Ion Tolerance of Saccharomyces cerevisiae Lacking the Ca\(^{2+}\)/CaM-Dependent Phosphatase (Calcineurin) Is Improved by Mutations in URE2 or PMA1

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ABSTRACT
Calcineurin is a conserved, Ca\(^{2+}\)/CaM-stimulated protein phosphatase required for Ca\(^{2+}\)-dependent signaling in many cell types. In yeast, calcineurin is essential for growth in high concentrations of Na\(^+\), Li\(^+\), Mn\(^{2+}\), and OH\(^-\), and for maintaining viability during prolonged treatment with mating pheromone. In contrast, the growth of calcineurin-mutant yeast is better than that of wild-type cells in the presence of high concentrations of Ca\(^{2+}\). We identified mutations that suppress multiple growth defects of calcineurin-deficient yeast (cnb1\(\Delta\) or cna1\(\Delta\) cna2\(\Delta\)). Mutations in URE2 suppress the sensitivity of calcineurin mutants to Na\(^+\), Li\(^+\), and Mn\(^{2+}\), and increase their survival during treatment with mating pheromone. ure2 mutations require both the transcription factor Glnc3p and the Na\(^+\) ATPase Pmr2p to confer Na\(^+\) and Li\(^+\) tolerance. Mutations in PMA1, which encodes the yeast plasma membrane H\(^{+}\)-ATPase, also suppress many growth defects of calcineurin mutants. pma1 mutants display growth phenotypes that are opposite to those of calcineurin mutants; they are resistant to Na\(^+\), Li\(^+\), and Mn\(^{2+}\), and sensitive to Ca\(^{2+}\). We also show that calcineurin mutants are sensitive to aminoglycoside antibiotics such as hygromycin B while pma1 mutants are more resistant than wild type. Furthermore, pma1 and calcineurin mutations have antagonistic effects on intracellular [Na\(^+\)] and [Ca\(^{2+}\)]. Finally, we show that yeast expressing a constitutively active allele of calcineurin display pma1-like phenotypes, and that membranes from these yeast have decreased levels of Pma1p activity. These studies further characterize the roles that URE2 and PMA1 play in regulating intracellular ion homeostasis.

The maintenance of ion gradients across lipid bilayers is a fundamental property of cells. Proper regulation of the proteins that mediate ion flux is necessary for cells to keep toxic ion concentrations low and to accumulate essential ions. In addition, ion gradients are used to store free energy in the form of an electrochemical potential (as in the case of the mitochondrial inner membrane) and to initiate signaling cascades (e.g., calcium spiking and transient membrane depolarizations; Tsien and Tsien 1991). Thus, knowledge of how cells regulate ion transport is critical for understanding the many cellular processes that depend on ion gradients. One difficulty with the study of ion homeostasis in vivo is that different ion transporters located in distinct intracellular locations are coordinately regulated. Genetic approaches available in the yeast Saccharomyces cerevisiae, however, make it an excellent system to identify ion transporters and their regulators. Although many transporters have been identified in S. cerevisiae, much less is known about the proteins that modulate transporter activity in yeast.

The yeast gene PMA1 encodes a plasma membrane H\(^{+}\)-ATPase that is conserved among plants and fungi and belongs to the same class of P-type ATPases as mammalian [Na\(^+\), K\(^+\)] and Ca\(^{2+}\)-ATPases. PMA1 is an essential gene whose product is required not only for cytosolic pH homeostasis, but also for maintaining the electrochemical potential at the plasma membrane (Goffeau and Slanyman 1981; Perlin et al. 1988; Serrano 1984). Yeast carrying mutations that reduce Pma1p activity are sensitive to low pH, resistant to the aminoglycoside antibiotic hygromycin B, and grow more slowly than wild type (McCusker et al. 1987). In addition, pma1 mutantsshown reduced uptake of many nutrients (Vallejo and Serrano 1989). Thus, Pma1p is important for pH homeostasis and for the transport of multiple nutrients/ions across the plasma membrane.
Pma1p activity increases severalfold in the presence of either glucose or acidic media (Eraso and Gancedo 1987; Serrano 1983). In response to glucose, transcription of PMA1 is induced and Pma1p level rises (Rao et al. 1993). At the same time, Pma1p also becomes hyperphosphorylated. This increase in phosphorylation of Pma1p correlates with a rise in its specific activity, while removal of glucose results in dephosphorylation and a decrease in activity (Chang and Slanyman 1991). Currently, no protein kinase or phosphatase has been shown to regulate Pma1p activity in vitro; however, incubation of Pma1p with acid phosphatase in vitro results in...
decreased activity (Kolarov et al. 1988). Thus, glucose modulates Pma1p activity by altering both its abundance and phosphorylation state.

The protein phosphatase calcineurin plays an important role in regulating ion homeostasis in the yeast S. cerevisiae. Calcineurin is a Ser/Thr protein phosphatase that is stimulated by Ca^{2+} and calmodulin, and it is highly conserved among mammals and yeast (Cyr et al. 1993; Cyr et al. 1992; Kroger et al. 1988; Kuro et al. 1991; Liu et al. 1991b). The immunosuppressant drugs FK506 and cyclosporin A are specific inhibitors of calcineurin and have been used to demonstrate its role in a variety of processes in several cell types (Liu 1993). Calcineurin is required for T-cell activation, neutrophil migration, and transcriptional induction of the glucagon gene in pancreatic islet cells (Lawson and Maxfield 1995; Liu et al. 1991a; Schwaninger et al. 1993). Calcineurin also affects a variety of ion fluxes in both plant and animal cells. Previous studies suggest that calcineurin may inhibit an inward-rectifying K^{+} current in plant guard cells and a weakly selective Ca^{2+} current in the plant tonoplast (Allen and Sanders 1995; Luan et al. 1993). In mammalian cells, calcineurin has been shown to shorten the duration of N-methyl-d-aspartate (NMDA) channel openings and to associate with the IP_{3} receptor to regulate Ca^{2+} flux through this channel (Cameron et al. 1995; Lieberman and Moody 1994).

While complete removal of calcineurin from yeast is not lethal, calcineurin mutants do exhibit altered growth in the presence of several ions. The growth of calcineurin-deficient yeast is inhibited by lower concentrations of Na^{+}, Li^{+}, and Mn^{2+} than that of wild-type cells (Farcasanu et al. 1995; Nakamura et al. 1993; Pozos et al. 1996). In contrast, yeast that lack calcineurin grow better in the presence of high concentrations of Ca^{2+} than wild type (Cunningham and Fink 1994; Tanida et al. 1995; Withee et al. 1997). One mechanism by which calcineurin modulates yeast ion tolerance is by regulating the abundance of P-type ATPases through activation of the transcription factor CRZ1/TCN1 (Matheos et al. 1997; Statopoulos and Cyr et al. 1997). Thus, the sensitivity of calcineurin-mutant yeast to Na^{+} and Li^{+} is caused in part by reduced expression of PM R2/ENA1, a gene encoding a plasma membrane Na^{+}-ATPase (Ferrando et al. 1995; Mendoza et al. 1994). Similarly, the low tolerance of calcineurin-deficient yeast to Mn^{2+} is caused, at least in part, by reduced transcription of PM R1, a Golgi-localized P-type ATPase (Antebi and Fink 1992; Cunningham and Fink 1994, 1996). The effect of calcineurin on Ca^{2+} homeostasis is complex and probably involves regulation of multiple targets. Although it is required for Ca^{2+}-dependent induction of PM C1, a gene that encodes a vacuolar Ca^{2+}-ATPase, the net effect of calcineurin is to decrease intracellular Ca^{2+} content (Cunningham and Fink 1994, 1996; Tanida et al. 1995; Withee et al. 1997). Thus, calcineurin regulates sequestration of Ca^{2+} into one or more intracellular compartments. Calcineurin is also required for viability when cells are exposed to yeast mating pheromone (Cyr et al. 1992; Withee et al. 1997; Moser et al. 1996).

We selected for mutations that allow growth of calcineurin-mutant strains on media containing a high concentration of NaCl. We identified recessive mutations in two complementation groups that, in addition to suppressing Na^{+} sensitivity, also suppress several other calcineurin-mutant phenotypes. One of these complementation groups is composed of alleles of URE2, a previously identified gene with homology to glutathione S-transferases (Coschigano and Magasanik 1991). Mutations in URE2 suppress the sensitivity of calcineurin-mutant yeast to Na^{+}, Li^{+}, and Mn^{2+}, and they increase their viability during exposure to mating pheromone. ure2 mutations require both the transcription factor Gln3p and the Na^{+} ATPase Pmr2p to confer Na^{+} and Li^{+} tolerance. The other complementation group is composed of alleles of PMA1, the plasma membrane H^{+}-ATPase. We report that mutations in PMA1 suppress multiple phenotypes of calcineurin-deficient yeast, including the loss of viability during incubation with mating pheromone. Furthermore, we show that mutations in PMA1 and calcineurin have opposite effects on the growth of yeast in the presence of particular cations. Specifically, pma1 mutants are resistant to Na^{+}, Li^{+}, Mn^{2+}, and aminoglycosides, but are sensitive to Ca^{2+}, while calcineurin mutants are sensitive to Na^{+}, Li^{+}, Mn^{2+}, and aminoglycosides, but are resistant to Ca^{2+}. We also report that pma1 and calcineurin mutations have opposite effects on the intracellular levels of Na^{+} and Ca^{2+}. Finally, we show that yeast expressing an activated allele of calcineurin exhibit reduced Pma1p activity.

**Materials and Methods**

**Yeast strains and media:** The yeast strains used in this study are listed in Table 1. Yeast were grown in either YPD (1% yeast extract, 2% Bacto-peptone, and 2% dextrose) or synthetic medium supplemented with twice the recommended amount of amino acids (Sherman et al. 1986). Where noted, NaCl was added at the indicated concentration. Cd^{2+}-containing medium was composed of 30 μM CdCl_{2} in synthetic medium. High Ca^{2+} medium was buffered to pH 5.5 with 50 mm succinate (to prevent precipitation), and CaCl_{2} was added to the indicated concentration. Low-pH medium was buffered with 50 mM succinate, and the pH was adjusted with either HCl or KOH.

**Isolation of suppressors of calcineurin-mutant yeast:** Independent cultures of MCY300-1 and DD12 (10 of each) were grown at 30°C to late log phase, and 2 x 10^{6} cells from each culture were incubated for 10 days at room temperature on YPD plates containing 1.2 mM NaCl. This concentration of NaCl completely inhibits the growth of calcineurin-deficient yeast. Na^{+}-tolerant mutants arose at a frequency of 5 x 10^{-6}. A total of 30 Na^{+}-tolerant mutants were further characterized.

**Phenotypic characterization:** The Na^{+}-tolerant calcineurin
TABLE 1
Strains used in this study

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<th>Strain</th>
<th>Genotype</th>
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<td>YPH 499</td>
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<td>Sikorski and Heiter (1989)</td>
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<td>DD12</td>
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mutants were divided into two distinct phenotypic classes. The first class, composed of 9 isolates, is resistant to Na\textsuperscript{+}, Li\textsuperscript{+}, and Mn\textsuperscript{2+}, and exhibits improved viability during incubation with mating pheromone. In addition, the growth of these mutants is extremely sensitive to Cd\textsuperscript{2+}. The second class, composed of 21 isolates, is resistant to Na\textsuperscript{+}, Li\textsuperscript{+}, and Mn\textsuperscript{2+}, shows improved viability in the presence of mating pheromone, and displays decreased Ca\textsuperscript{2+} tolerance. The growth of this second class is inhibited on synthetic media. This observation led us to investigate the growth of these mutants on low-pH media. We found that the Na\textsuperscript{+}-tolerant mutants from this second class are unable to grow on YPD buffered to pH 3.5 or below.

**Genetic analysis:** One isolate from class I and three from class II were characterized further. All four isolates were mated to the parental strain of opposite mating type (MCY100-2A), and the phenotypes of the resulting diploids were determined. We found that Cd\textsuperscript{2+} sensitivity of the class I isolate and low pH and Ca\textsuperscript{2+} sensitivity of the class II isolates are recessive. These diploids were sporulated, and the haploid segregants were assayed for resistance to Na\textsuperscript{+} and sensitivity to either Cd\textsuperscript{2+} or low pH. We found that Na\textsuperscript{+} resistance always segregated with sensitivity to either Cd\textsuperscript{2+} (class I) or low pH (class II), indicating that these mutants carry a single, recessive mutation that is responsible for the observed phenotypes. Next, the four isolates were mated in all possible pairwise combinations, and the resulting diploids were assayed for sensitivity to Cd\textsuperscript{2+}, low pH, and Ca\textsuperscript{2+}. On the basis of this analysis, the phenotypic classes were assigned to two complementation groups designated SCN1 and SCN2. These diploids were then sporulated, and the haploid segregants were assayed for Cd\textsuperscript{2+}, low pH, and Ca\textsuperscript{2+} sensitivity. Although scn1, scn2 haploids were recovered, the three scn2 mutations never demonstrated any...
recombination with each other in more than 40 tetrads analyzed. Therefore, linkage analysis confirmed the presence of two distinct complementation groups.

**Assays for ion tolerance and viability in mating pheromone:** Assays for ion tolerance were performed by three different methods: (1) Spot dilution tests were done by diluting saturated liquid cultures to 10^4 cells/ml and then spotting four serial 10-fold dilutions of each strain on the indicated medium. (2) Halo assays were performed by placing a sterile disk containing the indicated ion onto a lawn of yeast (2 × 10^6 cells) and incubating at 30°C for 3 days. (3) Liquid growth assays (shown in Figures 2, 3, and 7) were performed by diluting log-phase cultures to 4 × 10^4 cells/ml with YPD containing the indicated concentration of NaCl, CaCl_2, or buffered to the indicated pH using 50 mM succinate. Cultures were then incubated at 30°C with shaking for 12 hr, and they remained in log phase (i.e., <2 × 10^5 cells/ml) throughout the course of the experiment. Cell concentration was determined by measuring optical density at 600 nm (OD_600). For each culture, the percentage of growth represents the number of cell doublings achieved divided by the number of doublings achieved by an equivalent control culture incubated in (normal) YPD medium.

Viability in mating pheromone was determined by vital staining as described in Idia et al. (1990). Early log-phase cultures (10^6 cells/ml) in low pH media were treated with 10 µg/ml alpha factor. After a 5-hr incubation with alpha factor, cells were diluted 1:1 with a mixture of 0.2% methylene blue and 4% sodium citrate. The percentage of viable cells was determined immediately by bright-field microscopy.

**Disruption of URE2 and PMR2/ENA1:** A fragment for disruption of URE2 with the LEU2 gene was created as follows. A 4.0-kb Sall fragment containing URE2 was cut with Apal and NotI to remove 300 bases from the URE2 open reading frame. A three-way ligation was then performed with the pRS305 vector (containing the LEU2 gene), which was also digested with Apal and NotI. The resulting plasmid was then digested with Sall to linearize before transforming into the appropriate strains for disruption of URE2. URE2 disruptions (ure2Δ::LEU2) were verified in each strain by Southern analysis. The fragment for replacement of PMR2 with the LEU2 gene was a gift from Hans Rudolph (Wieland et al. 1995). PMR2 disruptions (pmr2Δ::LEU2) were verified in each strain by Southern analysis. Southern hybridizations were performed according to established protocols (Ausubel et al. 1987).

**Determination of intracellular Na^+:** Intracellular Na^+ was determined essentially as described previously (Ferrando et al. 1995). Cells were grown for at least 10 generations in YPD containing 0.7 mM NaCl (OD_600 final = 1), washed twice in 1.5 M cold sorbitol containing 20 mM MgCl_2, and collected by filtration through glass fiber filters (980-AH; Whatman, Clifton, NJ). Extracts were prepared by incubating cells in 20 mM MgCl_2 at 95°C for 30 min. Sodium concentrations in the clarified extracts were determined by atomic absorption spectroscopy (model 303; Perkin Elmer, Norwalk, CT).

**Determination of intracellular Ca^{2+}:** aCaCl_2 (Amersham, Arlington Heights, IL) was added to YPD to a specific activity of 1.2 × 10^6 cpn/µmol Ca^{2+} (assuming the [Ca^{2+}] of YPD is 300 µM (Dunn et al. 1994)). Radioactive YPD was then inoculated to an OD_600 of 0.05 with log-phase cultures, incubated at 30°C with agitation, and sampled at 6, 6.5, and 7 hr to determine Ca^{2+} content. The cellular [Ca^{2+}] was equivalent at all three time points, indicating that labeling had reached a steady-state level. At each time point, triplicate samples of 100 µl were collected on glass fiber filters (Whatman 934-AH). Samples were washed twice with 10 ml ice-cold buffer (100 mM Tris, pH 6.5, with 50 mM CaCl_2), allowed to dry, and the amount of aCa^{2+} in each sample was determined by liquid scintillation counting. Background radioactivity was assessed by addition of radioactive YPD to 100-µl cell aliquots, followed immediately by filtration and processing as described above. The total number of counts was normalized to cell number, and the background was subtracted to determine intracellular [Ca^{2+}].

**Membrane preparation, ATPase assays, and determination of Pma1p levels:** Total membrane fractions were prepared essentially as described (Chang and Slaiman 1991). Log-phase cells were pelleted, washed, and resuspended at 200 OD_600/ml in ice-cold lysis buffer (10 mM Tris, pH 7.4, 0.3 mM sorbitol, 0.1 mM NaCl, 5 mM MgCl_2, and protease inhibitors). Cells were then lysed at 4°C using glass beads (Ausubel et al. 1987). The lysate was centrifuged at 400 g for 5 min to remove unbroken cells. The total membrane fraction was then pelleted by centrifugation for 1 hr at 100,000 g at 4°C. The membrane pellet was washed twice and resuspended in 500 µl cold storage buffer (0.1 mM EDTA, 0.1 mM DTT, 10 mM Tris, pH 7.4, and 20% glycerol).

Studies from many groups have established that VO_2-sensitive ATPase activity is an accurate indicator of Pma1p activity. We determined VO_2-sensitive ATPase activity essentially as described previously (Serrano 1983). The membrane fraction to be assayed was added to 500 µl reaction buffer (50 mM MES, pH 6.5, 10 mM MgCl_2, 5 mM phosphoenolpyruvate, 5 mM NaATP, 5 mM NaN_3, and 18 µg/ml pyruvate kinase). The reaction was incubated at 30°C in the presence or absence of 100 µM NaVO_3. The reaction was stopped after 15 min by addition of a combination stop/color solution (0.34% ammonium molybdate, 0.86% H_2SO_4, 0.32% sodium dodecyl sulfate, and 1.36% ascorbic acid). After 45 min at room temperature, the OD_520 was determined for each sample. This value was converted to nanomoles of free phosphate liberated per minute by use of phosphate standards and subtraction of background (reaction buffer incubated at 30°C in the absence of membrane). Each sample was assayed in triplicate and normalized to total membrane protein.

Pma1p levels were determined in the same membrane fractions assayed above. Membrane fractions were separated on 8% SDS polyacrylamide gels and immunoblotted according to established protocols (Ausubel et al. 1987). We used an affinity-purified antibody specific for Pma1p that was generously provided by Carolyn Slaiman. The signal was visualized using enhanced chemiluminesence (ECL) and a phosphorimaging system and normalized to total protein loaded.

**RESULTS**

Identification of mutations that confer Na^+ tolerance upon calcineurin-deficient strains: We selected for mutations that permit the growth of calcineurin-mutant strains in the presence of 1.2 M NaCl (Figures 1 and 4A). Complementation and linkage analysis defined recessive mutations in two complementation groups named SCN1 and SCN2 for suppressor of calcineurin (see materials and methods). scn1 and SCN2 mutations were then assayed for suppression of other calcineurin-mutant phenotypes.

Characterization of scn1 mutants: scn1 mutations improve the growth of calcineurin-deficient yeast in the presence of Na^+, Li^+, or Mn^2+, and they increase the survival of calcineurin mutants during incubation with...
mating pheromone (Table 2, Figure 1). (Note that scn1 is identical to ure2-41, as shown below.) scn1 mutations do not alter either the high pH sensitivity or the Ca\(^{2+}\) resistance of calcineurin-deficient strains. In media containing high concentrations of Na\(^{+}\), Li\(^{+}\), or Mn\(^{2+}\), the growth of scn1 mutants is better than that of scn1 strains that lack calcineurin (cna\(\Delta\) cna\(\Delta\) scn1; Figure 1). Thus, the effect of calcineurin on cation tolerance is not abolished by scn1 mutations.

Although calcineurin is required for tolerance to Na\(^{+}\), Li\(^{+}\), and Mn\(^{2+}\), it does not affect resistance to all cations. For example, calcineurin-mutant yeast are not more sensitive than wild-type cells to Zn\(^{2+}\) or Cu\(^{2+}\). Similarly, scn1 mutations fail to alter the growth of yeast under these conditions (determined by halo assay, data not shown). Thus, scn1 mutations confer tolerance to the same subset of cations to which calcineurin mutants are sensitive. Finally, during our characterization of scn1 mutants, we observed that they displayed sensitivity to Cd\(^{2+}\) (Figure 1C). Although the growth of wild-type and calcineurin-mutant yeast is unaffected by the addition of 100 \(\mu\)M CdCl\(_2\), scn1 cells are unable to grow at concentrations \(>20\ \mu\)M (data not shown).

**SCN1 is the previously identified gene URE2:** We exploited the Cd\(^{2+}\) sensitivity of scn1 mutants to clone the SCN1 gene. scn1 calcineurin-mutant yeast (JY4-1) were transformed with a genomic library and plated on media containing 30 \(\mu\)M CdCl\(_2\). As stated above, the growth of scn1 mutants is completely inhibited by this concentration of Cd\(^{2+}\). A single plasmid that permits the growth of scn1 mutants on media containing Cd\(^{2+}\) was isolated, and the smallest fragment capable of conferring Cd\(^{2+}\) tolerance to scn1 mutants was determined. DNA sequencing of this fragment identified the previously characterized gene URE2 (Coschigano and Magasanik 1991). We constructed a deletion allele of URE2 (ure2\(\Delta\)::LEU2) and found that strains carrying this mutation exhibit the same phenotypes as scn1 mutants. ure2\(\Delta\)::LEU2 mutations confer sensitivity to Cd\(^{2+}\) and suppress the sensitivity of calcineurin-mutant yeast to Na\(^{+}\), Li\(^{+}\), and Mn\(^{2+}\); ure2\(\Delta\)::LEU2 yeast also fail to complement an scn1 mutant strain for any of the above phenotypes. Furthermore, dissection of 20 tetrads from a diploid heterozygous for scn1 and ure2\(\Delta\)::LEU2 yielded four Cd\(^{2+}\)-sensitive spores in every tetrad. SCN1, therefore, is the previously identified gene URE2. We have designated the scn1 mutation identified in this study as ure2-41. As shown above for ure2-41 mutants (Figure 1), ure2\(\Delta\)::LEU2 strains are more resistant to Na\(^{+}\), Li\(^{+}\), and Mn\(^{2+}\) than ure2\(\Delta\)::LEU2 strains that also lack calcineurin (data not shown). Thus, calcineurin modulates cation tolerance in yeast, even in the complete absence of the URE2 gene product.

**ure2 mutations require Pmr2p and the transcription factor Gln3p to confer tolerance to Na\(^{+}\):** The transcript level of PMR2/ENA1, which encodes a plasma membrane Na\(^{+}\)-ATPase, is induced severalfold in the presence of Na\(^{+}\) (Garcia-deblas et al. 1993; Haro et al. 1991; Wiel and 1995). Calcineurin and the transcription factor Crz1p/Tcn1p are required for full induction of Ca\(^{2+}\)-sensitive spores in every tetrad. SCN1, therefore, is the previously identified gene URE2. We have designated the scn1 mutation identified in this study as ure2-41. As shown above for ure2-41 mutants (Figure 1), ure2\(\Delta\)::LEU2 strains are more resistant to Na\(^{+}\), Li\(^{+}\), and Mn\(^{2+}\) than ure2\(\Delta\)::LEU2 strains that also lack calcineurin (data not shown). Thus, calcineurin modulates cation tolerance in yeast, even in the complete absence of the URE2 gene product.

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of PMR2/ENA1 mRNA in response to Na⁺, and yeast that lack calcineurin are sensitive to Na⁺ partly because of lower levels of the Na⁺-ATPase (Matheos et al. 1997; Mendoza et al. 1994; Stathopoulos and Cyert 1997). To investigate whether ure2 mutations require PMR2/ENA1 to confer Na⁺ tolerance, we examined the contribution of URE2 to Na⁺ tolerance in yeast that completely lack PMR2/ENA1 (see materials and methods). The growth of yeast that lack PMR2/ENA1 (pmr2Δ::LEU2) was reduced 65% in the presence of 300 mm NaCl (Figure 2A). The growth of yeast deficient for both PMR2/ENA1 and URE2 (pmr2Δ::LEU2 ure2-41) is also reduced ~65% in 300 mm NaCl (Figure 2A). Similarly, we observed the same reduction (72% in 300 mm NaCl) in yeast that lack both calcineurin and PMR2/ENA1 (cna1Δ cna2Δ pmr2Δ::LEU2), as well as in yeast that are deficient for calcineurin, URE2, and PMR2/ENA1 (cna1Δ cna2Δ ure2-41pmr2Δ::LEU2; Figure 2B). In the absence of PMR2/ENA1, therefore, ure2 mutations fail to confer Na⁺ tolerance to either calcineurin-mutant or wild-type yeast. These results indicate that URE2 may modulate Na⁺ resistance through regulation of PMR2/ENA1.

Previous studies demonstrated that mutations in URE2 lead to activation of the transcription factor Gln3p (Minehart and Magasanik 1991). We examined the effect of gln3 mutations on growth in media containing high concentrations of Na⁺. In media containing 800 mm NaCl, the growth of ure2Δ yeast was reduced ~10% relative to growth in media without added Na⁺, while the growth of both wild-type and ure2Δ gln3Δ double-mutant yeast was reduced by >50% (Figure 3). In addition, gln3Δ mutants and ure2Δgln3Δ double mutants showed a small increase in sensitivity to Na⁺ at the lower concentrations (Figure 3). Thus, GLN3 contributes to Na⁺ resistance, and ure2 mutations require GLN3 to confer increased tolerance to Na⁺. We also observed that the growth of ure2 mutants is improved by low concentrations of Na⁺ (Figure 3). This growth improvement is likely to be caused by an increase in osmotic strength, as it was also observed in media containing K⁺ or sorbitol. This effect is independent of the increased tolerance to high concentrations of Na⁺ observed for ure2 mutants (Figure 1). We also determined that ure2 mutants require GLN3 to exhibit other phenotypes we
observed, including resistance to Li\(^+\) and Mn\(^{2+}\) and sensitivity to Cd\(^{2+}\) (data not shown).

We investigated whether ure2 mutations compensate for a lack of calcineurin by increasing PMA1 transcript level. After treatment of wild-type yeast with 0.8 m NaCl for 30 min, the PMA1 transcript level increased ~20-fold. As previously reported, calcineurin-mutant yeast (cna1\(\Delta\) cna2\(\Delta\)) treated with the same concentration of Na\(^+\) displayed ~50% of the PM A1 transcript level seen in wild-type cells (Mendoza et al. 1994). ure2 mutants, however, exhibited no significant difference from wild type in PM A1 levels in either the presence or absence of Na\(^+\) (data not shown).

Characterization of scn2 mutants: scn2 mutations increase the resistance of calcineurin-deficient yeast to Na\(^+\), Li\(^+\), and Mn\(^{2+}\), and they improve the survival of calcineurin mutants in the presence of mating pheromone (Table 2, Figure 4). (Note that scn2 is identical to pma1-21, as shown below.) In addition, scn2 mutations reduce the enhanced growth of calcineurin mutants in the presence of Ca\(^{2+}\) (Figure 5C). scn2 mutants are more resistant to Na\(^+\), Li\(^+\), and Mn\(^{2+}\), and they are more sensitive to Ca\(^{2+}\) than scn2 mutants that also lack calcineurin (Figures 4 and 5C). Thus, calcineurin still modulates ion tolerance in the presence of scn2 mutations. scn2 mutations do not, however, suppress the high pH sensitivity of calcineurin-mutant yeast. As shown above, ure2, SCN2 does not have a significant effect on the growth of yeast in the presence of Zn\(^{2+}\) or Cu\(^{2+}\) (established using halo assays, data not shown).

**SCN2 is the previously characterized gene PMA1:** During our characterization of scn2 yeast, we observed that their growth was impaired on low pH media (see materials and methods and Figure 5B). This low pH sensitivity was used to clone the SCN2 gene. scn2 calcineurin-mutant yeast (JY10-2) were transformed with a genomic library and plated onto medium buffered to pH 3.5 (see materials and methods). The growth of scn2 mutants is completely inhibited at this pH. A single plasmid that allows JY10-2 to grow on low pH media was identified, and the smallest fragment from this plasmid that promotes growth of scn2 yeast on low pH media was determined. Hybridization of this fragment to an ordered collection of lambda phage representing the entire yeast genome localized it to an area on chromosome VII containing the PMA1 gene (Link and Olson 1991). scn2 mutations fail to complement the low pH sensitivity of previously characterized pma1 alleles (pma1-141 and pma1-105; McCusker et al. 1987). In addition, sporulation and dissection of a diploid heterozygous for scn2 and pma1-105 yielded four pH-sensitive spores from each of 50 tetrads. Thus, SCN2 is PMA1, which encodes the plasma membrane H\(^+\)-ATPase of S. cerevisiae (Serrano et al. 1986). We have assigned a pma1 allele designation to each member of the scn2 complementation group. Here we show our characterization of strains containing the pma1-21 mutation.

**Resistance to Na\(^+\), Li\(^+\), and Mn\(^{2+}\) and sensitivity to Ca\(^{2+}\) are conferred by mutations that reduce PMA1 function:** PMA1 is an essential gene. However, many mutations that reduce Pma1p activity have been identified, and they are known to cause sensitivity to low pH and resistance to the aminoglycoside antibiotic hygromycin B (McCusker et al. 1987). Like previously characterized pma1 alleles, the pma1 alleles identified in this study also cause sensitivity to low pH and resistance to aminoglycosides (Figures 4A and 5B). In addition, membranes from yeast carrying the pma1 alleles described here exhibit less Pma1p activity than the corresponding fraction from wild-type cells. For example, a membrane fraction from one pma1 mutant identified in this report (JY35116B) has a Pma1p ATPase activity of 0.11 ± 0.02 nmol PO4/\(\mu\)g protein/\(\min\) compared to 0.28 ± 0.02 nmol PO4/\(\mu\)g protein/\(\min\) for membranes from wild-type yeast (see materials and methods). A similar reduction in activity is exhibited by two other pma1 mutant strains identified in this study (data not shown). Thus, pma1 mutants isolated in a selection for Na\(^+\) tolerance have reduced Pma1p activity.

We further characterized previously identified pma1 mutants to determine if they exhibit phenotypes similar to pma1 mutants identified as suppressors of calcineurin-mutant phenotypes. We found that two pma1 alleles previously identified in a selection for hygromycin resistance, including resistance to Li\(^+\) and Mn\(^{2+}\) and sensitivity to Cd\(^{2+}\) (data not shown).

![Figure 3](image-url)---ure2 mutations require GLN3 to confer increased tolerance to Na\(^+\). Log-phase cultures were diluted to 4 × 10\(^6\) cells/ml in YPD containing 200, 400, or 800 mm NaCl, and were incubated with shaking at 30° for 10 hr. Percentage of growth represents the number of cell doublings in the indicated concentration of NaCl/number of doublings in its absence. Strains shown are wild type (YPH499, ○), gln3\(\Delta\) (JY128, ●), ure2\(\Delta\) (JY40, □), and ure2\(\Delta\) gln3\(\Delta\) (JY137, ■).
TABLE 2

Effect of calcineurin and suppressor mutations on growth under different conditions

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Na⁺</th>
<th>Li⁺</th>
<th>Mn²⁺</th>
<th>Ca²⁺</th>
<th>Hyg B</th>
<th>High pH</th>
<th>Alpha factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>87</td>
</tr>
<tr>
<td>cna1Δ or cna1Δ cna2Δ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ure2-41 (scn1)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>ure2-41 (scn1) cna1Δ cna2Δ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>46</td>
</tr>
<tr>
<td>pma1-21 (scn2)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>pma1-21 (scn2) cnb1Δ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>58</td>
</tr>
</tbody>
</table>

Notes:

a Assays for ion tolerance were performed as described in materials and methods. +, growth is equivalent to wild type; ++, growth is better than wild type; --, growth is weaker than wild type in media containing the specified ion.

b Survival during treatment with alpha factor (yeast mating pheromone) was assessed as described in materials and methods. Briefly, log phase cultures were treated with 10 μg/ml alpha factor for 6 hr, and the percentage of viability was determined by vital staining. Percentages refer to the number of viable cells/total cell number. Strains were WT, YPH499; cna1Δ, DD12; cna1Δ cna2Δ, MCY300-1; ure2-41, JY11002D; ure2-41 cna1Δ cna2Δ, JY4-1; pma1-21, JY314118A; and pma1-21 cnb1Δ, JY722A.

B-resistant mutants [pma1-141 and pma1-105 (McCusker et al. 1987)] confer resistance to Na⁺, Li⁺, and Mn²⁺, as well as sensitivity to Ca²⁺ (data not shown). Furthermore, both of these pma1 alleles confer resistance to Na⁺, Li⁺, and Mn²⁺, as well as sensitivity to Ca²⁺ upon calcineurin-mutant yeast. These results demonstrate that resistance to Na⁺, Li⁺, and Mn²⁺, as well as sensitivity to Ca²⁺, are phenotypes that result from many different mutations that reduce PMA1 function.

Calcineurin mutants are sensitive to hygromycin B:

Hygromycin B is a cationic aminoglycoside that inhibits translation. It has been shown that pma1 mutants have increased resistance to cationic drugs, such as hygromycin B and geneticin, but not to other inhibitors of translation, such as cycloheximide, which do not carry a net positive charge (McCusker et al. 1987). The increased resistance of pma1 mutants to cationic drugs is caused by reduced uptake, probably because of the decreased plasma membrane potential of pma1 mutants (Perlin et al. 1988; Vallejo and Serrano 1989). We found that the growth of calcineurin mutants is inhibited relative to that of wild-type cells on media containing 50 μg/ml hygromycin B (Figure 4A). Although calcineurin-deficient yeast are also more sensitive to geneticin, another aminoglycoside, they show the same degree of resistance as wild-type cells to cycloheximide (data not shown). Furthermore, pma1 calcineurin-deficient strains (pma1 cnb1Δ) are resistant to both hygromycin B and geneticin, demonstrating that pma1 mutations suppress the sensitivity of calcineurin mutant yeast to these cationic drugs (Figure 4A). Therefore, pma1 and calcineurin mutations confer antagonistic effects upon the growth of yeast in the presence of the cationic drugs hygromycin B and geneticin.

PMA1 and calcineurin have antagonistic effects on Na⁺ levels:

Calcineurin-mutant yeast accumulate more Na⁺ than wild-type when grown in high NaCl media (Nakamura et al. 1993). As shown above, mutations that
reduce Pma1p activity compensate for the requirement of calcineurin during growth on high Na\(^+\) (Figure 4A).

To investigate the effect of pma1 on Na\(^+\) homeostasis, we determined the Na\(^+\) content of yeast grown in the presence of 0.7 m NaCl. Wild-type yeast accumulated Na\(^+\) to a concentration of 120 mm, while a calcineurin mutant (cnb1Δ) accumulated to \(\sim 210\) mm under the same conditions (Figure 6A). In contrast, pma1 mutations reduce the level of intracellular Na\(^+\) in both calcineurin-mutant and wild-type yeast. Yeast with pma1 mutations accumulated 70 mm Na\(^+\) compared to 120 mm in wild type (Figure 6A). Likewise, yeast deficient for both calcineurin and PMA1 (cnb1Δ pma1) had an internal Na\(^+\) concentration of 110 mm compared to 210 mm for yeast that lack calcineurin (cnb1Δ; Figure 6A). These results demonstrate that pma1 mutants display increased Na\(^+\) tolerance because of reduced Na\(^+\) levels. Thus, calcineurin and PMA1 have opposite effects on both Na\(^+\) tolerance and content. Previous studies demonstrated that calcineurin promotes decreased intracellular Na\(^+\) levels by inducing transcription of the plasma membrane Na\(^+\)-ATPase PMR2/ENA1 (Mendoza et al. 1994). In contrast, pma1 mutations do not confer Na\(^+\) tolerance through PMR2/ENA1. pma1 mutations still confer Na\(^+\) tolerance upon yeast, even in the absence of PMR2/ENA1 (Figure 2, C and D). In addition, pma1 mutants do not exhibit greater induction of PMR2/ENA1 mRNA than wild-type cells after treatment with 0.8 m NaCl (data not shown).

PMA1 and calcineurin also have antagonistic effects on Ca\(^{2+}\) homeostasis: Previous work demonstrated that calcineurin inhibits Ca\(^{2+}\) sequestration into one or more intracellular compartments (Tanida et al. 1995). In this report, we demonstrate that pma1 mutants are sensitive to Ca\(^{2+}\). We determined the effect of pma1 mutations on intracellular [Ca\(^{2+}\)] to investigate the cause of this sensitivity. When grown in YPD, calcineurin-mutant yeast contain approximately twofold more Ca\(^{2+}\) than wild-type cells (Tanida et al. 1995; Withee et al. 1997; Figure 6B). In contrast, pma1 mutations reduce the level of intracellular Ca\(^{2+}\); pma1 mutants accumulate approximately fourfold less Ca\(^{2+}\) than wild-type cells, while yeast deficient for both calcineurin and PMA1 (cnb1Δ pma1) contain 1.7-fold less Ca\(^{2+}\) than yeast that lack only calcineurin (cnb1Δ) (Figure 6B). Thus, pma1 mutants display decreased intracellular [Ca\(^{2+}\)] and increased Ca\(^{2+}\) sensitivity, while deletion of calcineurin has the opposite effect on both Ca\(^{2+}\) content and tolerance (Figures 5C and 6B).

Overexpression of constitutively active calcineurin confers sensitivity to low pH: This report demonstrates that pma1 and calcineurin mutations confer opposite effects upon growth in the presence of particular cations. Therefore, we examined whether expressing a constitutively active allele of calcineurin would cause phenotypes similar to pma1 mutations. We introduced a plasmid into wild-type yeast to direct high-level expression of a constitutively active, Ca\(^{2+}\)-calmodulin-independent form of calcineurin (Cna2Δp; Withee et al. 1997). Cna2Δp lacks both the calmodulin-binding site and the autoinhibitory domain, which renders the full-length enzyme dependent on Ca\(^{2+}\) and calmodulin for activity (Cyert et al. 1991; Hubbard and Klee 1989; O’Keefe et al. 1992). A hallmark of pma1 mutant strains is that their growth is inhibited at low pH. In media buffered to pH 4.0, the relative growth of yeast containing a plasmid vector without an insert (pVT-L) is indistinguishable from that of yeast containing a plasmid that directs high-level expression of Cna2Δp (Figure 7). However, in media buffered to pH 3.0, the growth of cells expressing Cna2Δp is reduced by \(\sim 70\%\), compared to a 40% reduction for cells containing the
Figure 7.—The growth of yeast expressing high levels of a Ca\(^{2+}\)/CaM-independent allele of calcineurin is inhibited by low pH. Wild-type yeast (YPH499) were transformed with a plasmid (pVT-LCna2\(^{D}\)p, □) encoding a Ca\(^{2+}\)/CaM-independent allele of calcineurin or the corresponding vector without an insert (pVT-L, ○). Log-phase yeast were inoculated to a final OD\(_{600}\) of 0.05 into synthetic media lacking leucine to select for plasmids, buffered to pH 5.0, 4.0, 3.0, or 2.5, and grown at 30° with shaking (see materials and methods). After 12 hr of growth, the OD\(_{600}\) was determined for each culture. Percentage of growth represents the number of cell doublings at the indicated pH/number of doublings at pH 5.0. The data shown are the average of results from duplicate cultures in a single experiment. The experiment was repeated twice with similar results.

Yeast expressing high levels of constitutively active calcineurin display decreased Pma1p activity: To further investigate the effect of calcineurin on Pma1p, we assayed Pma1p activity in membranes from yeast overexpressing Cna2\(^{D}\)p (see materials and methods). Membranes from yeast expressing high levels of Cna2\(^{D}\)p have 45% less Pma1p activity than membranes from yeast lacking Cna2\(^{D}\)p (Figure 8A). Next, we determined the effect of Cna2\(^{D}\)p on the abundance of Pma1p. Membranes from yeast expressing high levels of Cna2\(^{D}\)p have ~20% less Pma1p than membranes from yeast that lack Cna2\(^{D}\)p (Figure 8B and C). Thus, expression of high levels of Cna2\(^{D}\)p reduces both the activity and amount of Pma1p. We also compared Pma1p activity in membranes from wild-type and calcineurin-mutant yeast, but we were unable to detect any difference in ATPase activity between these preparations (data not shown).

DISCUSSION

Studies from many groups have demonstrated that yeast calcineurin plays a central role in regulating ion homeostasis. Yeast that lack calcineurin are sensitive to vector control. Likewise, in media of pH 2.5, the growth of cells overexpressing Cna2\(^{D}\)p is compromised by 80% vs. a reduction of ~50% for cells containing vector (Figure 7).
and Mn$^{2+}$ resistance to strains that lack calcineurin. \(pma1\) mutations suppress the sensitivity to Na$^+$, Li$^+$, Mn$^{2+}$, and aminoglycosides, and they reverse the Ca$^{2+}$ tolerance of calcineurin-mutant yeast. In addition, mutations in \(PMA1\) or \(URE2\) improve the viability of calcineurin-mutant yeast in the presence of mating pheromone. These observations demonstrate that both \(URE2\) and \(PMA1\) affect cation tolerance in \(S.\ \text{cerevisiae}\) and may provide information about calcineurin's role in the yeast response to ion stress.

**Role of \(URE2\) in ion homeostasis:** The previously characterized gene \(URE2\) shares sequence homology with glutathione S-transferases (Coschigano and Magasanik 1991). \(URE2\) was first identified by a mutation that relieved the transcriptional repression of several genes involved in nitrogen utilization (Drillien et al. 1973). \(URE2\) represses the transcription of these genes by inhibiting the activity of Gln3p, a DNA-binding protein required for increased transcription of several nitrogen-regulated genes (Courchesne and Magasanik 1988; Minehart and Magasanik 1991). Although the manner in which \(Ure2p\) inhibits Gln3p is unknown, it does not involve changes in GLN3 transcript level or Gln3p abundance. This report demonstrates that \(URE2\) also affects ion tolerance. Furthermore, mutations in \(URE2\) provide resistance to the same set of cations to which calcineurin-deficient strains are sensitive, but they do not confer general resistance to all toxic cations.

What is the relationship among calcineurin, \(URE2\), and the yeast response to ion stress? Previous work showed that the mRNA level of a plasma membrane Na$^+$-ATPase (PMR2/ENA1) is induced by Na$^+$ or Li$^+$ and that full induction requires calcineurin and the transcription factor Crz1p/Tcn1p (Garciadeblas et al. 1992; Mendoza et al. 1994; Stathopoulos and Cyert 1997; Mathews et al. 1997). We demonstrate here that mutations in \(URE2\) require the presence of PMR2/ENA1 to confer Na$^+$ resistance to yeast cells. This finding suggests that the effect of \(URE2\) on Na$^+$ and Li$^+$ homeostasis is via regulation of PMR2/ENA1. Because of the relationship between \(Ure2p\) and Gln3p discussed above, it seems likely that activation of Gln3p is responsible for the Na$^+$, Li$^+$, and Mn$^{2+}$ resistance of \(ure2\) mutant yeast. Consistent with this, \(ure2\) mutants require GLN3 to exhibit increased tolerance to Na$^+$, Li$^+$, and Mn$^{2+}$. The requirements for \(PMR2\) and the transcription factor \(GLN3\) imply that \(ure2\) mutants exhibit increased Na$^+$ tolerance because of elevated levels of \(PMR2\). In support of this, multiple copies of the upstream activation sequence through which Gln3p acts (GATAA) are present upstream of \(PMR2\) (Cunningham et al. 1996). However, we did not observe a significant rise in \(PMR2\) mRNA levels in \(ure2\) mutants. Therefore, activation of Gln3p in \(ure2\) mutants may induce a change in Pmr2p activity through an unknown effector. Alternatively, \(ure2\) mutants may exhibit an increase in \(PMR2\) expression that is responsible for the observed Na$^+$ tolerance but is below the limit of our detection. We also report that
ure2 mutations confer sensitivity to Cd\(^{2+}\) in the presence and absence of calcineurin. However, ure2 gln3 double mutants show the same sensitivity to Cd\(^{2+}\) as wild-type yeast. This requirement for GLN3 suggests that it is the transcriptional activation of a target(s) that is responsible for the Cd\(^{2+}\) sensitivity of ure2 mutants.

We also show that calcineurin increases tolerance to Na\(^{+}\) and Li\(^{+}\) in the complete absence of the URE2 gene product. In addition, calcineurin increases the tolerance to Mn\(^{2+}\) and improves the viability of yeast during incubation with mating pheromone in strains that lack URE2. Thus, it is unlikely that calcineurin promotes cation tolerance and survival during treatment with pheromone through regulation of URE2. On the other hand, ure2 mutations cause resistance to Na\(^{+}\), Li\(^{+}\), and Mn\(^{2+}\) in yeast that lack calcineurin, making it unlikely that URE2 acts upstream of calcineurin. Instead, we propose that URE2 and calcineurin act independently to modulate the yeast response to ion stress.

**Role of PMA1 in ion homeostasis:** The yeast gene PMA1 encodes a plasma membrane H\(^{+}\)-ATPase that is conserved among higher plants and fungi and belongs to the same class of P-type ATPases as mammalian [Na\(^{+}\), K\(^{+}\)] and Ca\(^{2+}\)-ATPases (Serrano et al. 1986). PMA1 is an essential gene whose product is required not only for cytosolic pH homeostasis, but also for maintaining the electrochemical potential at the plasma membrane. Previous studies showed that mutations that reduce Pma1p activity confer slow growth, sensitivity to low pH, resistance to the aminoglycoside antibiotic hygromycin B, and more recently, resistance to Na\(^{+}\) (McCusker et al. 1987; Nass et al. 1997; Vallejo and Serrano 1989). In addition, pma1 mutants exhibit reduced uptake of many nutrients (including several amino acids) and hygromycin B (Vallejo and Serrano 1989). Thus, Pma1p is important for the transport of multiple nutrients/ions across the plasma membrane. This report further characterizes the effect of PMA1 on cation tolerance. Specifically, we report for the first time that pma1 and calcineurin mutations have opposite effects on yeast growth in the presence of a particular set of cations: pma1 mutants are resistant to Na\(^{+}\), Li\(^{+}\), Mn\(^{2+}\), and aminoglycosides, and are sensitive to Ca\(^{2+}\); while yeast lacking calcineurin are sensitive to Na\(^{+}\), Li\(^{+}\), Mn\(^{2+}\), and aminoglycosides, and are resistant to Ca\(^{2+}\). It seems likely that the Na\(^{+}\), Li\(^{+}\), and Mn\(^{2+}\) tolerance of pma1 mutants results from reduced uptake of these cations. In support of this proposal, we show that pma1 mutations reduce intracellular Na\(^{+}\) levels and confer Na\(^{+}\) resistance, even in the absence of export by the plasma membrane Na\(^{+}\)-ATPase. The decreased uptake of these cations may be caused by depolarization of the plasma membrane, reduction of the plasma membrane pH gradient, or both. These observations are consistent with the recent results of Nass et al. (1997), who show that mutations that reduce the activity of Pma1p confer increased tolerance to and decreased accumulation of Na\(^{+}\), although these authors further suggest that decreased Pma1p activity leads to increased Na\(^{+}\) sequestration via a vacuolar Na\(^{+}\)/H\(^{+}\) exchanger.

pma1 mutations also confer sensitivity to Ca\(^{2+}\) and a corresponding decrease of intracellular Ca\(^{2+}\) levels. This suggests that Pma1p activity is required for effective Ca\(^{2+}\) sequestration into one or more intracellular compartments. The pH gradient maintained at the vacuolar membrane (\(\Delta pH_v\)) is important for vacuolar Ca\(^{2+}\) sequestration, and perturbations that lower \(\Delta pH_v\) decrease vacuolar Ca\(^{2+}\) accumulation (Dunn et al. 1994; Ohsumi and Anraku 1983; Ohya et al. 1991). Because decreased Pma1p activity promotes acidification of the cytosol (Portillo and Serrano 1989), it may also reduce \(\Delta pH_v\). Thus, inhibition of Pma1p may reduce Ca\(^{2+}\) sequestration by lowering \(\Delta pH\) at the vacuolar membrane. Alternatively, pma1 mutations may reduce uptake at the plasma membrane of a factor important for Ca\(^{2+}\) sequestration.

**Relationship between calcineurin, PMA1 and ion homeostasis:** The observation that mutations in pma1 and calcineurin confer opposite effects upon the growth of yeast under conditions of ion stress suggests that calcineurin may negatively regulate Pma1p in vivo. Consistent with this possibility, we report that expressing high levels of a Ca\(^{2+}\)/CaM-independent allele of calcineurin (Cna2p) inhibits the growth of yeast at low pH, which is a hallmark of cells with reduced Pma1p activity. We also show that expression of Cna2p reduces Pma1p ATPase activity in membrane fractions. However, our data are also consistent with the possibility that calcineurin and Pma1p have antagonistic effects upon ion tolerance through independent mechanisms. In fact, several observations support this possibility. First, we were unable to observe an increase in Pma1p activity in calcineurin-deficient yeast. Second, the Pma1p ATPase activity of membrane fractions from calcineurin-mutant yeast was not affected by addition of soluble extracts from cells expressing Cna2p. Finally, neither purified yeast nor bovine calcineurin changed the Pma1p ATPase activity of yeast membrane fractions (J. L. Withee and M. S. Cyert, unpublished results).

To further investigate the relationship between calcineurin and Pma1p, we determined whether calcineurin-mutant yeast are sensitive to aminoglycosides. We report that calcineurin is required for resistance to cationic drugs, such as hygromycin B and G418, but not to other inhibitors of protein synthesis, such as cycloheximide. Calcineurin, therefore, either affects an intracellular target that is important for drug action or promotes reduced accumulation or increased sequestration of these compounds. It seems unlikely that this resistance is mediated directly through calcineurin's known effect on the transcript levels of the P-type ATPases PMR2/ENA1, PMR1, and PCM1. However, the resistance to cationic aminoglycosides is consistent with the proposal that PMA1 and calcineurin act antagonistically but independently on a common cellular target. Mutations that reduce Pma1p activity cause resistance to cat-
Yeast Ion Tolerance

The yeast responses to mating pheromone and ion stress are related: Calcineurin is required to maintain viability during prolonged incubation with mating pheromone and is activated by the Ca\(^{2+}\) influx associated with the pheromone response (Cyert and Thorner 1992; Moser et al. 1996; Withee et al. 1997). We report here that pma1 and ure2 mutations confer Na\(^+\) resistance upon yeast cells through different mechanisms. However, mutations in either URE2 or PMA1 also increase the viability of calcineurin mutants in the presence of mating pheromone. These observations suggest a connection between the responses to ion stress and mating pheromone. Recent studies demonstrated that calcineurin mutants display aberrant vacuole morphology during treatment with mating pheromone (Withee et al. 1997). It may be that abnormalities in cation transport are somehow responsible for the errant vacuole morphology observed in calcineurin mutants under these conditions.

Conclusion: We have demonstrated that URE2 modulates tolerance to Na\(^+\), Li\(^+\), and Mn\(^{2+}\) and that ure2 mutations require the Na\(^+\)-ATPase PMR2/ENA1 and the transcription factor Gin3p to confer increased Na\(^+\) tolerance. We also report that PMA1 modulates tolerance to Na\(^+\), Li\(^+\), Mn\(^{2+}\), and Ca\(^{2+}\). This study has further characterized the roles of PMA1 and URE2 in the yeast response to ion stress and expanded our understanding of the regulation of cation transport in S. cerevisiae.

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