine inhibits hypotonic-downshift induced

Ca^{2+} influx. If Ca^{2+} influx is mediated by a specific protein or protein complex, then dodecylamine may bind to this protein to block Ca^{2+} influx. Alternatively, dodecylamine may inhibit divalent influx into the cell by binding to the plasma membrane and changing its surface charge or other physical properties. Dodecylamine does not inhibit Ca^{2+} accumulation by the vacuole (the main intracellular Ca^{2+} storage site) indicating that it acts on the plasma membrane to block cellular Ca^{2+} accumulation.

Dodecylamine (25 μM) blocks 75% of the hypotonic downshift-induced Ca^{2+} influx but only 25% of the Ca^{2+} influx into cells growing in YPD. While 10 mM Mg^{2+} is a poor inhibitor of hypotonic downshift-induced Ca^{2+} accumulation, it blocks 90% of Ca^{2+} accumulation by exponentially growing cells. These observations suggest that about 10–35% of the Ca^{2+} influx into cells growing in YPD may be mediated by the same mechanism that mediates hypotonic downshift-induced Ca^{2+} influx. This Ca^{2+} influx may be spatially and temporally localized, for example concentrated at sites of active cell growth. In the apical growth of fungal hyphae, both proton and Ca^{2+} gradients may be established from the growth tip along the hyphal axis (Harold, 1994). These gradients may be generated by Ca^{2+} and proton influx induced by increases in membrane tension at the hyphal apex.

Identification of proteins that mediate hypotonic downshift-induced Ca^{2+} influx would be facilitated by the identification of genes required for hypotonic-induced cation influx. The 10-fold increase in Cd^{2+} toxicity observed during hypotonic downshift suggests a possible protocol for the enrichment of mutants defective in hypotonic downshift-induced cation fluxes. Mutant cells that are more resistant to an acute exposure to high Cd^{2+} during a hypotonic downshift might identify proteins that mediate Cd^{2+} influx.

The physiological function of increased ion flux during hypotonic downshift or cell growth is not known. High or low Ca^{2+} concentrations do not appear to affect the ability of cells to survive a hypotonic downshift. Rapid ion fluxes may limit membrane damage induced by membrane tension and expansion by decreasing the osmotic gradient across the plasma membrane. By increasing ion permeability, the efflux of ions from the cell could rapidly reduce intracellular osmolarity.

Ca^{2+} influx initiates signalling pathways that activate cell wall synthesis and ion transport systems. Ca^{2+} induces the expression of P-type ATPases that are required to prevent accumulation of toxic levels of Mn^{2+}, Ca^{2+} and Na^{+} (Haro, Garciadeblas & Rodrigues-Navarro 1992; Cunningham & Fink, 1994, 1996; Lapinskas, et al., 1995). Ca^{2+} activates K^{+} channels in the plasma membrane which could act to repolarize the plasma membrane (Bertl, Gradmann & Slayman, 1992). Ca^{2+} also induces expression of *FKS2* which encodes a 1,3-β glucan synthetase which catalyzes synthesis of the main structural component of the cell wall (Mazur et al., 1995). Ca^{2+} might therefore be regarded as a major SOS signal for the cell to respond the consequences of increased membrane tension.

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