Internal Ca²⁺ release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue

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Calcium ions, present inside all eukaryotic cells, are important second messengers in the transduction of biological signals. In mammalian cells, the release of Ca²⁺ from intracellular compartments is required for signaling and involves the regulated opening of ryanodine and inositol-1,4,5-trisphosphate (IP₃) receptors. However, in budding yeast, no signaling pathway has been shown to involve Ca²⁺ release from internal stores, and no homologues of ryanodine or IP₃ receptors exist in the genome. Here we show that hyperosmotic shock provokes a transient increase in cytosolic Ca²⁺ in vivo. Vacuolar Ca²⁺, which is the major intracellular Ca²⁺ store in yeast, is required for this response, whereas extracellular Ca²⁺ is not. We aimed to identify the channel responsible for this regulated vacuolar Ca²⁺ release. Here we report that Yvc1p, a vacuolar membrane protein with homology to transient receptor potential (TRP) channels, mediates the hyperosmolarity induced Ca²⁺ release. After this release, low cytosolic Ca²⁺ is restored and vacuolar Ca²⁺ is replenished through the activity of Vcx1p, a Ca²⁺/H⁺ exchanger. These studies reveal a novel mechanism of internal Ca²⁺ release and establish a new function for TRP channels.

Introduction

Eukaryotic cells can sense a wide variety of environmental stresses, including changes in temperature, pH, osmolarity, and nutrient availability. They respond to these changes through a variety of signal transduction mechanisms, including activation of Ca²⁺-dependent signaling pathways. In mammalian cells, various stimuli are known to induce the release of Ca²⁺ from the endoplasmic or sarcoplasmic reticulum, the primary Ca²⁺ stores. In yeast, the Ca²⁺ concentration in the cytosol ($[Ca^{2+}]_{cyt}$)* has been shown to increase in response to the mating pheromone α factor (Iida et al., 1990), hypotonic shock (Batiza et al., 1996, Beeler et al., 1997), and addition of glucose to starving cells (Nakajima-Shimada et al., 1991). However, none of these increases in $[Ca^{2+}]_{cyt}$ has been shown to depend on internal Ca²⁺ release, as opposed to influx from the external media. In the case of hypotonic shock, increases in $[Ca^{2+}]_{cyt}$ are partially independent from external Ca²⁺, but there is no direct evidence of internal Ca²⁺ release (Batiza et al., 1996). Therefore, although yeast possesses many of the conserved elements involved in Ca²⁺ signaling (i.e., calmodulin, adenylate cyclase, and various protein kinases), signaling through internal Ca²⁺ release is still speculative in yeast.

If internal Ca²⁺ release exists in yeast, the vacuole is likely to be involved in this function, as it plays a major role in Ca²⁺ homeostasis. Indeed, free Ca²⁺ concentration in the yeast vacuole reaches 1.3 mM, compared with only 10 μM in the endoplasmic reticulum (Halachmi and Eilam, 1989; Strayle et al., 1999). Therefore, the yeast vacuole is the functional counterpart of the mammalian endoplasmic and sarcoplasmic reticulum for Ca²⁺ storage. Two transporters play complementary roles in sequestering Ca²⁺ into the vacuole: (a) Vcx1p, a low-affinity Ca²⁺/H⁺ exchanger that rapidly sequesters Ca²⁺ into the vacuole; and (b) Pmc1p, a high-affinity Ca²⁺ ATPase required for maintaining low $[Ca^{2+}]_{cyt}$ (Cunningham and Fink, 1994, 1996; Pozos et al., 1996; Miseta et al., 1999). It has been reported that vacuolar membrane vesicles could release Ca²⁺ in the presence of IP3 (Belde et al., 1993); however, the mechanism and the physiological relevance of this effect have not been addressed. Although Ca²⁺ influx into the vacuole has been well characterized, no protein has been shown to effect vacuolar Ca²⁺ release.

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*Abbreviations used in this paper: $[Ca^{2+}]_{cyt}$, concentration of Ca²⁺ in the cytosol; GFP, green fluorescent protein; HA, hemagglutinin; HOG, high-osmolarity glycerol; IP3, inositol-1,4,5-trisphosphate; TRP, transient receptor potential.

Key words: calcium signaling; ion channels; osmotic pressure; vacuoles; *Saccharomyces cerevisiae*

All cells must repeatedly adapt to hypertonic shock caused by variations in water availability or solutes concentration. Yeast cells are particularly exposed to such changes, and therefore have developed multiple responses to hypertonic stress. Within minutes, cells shrink and the cytoskeleton disassembles (Morris et al., 1986; Chowdhury et al., 1992). Adaptation to these new conditions requires transcriptional induction of stress-responsive genes, as well as the accumulation of intracellular glycerol (Brown et al., 1986; Albertyn et al., 1994; Hirayama et al., 1995; Tamás et al., 1999). This transcriptional activation is mediated in part by the high-osmolarity glycerol (HOG) response pathway, which is composed of a mitogen-activated kinase cascade regulated by at least two independent osmosensors (Brewster et al., 1993; Posas et al., 1998).

Although the response to hypertonic shock has been intensively studied, whether it involves Ca^{2+} signaling is unknown. In addressing this question, we show that: (a) hypertonic shock induces a transient increase in cytosolic Ca^{2+} concentrations; (b) the Ca^{2+} flux comes from the vacuole; and (c) Yvc1p, a homologue of transient receptor potential (TRP) channels, is required for this release. Yvc1p has recently been cloned by Palmer et al. (2001), and has been shown to be a cation-selective channel that can conduct Ca^{2+}, K^+, or Na^+. This conductance had been previously characterized by electrophysiological methods (Wada et al., 1987; Bertl and Slayman, 1990, 1992; Bertl et al., 1992). The electrophysiological properties of Yvc1p and its presence in the vacuolar fraction suggested that Yvc1p could be a vacuolar Ca^{2+} channel. In this study we verify this hypothesis using living cells; we demonstrate in vivo, for the first time, that Yvc1p is a vacuolar channel that mediates Ca^{2+} release in response to hyperosmotic stress.

## Results and discussion

To analyze the effect of hypertonic shock on $[Ca^{2+}]_{cyt}$, we added media containing high NaCl, KCl, or sorbitol to cells expressing the cytosolic, luminescent Ca^{2+} reporter aequorin

(Nakajima-Shimada et al., 1991; Batiza et al., 1996), and monitored luminescence (Fig. 1 a). All of these treatments induced an increase in $[Ca^{2+}]_{cyt}$, which peaked ∼1 min after the hypertonic shock. $[Ca^{2+}]_{cyt}$ rapidly decreased and returned to its basal level by 5 min. In comparison, addition of $CaCl_2$ to the extracellular medium induced a sudden increase in $[Ca^{2+}]_{cyt}$ that peaked within the first second and then decreased rapidly (Fig. 1 a) (Miseta et al., 1999). As shown previously, this decrease is due to Ca^{2+} sequestration into the vacuole (Miseta et al., 1999). These experiments show that hyperosmotic shock induces a transient increase in cytosolic Ca^{2+}, and that the timing of this response is slower than that induced by simple addition of external Ca^{2+}.

To further investigate this novel Ca^{2+} response, we examined whether the hyperosmolarity induced Ca^{2+} flux comes from an external or an internal source. We repeated these experiments using media containing the Ca^{2+} chelators EGTA or 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA), as well as using low Ca^{2+} SD medium (see Materials and methods), and observed no differences under these conditions (unpublished data). This strongly suggests that external Ca^{2+} is not required for the observed cytosolic Ca^{2+} peak. Next, we asked if the Ca^{2+} flux is released from internal stores. Because the vacuole plays an important role in Ca^{2+} storage and homeostasis, we investigated the hyperosmolarity induced Ca^{2+} flux in mutants with defects in vacuolar Ca^{2+} storage. In a $pmc1\Delta vcx1\Delta$ strain lacking both transporters for vacuolar Ca^{2+} storage, Ca^{2+} is not sequestered in the vacuole, and consequently, vacuolar $[Ca^{2+}]$ is dramatically reduced (Cunningham and Fink, 1996; Pozos et al., 1996). Therefore, if the hyperosmolarity induced Ca^{2+} flux comes from the vacuole, we expect it to be reduced in this strain. Wild-type, $pmc1\Delta$, $vcx1\Delta$, and $pmc1\Delta vcx1\Delta$ strains were subjected to high osmolarity shock (0.8 M NaCl). Strikingly, the Ca^{2+} increase was completely absent in the $pmc1\Delta vcx1\Delta$ strain (Fig. 1 b). In contrast, the single mutant $pmc1\Delta$ had a Ca^{2+} peak comparable to wild-type strain, and the Ca^{2+} response was increased in the $vcx1\Delta$ strain (Fig. 1 b). This last observation confirms that

Figure 1. **Changes in $[Ca^{2+}]_{cyt}$ in response to hypertonic shock.** (a) Luminescence response in a wild-type strain (YPH499) after addition (Time = 0) of 0.1 M $CaCl_2$ (gray), or after hypertonic shock treatments: 0.8 M NaCl (red); 0.9 M KCl (yellow); and 0.7 M sorbitol (blue). As measured with an osmometer, the osmolality of media with 0.8 M NaCl is the same as with 0.9 M KCl, and is ∼1.2-fold higher than with 0.7 M sorbitol. (b) Luminescence response after treatment with 0.8 M NaCl in wild-type (YPH499, black), $pmc1\Delta$ (TPYp, blue), $vcx1\Delta$ (KKY127, purple), and $pmc1\Delta vcx1\Delta$ (KKY124, pink) strains. All strains were transformed with PEVP11/AEQ.
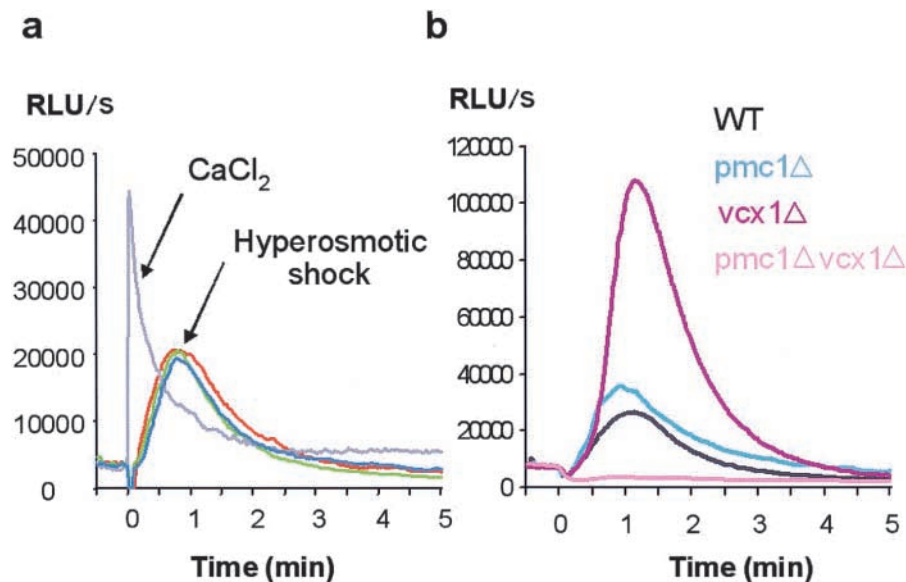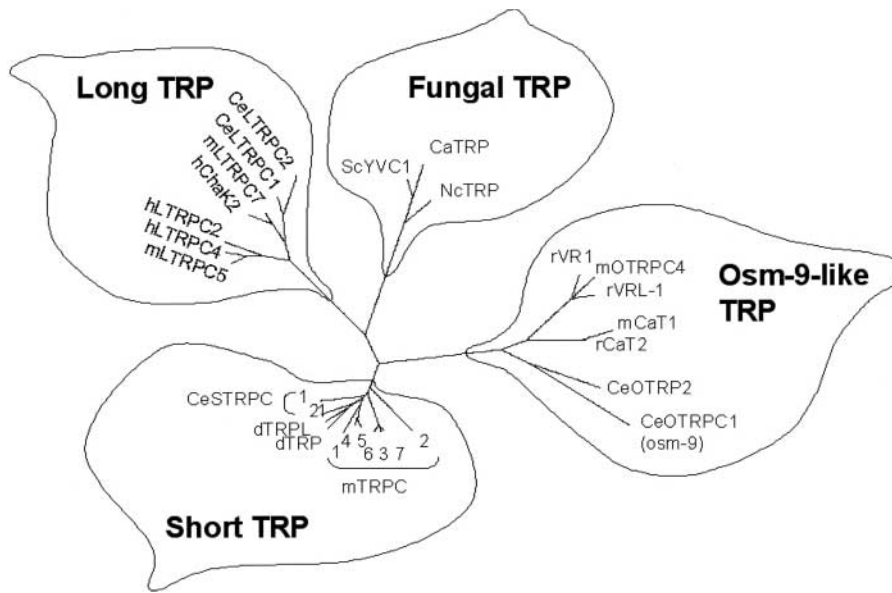
Figure 2. **Phylogenetic tree of TRP family of ion channels displaying the new fungal subfamily.** Ca, *C. albicans*; Ce, *C. elegans*; d, *D. melanogaster*; h, human; m, mouse; Nc, *N. crassa*; r, rat; Sc, *S. cerevisiae*; and TRP homologues cluster into four subfamilies.

Vcx1p plays a critical role in rapidly sequestering a sudden pulse of cytosolic Ca^{2+} into the vacuole. Indeed, *vcx1Δ* cells also display a delay in restoring low $[Ca^{2+}]_{cyt}$ after addition of extracellular Ca^{2+} (Miseta et al., 1999). Together, these results strongly suggest that the hyperosmolarity induced Ca^{2+} flux is generated by release of Ca^{2+} from the vacuole. This is the first time that Ca^{2+} release from the vacuole has been shown in vivo in yeast in response to a specific signal.

As a next step, we aimed to identify the channel responsible for this Ca^{2+} release. We examined the yeast genome for putative Ca^{2+} channels and found a candidate ORF, recently characterized as *YVC1*, that shows significant homology to the TRP family of ion channels (Palmer et al., 2001). The first TRP channel was discovered in *Drosophila melanogaster* and is required for phototransduction (Montell and Rubin, 1989). Multiple homologues have since been identified in mammals, *Xenopus*, squid, and worms, and are involved in such diverse sensory functions as pain, heat, olfaction, and osmolarity signaling; they may also be involved in replenishing intracellular Ca^{2+} stores (Putney and McKay, 1999; Harteneck et al., 2000; Clapham et al., 2001). TRP channels have been the subject of intense investigation recently, yet their gating mechanisms and biological role are not fully understood (Harteneck et al., 2000; Clapham et al., 2001; Montell, 2001). The discovery of a TRP homologue in *Saccharomyces cerevisiae* prompted us to search other fungal genomes for YVC1 homologues. We found a single homologue in *Candida albicans*, *Neurospora crassa*, and in 5 of the 14 hemiascomycetous yeast genomes that have been partially sequenced (Souciet et al., 2000). Next, we analyzed the phylogenetic relationship between these new fungal TRP channels and animal TRPs from worm and mammals (Fig. 2). The resulting tree shows that the newly defined cluster of fungal TRPs forms a distinct subfamily (Fig. 2), in addition to the previously described Short, Osm-like, and Long subfamilies (Harteneck et al., 2000; Clapham et al., 2001), also defined, respectively, as TRPC, TRPV, and TRPM subfamilies (Montell, 2001).

As a first step toward characterizing yeast Yvc1p, we determined its localization in vivo using a COOH-terminal green fluorescent protein (GFP) fusion. Interestingly, Yvc1–GFP was specifically localized to the vacuolar membrane (Fig. 3 a). This localization of the yeast TRP homologue is in contrast to other TRP channels studied thus far, which localize to the plasma membrane (Pollock et al., 1995; McKay et al., 2000; Xu and Beech, 2001). Next, we characterized the effect of Yvc1p levels on yeast cell growth. Although *yvc1Δ* had no apparent growth defects, cells expressing high levels of Yvc1p were extremely sensitive to the presence of $CaCl_2$ in the medium (Fig. 3 b). Furthermore, this sensitivity was Ca^{2+}-specific, as $MgCl_2$ at the same concentration did not affect growth (Fig. 3 b), and cells overexpressing *YVC1* did not show increased sensitivity to NaCl or KCl (0.6 to 1.2 M) (unpublished data). This Ca^{2+} sensitivity strongly suggests that Yvc1p, like some other TRP channels, participates in Ca^{2+} homeostasis and acts to increase cytosolic $[Ca^{2+}]$. Based on this finding, as well as its localization to the vacuolar membrane, Yvc1p is a good candidate for a Ca^{2+} channel that mediates vacuolar Ca^{2+} release. This hypothesis is also consistent with the electrophysiological properties of YVC1, which has been shown to be permeable to Ca^{2+}, among other cations (Bertl and Slayman, 1990, 1992; Bertl et al., 1992; Palmer et al., 2001).

We tested whether *YVC1* was involved in the hyperosmolarity induced Ca^{2+} increase by examining $[Ca^{2+}]_{cyt}$ in cells lacking or overexpressing *YVC1*. The *yvc1Δ* strain displayed no significant increase in $[Ca^{2+}]_{cyt}$ after hypertonic treatment (Fig. 3 c). In contrast, *YVC1* overexpression greatly enhanced the magnitude of the Ca^{2+} peak induced by high osmolarity (Fig. 3 c). These results indicate that Yvc1p mediates increased $[Ca^{2+}]_{cyt}$ in response to hypertonic shock. To confirm that this *YVC1*-mediated Ca^{2+} release is dependent on vacuolar Ca^{2+}, we examined $[Ca^{2+}]_{cyt}$ in *pmc1Δ*, *vcx1Δ* and *pmc1Δvcx1Δ* strains carrying a *yvc1Δ* allele or overexpressing *YVC1*. The *pmc1Δvcx1Δ* strain showed no vacuolar Ca^{2+} release even when *YVC1* was overexpressed (Fig. 3 d). This is likely due to low vacuolar $[Ca^{2+}]$, and shows that
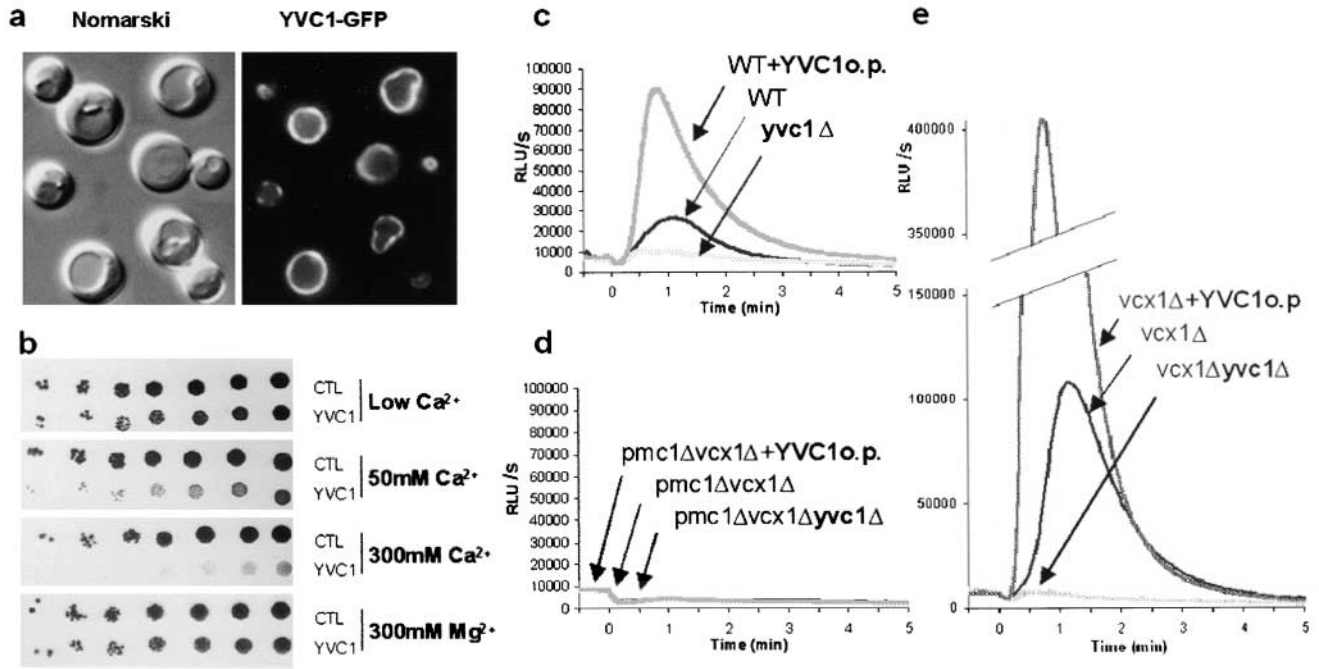
Figure 3. **Functional characterisation of Yvc1p.** (a) Yvc1–GFP localization to the vacuolar membrane, as visualized by fluorescence microscopy using an FITC filter. (b) *YVC1* overexpression causes Ca^{2+} sensitivity, as shown by serial fivefold dilutions of wild-type (YPH499) strain transformed with a control (CTL) or the pYVC1-L-HA plasmid allowing high expression levels. Because *YVC1*-expressing cells grew slightly more slowly than control cells on regular media (unpublished data), low Ca^{2+} SD medium (see Materials and methods) was used to perform this experiment in order to have identical growth on the control plate. (c) *YVC1* is required for vacuolar Ca^{2+} release in response to hypertonic shock. Luminescence response of the wild-type strain (YPH499), carrying pYVC1-U for *YVC1* overexpression (WT+YVC1o.p.) or a control plasmid (WT), and of the *yvc1Δ* strain (VDY23) carrying a control plasmid (yvc1Δ). (d) Same experiment as c in *pmc1Δvcx1Δ* (KKY124) and *pmc1Δvcx1Δyvc1Δ* (VDY40) strains. (e) Same experiment as c in *vcx1Δ* (KKY127) and *vcx1Δyvc1Δ* (VDY31) strains.

YVC1-dependent Ca^{2+} release comes from the vacuole. As expected, changes in $[Ca^{2+}]_{cyt}$ observed in the *Δpmc1* background lacking or overexpressing *YVC1* were equivalent to those seen in the wild-type strain (unpublished data). In a *vcx1Δ* background, deletion of *YVC1* completely eliminated the Ca^{2+} increase induced by hypertonic shock (Fig. 3 e). In contrast, overexpression of *YVC1* in the *vcx1Δ* strain caused a dramatic increase in this Ca^{2+} response (Fig. 3 e). Thus, overexpression of *YVC1* and mutational inactivation of *VCX1* both increase the amplitude of the hyperosmolarity induced Ca^{2+} peak, and these two effects are additive. These observations underscore the importance of Vcx1p in antagonizing and potentially modulating *YVC1*-dependent Ca^{2+} release. Together, these results show that following hypertonic shock, Yvc1p effects Ca^{2+} release from the vacuole into the cytosol, and that this release is followed by rapid Ca^{2+} sequestration into the vacuole by Vcx1p (Fig. 4).

Yeast actively sequester Ca^{2+} in their vacuole. In these studies we establish that, as in other eukaryotic cells, this Ca^{2+} can be released into the cytosol in response to external stimuli. We also show that this release is followed by refilling of the internal store. However, two key questions remain: (a) What leads to Yvc1p channel opening?; and (b) What are the physiological consequences of Ca^{2+} release? We investigated whether other environmental changes besides hypertonic shock induced Ca^{2+} release by Yvc1p. First, we found that the Ca^{2+} peak induced by injection of extracellular Ca^{2+} (Fig. 1 a) was not affected by *YVC1* deletion or overexpression (unpublished data). Thus, in vivo, a brief increase

in $[Ca^{2+}]_{cyt}$ is apparently not sufficient to trigger Yvc1p opening, although the YVC1 cation conductance observed in isolated vacuoles is activated by Ca^{2+} (Wada et al., 1987;
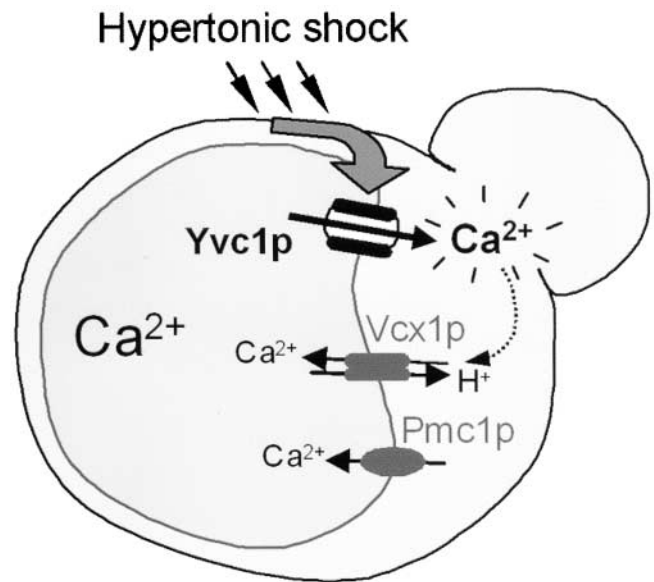

Figure 4. **Model for vacuolar Ca^{2+} release and sequestration.** Hypertonic shock induces vacuolar Ca^{2+} release by YVC1, which requires the presence of at least one of the Ca^{2+} transporters Pmc1p or Vcx1p. After Ca^{2+} release, Vcx1p rapidly sequesters Ca^{2+} into the vacuole and decreases cytosolic $[Ca^{2+}]$.

Bertl and Slayman, 1990, 1992; Bertl et al., 1992; Palmer et al., 2001). Other conditions, such as hypotonic shock or the addition of 0.03% SDS or 7% ethanol, also induced a transient increase in cytosolic Ca²⁺ (Batiza et al., 1996; unpublished data); however, *YVC1* was similarly not required for these Ca²⁺ peaks (unpublished data). Therefore, the response of Yvc1p to hypertonic shock appears to be specific. We are currently investigating the role of *YVC1*-mediated Ca²⁺ release in hypertonic stress signaling. The signaling pathway activated by hypertonic shock has been well characterized in yeast, and is composed of the HOG mitogen-activated kinase cascade (Posas et al., 1998). Further studies will examine the relationship between components of the HOG pathway and the Ca²⁺ increase mediated by *YVC1*.

In conclusion, we show that internal Ca²⁺ release in yeast is mediated by a novel class of Ca²⁺ release channel, which is unrelated to IP₃ or ryanodine receptors. Instead, this release requires a homologue of the TRP family of ion channels, Yvc1p. Like TRP channels in multicellular organisms, YVC1 acts in sensory transduction. However, YVC1 is the first TRP channel homologue shown to mediate Ca²⁺ release from an intracellular store.

Materials and methods

Yeast strains and media
Strains were isogenic to YPH499 (*MATa ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 leu2-Δ1*) (Sikorski and Hieter, 1989). TPYp is *Matα pmc1Δ::TRP1*; KKY127 is *Mata vcx1Δ*; KKY124 is *Matα pmc1Δ::TRP1 vcx1Δ*; VDY23 is *MATa yvc1Δ::Kanᴿ*; VDY25 is *Matα pmc1Δ::TRP1 yvc1Δ::Kanᴿ*; VDY31 is *Mata vcx1Δ yvc1Δ::Kanᴿ*; and VDY40 is *Matα pmc1Δ::TRP1 vcx1Δ yvc1Δ::Kanᴿ*. SD medium (Sherman et al., 1986) contained twice the recommended levels of supplements with 3.5 g of ammonium chloride per liter substituted for ammonium sulfate. Low Ca²⁺ SD medium ([Ca²⁺] ~ 0.24 μM) was the SD medium in which CaCl₂ was omitted and calcium pantothenate was replaced by sodium pantothenate as described previously (Iida et al., 1994).

Vector construction and gene deletion
YVC1–GFP plasmid (VDp88) was constructed by cloning a PCR fragment containing *YVC1* and 737 pb upstream sequence into the SacI/NheI sites of pGRU2, provided by Bertrand Daignan-Fornier (Institut de Biochemie et Génétique Cellulaires, Bordeaux, France). Hemagglutinin (HA)-tagged *YVC1* overexpression plasmids were constructed by a two-step PCR: PCR-amplified 3× (HA) was used as a downstream primer to amplify *YVC1* (YOR087/088W). This fragment was cut by Xho/BglII and cloned into Xho/Bam sites in pVT100L or pVT100U (Vernet et al., 1987), leading to pYVC1-HA-L for high expression of *YVC1*, and pYVC1-HA-U, for moderate expression, respectively. We verified that full-length Yvc1-HA protein (~78 kD) was expressed in yeast by Western blotting. pYVC1-U, used for aequorin experiments, is a nontagged version of pYVC1-HA-U: the HA tag was removed by a NotI digest and self-religation. To delete *YVC1*, a fragment comprising ORF *YOR087/088* and 736 bp of upstream sequence was cloned between the XhoI and BamHI sites of pcDNA3.1 (Invitrogen). This plasmid was then cut by EcoRI, which removed the sequence from position −41 to +1780 from the ATG, and a cassette containing the *kanʳ* gene was inserted into this site. The resulting plasmid was used to amplify a deletion cassette that was used to transform yeast. Kanamycin-resistant colonies were selected and *Δyvc1* knockout was checked by PCR as described previously (Güldener et al., 1996).

Aequorin experiments
Yeast carrying the PEVP11/AEQ plasmid, provided by Patrick H. Masson (University of Wisconsin-Madison, Madison, WI) (Batiza et al., 1996) were inoculated from a saturated overnight culture to OD₆₀₀ = 0.5 in SD media with 2 μM coelenterazine, and were grown overnight at room temperature to reconstitute aequorin from apoaequorin. For each experiment, an aliquot of 250 μl (OD₆₀₀ = 2–3) was harvested. Cells were resuspended in 100 μl SD media and transferred to luminometer tubes. The baseline lumi-

nescence was recorded every second for 30 s (1-s integration) using a Berthold LB9507 luminometer, and was reported in relative luminescence units/s. Hypertonic shock was performed by adding 100 μl SD containing twice the desired final concentration of sorbitol, KCl, or NaCl. To ensure that total reconstituted aequorin was not limiting in our assays, we measured the maximal luminescence after addition of 0.1% digitonin. The maximal luminescence was 4,000,000 relative luminescence units or more, which is 10× higher than the highest signal observed in our assays. Because light units cannot be accurately converted into intracellular Ca²⁺ concentrations, our results are presented as relative quantities.

Phylogenetic tree
Evolutionary distances between peptide sequences aligned with ClustalW were calculated with the PHILIP protdist software (Felsenstein, 1993), and the tree was subsequently plotted by the neighbor-joining method (Saitou and Nei, 1987). The GenBank accession numbers for the proteins used are the following (available at GenBank/EMBL/DDBJ accession no.): ScYVC1 (*S. cerevisiae*, YOR087/088w) (Palmer et al., 2001); CaTRP (sequence 11894–9852 from *Candida albicans* genome contig 1.802); NcTRP (sequence 4727–6751 from *N. crassa* genome contig 6–2259); rVR1 (T09054); mOTRPC4 (AAG17543); rVRL-1 (NP_035836); mCaT1 (BAA99538); rCaT2 (BAA99541); CeOTRPC2 (CAA96644); CeOTRPC1 (T37241); CeLTRPC2 (CAA92726); CeLTRPC1 (CAB02303); mLTRPC7 (AAK57433); hChaK2 (AAK31202); hLTRPC2 (NP_003298); hLTRPC4 (NP_060106); mLTRPC5 (AAF98120);CeSTRPC1 (AAA28168); CeSTRPC2 (AAK21447); dTRPL (P48994); dTRP (P19334); mTRPC1 (AAB50622); mTRPC4 (AAC05179); mTRPC5 (AAC13550); mTRPC6 (AAC06146); mTRPC3 (NP_062383); mTRPC7 (AAD42069); and mTRPC2 (AAG29950).

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