Cell Calcium xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Ca^{2+} homeostasis in the budding yeast *Saccharomyces cerevisiae*: Impact of ER/Golgi Ca^{2+} storage

Petra D'hooge[a], Catherina Coun[b], Vincent Van Eyck[a], Liesbeth Faes[a], Ruben Ghillebert[b], Lore Mariën[a], Joris Winderickx[b,*], Geert Callewaert[a,**]

[a] *The Yeast Hub Lab, KU Leuven, Campus Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium*
[b] *Functional Biology, Department of Biology, KU Leuven, Kasteelpark Arenberg 31, 3001 Heverlee, Belgium*

ARTICLE INFO

Article history:
Received 10 February 2015
Received in revised form 11 May 2015
Accepted 26 May 2015
Available online xxx

Keywords:
Calcium homeostasis
Vacuole
ER
Golgi
Saccharomyces cerevisiae

ABSTRACT

Yeast has proven to be a powerful tool to elucidate the molecular aspects of several biological processes in higher eukaryotes. As in mammalian cells, yeast intracellular Ca^{2+} signalling is crucial for a myriad of biological processes. Yeast cells also bear homologs of the major components of the Ca^{2+} signalling toolkit in mammalian cells, including channels, co-transporters and pumps. Using yeast single- and multiple-gene deletion strains of various plasma membrane and organellar Ca^{2+} transporters, combined with manipulations to estimate intracellular Ca^{2+} storage, we evaluated the contribution of individual transport systems to intracellular Ca^{2+} homeostasis. Yeast strains lacking Pmr1 and/or Cod1, two ion pumps implicated in ER/Golgi Ca^{2+} homeostasis, displayed a fragmented vacuolar phenotype and showed increased vacuolar Ca^{2+} uptake and Ca^{2+} influx across the plasma membrane. In the *pmr1Δ* strain, these effects were insensitive to calcineurin activity, independent of Cch1/Mid1 Ca^{2+} channels and Pmc1 but required Vcx1. By contrast, in the *cod1Δ* strain increased vacuolar Ca^{2+} uptake was not affected by Vcx1 deletion but was largely dependent on Pmc1 activity. Our analysis further corroborates the distinct roles of Vcx1 and Pmc1 in vacuolar Ca^{2+} uptake and point to the existence of not-yet identified Ca^{2+} influx pathways.

1. Introduction

As in all eukaryotic cells, yeast cells employ calcium signalling mechanisms to regulate a wide variety of cellular processes including cell-cycle progression, mating, protein processing, responses to hypotonic stress, maintenance of intracellular pH [1–8] and adequate nutritional and metabolic signalling [9–12]. Moreover, the toolkit of Ca^{2+} signalling proteins in yeast and mammalian cells is remarkably similar. Cytosolic free Ca^{2+} concentration in yeast cells is delicately regulated and maintained at low levels (50–200 nM) through the action of different Ca^{2+} transporters including channels, co-transporters and pumps and some of these Ca^{2+} transporters are evolutionarily conserved from yeast to human [13–15].

Extracellular Ca^{2+} can enter the cytosol through several Ca^{2+} influx pathways. The best characterized pathway is a high affinity, low capacity influx system composed of Cch1 and Mid1, the yeast homologue of voltage-gated Ca^{2+} channels [16–23]. In fungal and yeast cells the Cch1/Mid1 complex, however, primarily functions as a store-operated Ca^{2+} channel that is activated upon depletion of secretory Ca^{2+} [18,24]. Several other Ca^{2+} influx pathways have been reported, but are not fully characterized at the molecular level yet. These include a low affinity, high capacity Ca^{2+} channel involved in pheromone-signalling [4,7], two transporters referred to as X and M involved in Ca^{2+} influx in non-stimulated yeast cells grown under standard conditions [13] and a glucose-induced Ca^{2+} influx or GIC pathway that provides influx when Cch1/Mid1 channels are inactivated [25]. Following Ca^{2+} influx, cytosolic Ca^{2+} is mainly transported into the vacuole, the topologically equivalent of the mammalian lysosome [26]. The vacuole contains approximately ≥95% of total cellular Ca^{2+}, largely in complex with inorganic polyphosphate. Vacuolar Ca^{2+} sequestration is accomplished by the Ca^{2+} ATPase Pmc1 [27,28] and the Ca^{2+}/H^+ exchanger Vcx1 [29]. Upon hypotonic and mechanical stress [30,31], Ca^{2+} can be released from the vacuole through the Yvc1 channel, a homolog of mammalian TRPC-type Ca^{2+} channels [32]. In addition to the vacuole, the ER and Golgi apparatus also play a crucial role in maintaining proper Ca^{2+} homeostasis in yeast cells. Three transporters, the Ca^{2+}-ATPase Pmr1 [33] and presumable the ATPase Cod1 [34] and the Ca^{2+}/H^+ exchanger Gdt1 [35,36] function together in ER/Golgi

* Corresponding author. Tel.: +32 16 321516
** Corresponding author. Tel.: +32 56 246224.
E-mail addresses: joris.winderickx@bio.kuleuven.be (J. Winderickx), geert.callewaert@med.kuleuven.be (G. Callewaert).

http://dx.doi.org/10.1016/j.ceca.2015.05.004
0143-4160/

Ca^{2+} sequestration. Pmr1 is a Secretory Pathway Ca^{2+} ATPase (SPCA) that transports Ca^{2+} and Mn^{2+} into the secretory pathway compartments. Transport of Ca^{2+} (and Mn^{2+}) into the lumen of ER/Golgi is crucial for the correct folding and processing of proteins during their transport through the secretory pathway [37–39].

Re-addition of glucose to carbohydrate starved yeast cells results in a variety of effects including increased turnover of phosphatidyl inositol, activation of the Ras-adenylate cyclase pathway [40], activation of the plasma membrane H^+-ATPase [41], intracellular acidification due to glycolysis, which leads to the activation of the vacuolar H^+ ATPase [9,42,43] and a transient elevation of cytosolic Ca^{2+} or TECC response [10,12]. This TECC response has been shown to depend on glucose uptake and phosphorylation [11].

Despite extensive knowledge about the molecular identity and individual function of most Ca^{2+} transporters, we are still far from a full understanding of the complexity that governs Ca^{2+} homeostasis. In this study, we extend previous studies by analysing Ca^{2+} responses in *Saccharomyces cerevisiae* expressing the Ca^{2+} dependent photoprotein aequorin. The relative importance of individual Ca^{2+} transport systems was evaluated using single- and multiple-gene deletion strains of Ca^{2+} transporters in conjunction with a novel experimental approach to estimate intracellular Ca^{2+} storage. We show that deletion of the ER/Golgi transporters Pmr1 and Cod1 promotes vacuolar fragmentation and markedly enhances Ca^{2+} storage and Ca^{2+} influx whereas deletion of the vacuolar transporters Vcx1 and Pmc1 leads to an overall decrease in Ca^{2+} storage.

2. Materials and methods

2.1. Yeast strains, plasmids, and media

In this study we used the yeast *S. cerevisiae* BY4741 (Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and different deletion strains (Euroscarf, Frankfurt, Germany). Multiple deletion strains were generated by standard genetic crossing, and verified by PCR. YPD medium containing 2% peptone, 1% yeast extract and 2% glucose was used for growth and maintenance of yeast cells. Synthetic complete medium (Sc) containing 0.19% yeast nitrogen base without amino acids, 0.5% ammonium sulphate supplemented with synthetic drop-out amino-acid (Leu and Ura)/nucleotide mixture and 2% glucose (SD) was used for the selection, growth and maintenance of transformed yeast strains. Transformation of yeast cells was performed following the lithium/polyethylene glycol method [44]. To monitor cytosolic Ca^{2+} levels, strains were transformed with pYX212 vector encoding cytosolic aequorin (pYX212-cytAEQ) (kind gift from E. Martegani, Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy) [45]. Furthermore, in order to use the yeast strains as a model to study the effects of expression of heterologous proteins on Ca^{2+} homeostasis at later stages, all yeast strains were now already transformed with an empty control vector (pGGE181-EV).

2.2. Determination of growth profiles

To evaluate the effect of the different deletions on growth, the growth profiles of yeast were determined (Sup. Fig. 1). All yeast strains transformed with the pYX212-cytAEQ and PGGE181-EV were grown overnight. These cultures were used to inoculate new cultures at a starting OD_{600} of 0.05. Growth was quantified by measuring the OD_{600} over 70 h period (plate reader Multiskan Go, Thermo Scientific). Absorbance data were averaged across at least 18 replicates for each strain and plotted as a function of time.

2.3. Cytosolic Ca^{2+} measurements using aequorin

Cytosolic Ca^{2+} levels ($[Ca^{2+}]_{in}$) were measured in populations of yeast cells expressing aequorin essentially as described [46,47]. Briefly, yeast cells were transformed with the pYX212 encoding apoaequorin gene under the control of the TPI promoter and the pGGE181-EV and grown in selective medium with 2% glucose. Cells taken from stationary-phase pre-cultures were used to inoculate a new culture. When cultures reached an OD_{600} of ±2–3, two OD_{600} units of cells were plated on concanavaline A coated coverslips and incubated at 30 °C for 1 h. Cells were subsequently washed with 0.1 M 2-(N-morpholino) ethanesulphonic acid (MES)/Tris, pH 6.5, which is a nutrient free buffer, and again incubated for 1 hr at 30 °C with 0.1 M MES/Tris pH 6.5 supplemented with 5 μM wild-type coelenterazine (Promega) to charge aequorin. Excess of coelenterazine was removed by washing the cells 3 times with 0.1 M MES/Tris pH 6.5 and coverslips were mounted in a thermostated perfusion chamber (30 °C). Glucose-starved yeast cells were initially perfused with 0.1 M MES/Tris pH 6.5, followed by 0.1 M MES/Tris pH 6.5 supplemented with 10 mM $CaCl_2$ (referred to as Ca^{2+} pulse). Cells were then stimulated by addition of 80 mM glucose to induce a transient elevation of cytosolic Ca^{2+} (referred to as TECC response) [10–12]. To estimate intracellular Ca^{2+} storage following the Ca^{2+} pulse (referred to as Ca^{2+} pulse release) or the TECC response (referred to as TECC release), cells were exposed for 90 s to a Ca^{2+}-free medium containing (in mM): 200 KCl, 100 NaCl, 3 EGTA, 20 Hepes/KOH pH 6.8 and then subsequently permeabilized with 0.5% Triton X-100 in the same medium. At the end of all experiment, cells were perfused in a Ca^{2+}-rich hypotonic medium (10 mM $CaCl_2$ in H_2O). Photons emitted as a result of Ca^{2+} binding to charged aequorin were detected by a photon-counting tube (Type H3460-04, Hamamatsu Photonics, Japan) that was positioned about 2 cm above the cells. Light impulses were discriminated, prescaled and counted with a PC-based 32-bit counter/timer board (PCI-6601, National Instruments Corporation, Austin, TX, USA). The number of impulses occurring during a 1 s time interval was monitored with custom-built software. The recorded aequorin luminescence data were calibrated offline into cytosolic Ca^{2+} ($[Ca^{2+}]_{in}$) values using the following algorithm $[Ca^{2+}]_{in} = ((L/L_{max})^{1/3} + [118(L/L_{max})^{1/3} - 1)/(7 \times 10^6 - [7 \times 10^6 (L/L_{max})^{1/3})])$ where L is the luminescence intensity at any time point and L_{max} is the integrated luminescence [48].

In selected experiments, cells were preincubated for 2 or 43 hrs with 20 μg/ml cyclosporine A (CsA – Sigma–Aldrich) or 4 μg/ml FK506 (Invivogen). CsA was dissolved in ethanol, FK506 in DMSO and results were compared to cells pretreated with vehicle alone.

2.4. Yeast vacuolar staining

Vacuolar membranes were labelled by GFP-tagged Vph1p, a transmembrane subunit of the vacuolar proton ATPase. GFP-Vph1p expressing cells (OD_{600} of ±1.2 incubated in 0.1 M MES/Tris pH 6.5 supplemented with 10 mM external Ca^{2+}) were visualized with a Zeiss LSM 710 laser scanning microscope using a 100× high NA objective, and image processing was done using ZEN software. Vacuole morphology was quantified in >250 cells of each yeast strain and scored as either having one to two vacuoles or >two vacuoles.

2.5. Statistical analysis and curve fitting

Ca^{2+} values are expressed as mean ± standard error of the mean (SEM) and n is the number of experiments performed. Comparisons between two groups were carried out using an unpaired *t*-test. $P < 0.001$ (marked by one asterisk) indicates statistical significance.

Please cite this article in press as: P. D'hooge, et al., Ca^{2+} homeostasis in the budding yeast *Saccharomyces cerevisiae*: Impact of ER/Golgi Ca^{2+} storage, Cell Calcium (2015), http://dx.doi.org/10.1016/j.ceca.2015.05.004