Glucose-induced calcium influx in budding yeast involves a novel calcium transport system and can activate calcineurin

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1. Introduction

As in all eukaryotic cells, in budding yeast cytosolic calcium concentration is typically maintained at low levels (sub-micromolar) by calcium homeostasis mechanisms. Stimulus-dependent opening of Ca^{2+} channels in plasma membrane and/or in internal compartments triggers a rapid increase in calcium concentration in the cytosol, which represents a signal with specific spatial and temporal dynamics. In yeast, calcium signalling is essential for survival during conjugation, ion stress resistance, cell cycle progression, osmotic shock, vacuoles fusion [1–4].

At least two independent calcium influx systems were identified and characterized for calcium influx during mating process: a high affinity, low capacity, influx system (HACS), composed essentially of Mid1 and Cch1 proteins, and a low-affinity, high capacity, influx system (LACS), not yet fully characterized at the molecular level.

In minimal medium, HACS is primarily responsible for pheromone induced calcium response, but in rich media HACS is strongly inhibited by calcineurin and LACS becomes essential for this response [5,6].

Cch1 is homologous to the α1 catalytic subunits of L-type voltage-gated Ca^{2+} channels in mammals, and Mid1 appears to function as a regulatory subunit in yeast [7]. Cch1 and Mid1 are both required for HACS to be stimulated (up to 25-fold) in situations causing depletion of secretory Ca^{2+} pools, in a regulatory mechanism related to Capacitative Calcium Entry (CCE) in animal cells. Cch1 and Mid1 are likely both parts of the same calcium ion channel, with Cch1 forming the pore and Mid1 acting as a regulatory subunit. Interestingly, recent evidence has suggested that Mid1 might be a stretch response activator of calcium influx, initiating uptake in response to cell swelling [8]. HACS is stimulated also in Golgi Ca^{2+} pump pmr1 mutants and in wild-type strains overexpressing either the vacuolar Ca^{2+}-ATPase Pmc1 or the vacuolar H^{+}/Ca^{2+} exchanger Vcx1 [9], in response to depolarization and to hypotonic shock [10], in case of ER stress or depletion of Ca^{2+} stores in the ER/secretory pathway [11], and is required for cold stress and iron tolerance [12]. Calcineurin is known to inhibit channel activity, possibly through direct dephosphorylation. Only Fig. 1 protein has been identified up to now as a regulator or a component of LACS [13]. Fig. 1 is N-glycosylated and localizes to the plasma membrane via its four predicted transmembrane helices.
characteristic of the PMP-22/EMP/MP20/Claudin protein superfamily. Fig. 1, whose expression is strongly induced upon activation of pheromone signalling pathway, promotes Ca\(^{2+}\) influx and elevation of cytosolic free Ca\(^{2+}\) concentration upon exposition to mating factor [13] and promotes mating factor-dependent programmed cell death independently of Ca\(^{2+}\) [14], but up to now any calcium transport activity has been reported for this protein.

Saccharomyces cerevisiae responds with a transient elevation in cytosolic calcium not only to exposure to pheromones, but also to addition of hexoses after carbon source limitation. The glucose induced calcium increase requires glucose phosphorylation [19]. GIC response is mediated mainly by an influx of calcium from the extracellular space, which was reported to require Mid1 channel in synthetic minimal medium [18] but not in rich media [19]. Here we show that an additional channel exists in yeast.

### 2. Materials and methods

#### 2.1. Strains and cultural conditions

Cells were grown in YPD, containing 2% glucose, 2% tryptone, 1% yeast extract and 2% agar by Biolife, USA, supplemented with 50 mg/l adenine, or in minimal medium, containing 2% glucose, 0.67% Yeast Nitrogen Base (Difco, USA) and supplemented with 50 mg/l adenine, histidine, tryptophan and leucine, with shaking, at 30 °C. Selective solid media were prepared with complete supplemented synthetic medium, containing 2% glucose, 0.67% YNB, the appropriate CSM drop-out (BIO101, USA) according to supplier instructions, 2.5% agar (Biolife, USA), supplemented with 50 mg/l tryptophan. Cell density was determined by measuring optical density (OD\(_{600}\)) or by Coulter Counter (Coulter Electronics Z2).

#### 2.2. Plasmids and strains construction

YVC1, FIG1 and CNB1 genes were deleted in wild-type and mutant strains (Table 1) using a disruption cassette generated by PCR according to the method first described by Wach et al. [25], using pFA6a-His3MX6 plasmid [26], containing an expression cassette for the heterologous marker his\(^{+}\) from Schizosaccharomyces, as a template (primers ATTACCAGGCGACATTCGGAGTCCTCCTTTCGATCAGCTATGACATGAAATTAACGGATCCCCGGGTATTA and TTTTTATTTTAACTGGATCAAGGAATGCAATAAACGGATCCCCGGGTTAATTAA and the integration of the cassette and disruption at the deletion). The deleted strains were selected on synthetic complete medium lacking histidine and the integration of the cassette disruption in the correct locus was verified by PCR.

YVC1 and CNB1 deletion in the mid1::LEU2 cch1::TRP1 fig1::Sp\(^{+}\) mutant strain were performed by using a disruption cassette generated by PCR using pFA6a-KanMX4 [26] as template and the same primers described above. The deleted strains were selected on YPD medium with 500 μg/ml G418 and 400 μg/ml G418 respectively and the integration of the cassette and disruption at the correct locus were verified by PCR.

For luminescence assays yeast cells were transformed by lithium acetate method with the multicopy pVTU-AEQ plasmid [19]. For β-galactosidase assays yeast cells were transformed with multicopy pAM366 plasmid kindly provided by M. Cyert (Stanford University) [27], containing 4xCDRE:λacZ reporter.

#### 2.3. Bioluminescence assay

Exponentially growing cells (5–6 × 10\(^{6}\) cells/ml) in YPD or in minimal medium at 30 °C, were harvested by filtration on nitrocellulose filters (Millipore, pore sizes 0.22 μm), washed twice with cold water (1 volume/wash) and then resuspended in 0.1 M 2-(N-morpholino)-ethanesulphonic acid (MES)/Tris, pH 6.5 (MES/Tris buffer) at a density of about 10\(^{7}\) cells/ml. The bioluminescent assay was performed as described previously [17]. Briefly cells were incubated for 1 h at room temperature, collected by centrifugation at 4000 rpm for 10 min and concentrated in the same buffer at a density of 2.5 × 10\(^{8}\) cells/ml, they were then collected by centrifugation at 7500 rpm for 2 min and resuspended in 10 μl of MES/Tris buffer for each treatment. To
reconstitute functional aequorin, 50 µM coelenterazine (Molecular Probes; stock solution 1 µg/µl dissolved in 99.5% methanol, conserved in the dark at −20 °C) was added to the cell suspension and the cells were incubated for 20 min at room temperature in the dark. Excess coelenterazine was removed by washing cells three times with 0.1 M MES/Tris buffer pH 6.5 (200 µl/wash) and by centrifugation at 7000 rpm for 1 min. For each treatment, 200 µl of the cell suspension (in 0.1 M MES/Tris buffer pH 6.5) were transferred to a luminometer tube, in the presence of solution of CaCl₂ of the calcium chelator ethylene glycol tetraacetic acid (EGTA) and of the indicated metals (stock solutions prepared in 0.1 M MES/Tris buffer pH 6.5) or inhibitors (gadolinium chloride hexahydrate, Nifedipine dissolved in DMSO, Verapamil hydrochloride, Sigma–Aldrich), at the final concentrations indicated in the figures. Light emission was monitored with a Berthold Lumat LB 9507 at intervals of 10 s for 1 min before and for at least 6 min after the addition of glucose to a final concentration of 100 mM, and reported in relative luminescence units/s (RLU/s).

At the end of each experiment aequorin expression and activity was tested by lysing cells with 0.5% Triton X-100 in the presence of 10 mM CaCl₂ (stock solution 0.1 M in MES/Tris buffer pH 6.5) monitoring light emission for 18 min. This maximum intensity was used to normalize light emission according to the amount of aequorin expressed.

Free calcium concentration in the presence of chelators was determined using an on-line available MaxChelator program [28]. Dose–response curves were constructed by calculating the initial shape of the peak in light emission curve, and plotting it in a semi-log plot as a function of external calcium concentration. Apparent $K_M$ was calculated by fitting the curve with a Hill function in the form $y = y_0 + \left(\frac{y_{max} - y_0}{K_M + [Ca^{2+}]^n}\right)$. Competition curves were constructed by plotting the percentage of inhibition of the maximal response as a function of the concentration of the added competitive metal. Apparent $IC_{50}$ was calculated by fitting the curve with a Hill equation in the form $I(Me) = \left[1 + \left(\frac{IC_{50}}{[Me]}\right)\right]^{-1}$, where $IC_{50}$ is the concentration at half maximal block, and $n$ is the Hill coefficient. $K_M$ was calculated by plotting apparent $K_M$ values, calculated in the presence of different concentrations of metal on Lineweaver–Burk plots, on inhibitor concentration, and fitting them to the linear equation: apparent $K_M = K_M(1 + [I]/K_I)$.

### 2.4. Calcineurin reporter assay

Exponentially growing cells (5–6 × 10⁶ cells/ml) in YPD or minimal medium, with or without addition of either 3 mM cAMP, 1 mM CaCl₂ or 1 mM EGTA when indicated, were harvested by filtration on nitrocellulose filters (Millipore, pore sizes 0.22 µm), washed twice with cold water (1 volume/wash) and then resuspended in 0.1 M 2-(N-morpholino)-ethanesulphonic acid (MES)/Tris, pH 6.5 (MES/Tris buffer) at a cell density of 1.25 × 10⁷ cells/ml. To measure the β-galactosidase activity in exponentially growing cells, the method described by Kiechle et al. [29] was used. Briefly, 200 µl of the cell suspension (‘exponentially growing’ treatment) or 1 mM ethylene glycol tetraacetic acid (EGTA) (‘EGTA + glc’ treatment) and processed as described above.

For detection, all samples were simultaneously thawed, a 100 µl-aliquot was removed and used to determine the $OD_{600}$. The remaining cells were incubated for 1 h at 37 °C with 100 µL zymolyase solution (zymolyase T20, ICN, USA; 0.5 mg/ml in Z-buffer). To measure β-galactosidase activity, 100 µl of 4 mg/ml CPRG (chlorophenol-red-β-D-galactopyranoside, Sigma–Aldrich) in Z-buffer were added to each sample and after incubation for suitable time at 37 °C the reaction was stopped with 200 µl of 1 M Na₂CO₃. Then samples were centrifuged at 13000 rpm for 15 min and the supernatant was read at 574 nm. β-galactosidase activity was calculated as follows and expressed in Miller Units (MU): $MU = \left[\frac{(OD_{574} - OD_{574} \text{ ctrl})}{(OD_{600} \times t_{inc})}\right] \times 1000$. Each value is the average of two independent extracts of the same strain. The experiments were repeated at least three times giving similar results.

### 3. Results

#### 3.1. Glucose-induced calcium (GIC) signalling is modulated by nutrients

Glucose addition to glucose-deprived cells induces a calcium peak reaching its maximum within 60s in presence of extracellular calcium [15,17]. The timing of this response is peculiar in K601...
strain, where two peaks are usually observed, the first and generally higher at 60s and a second one at 180s, but this is only typical for this genetic background. The absence of extracellular calcium reduces calcium peak to a very faint signal, indicating that calcium response depends mainly on calcium influx from the external medium. This response is also depending on external calcium concentration (Fig. 1A).

While Mid1/CCh1 transporter was characterized, in cells over-expressing both the proteins, as a unique channel with a $K_M$ of 12 $\mu$M [30], LACS system was reported with a $K_M$ of ~3 mM in response to pheromones [5]. Glucose-induced calcium response is unlikely involving any such low affinity system: though the quite large standard deviation, intrinsically related to bioluminescence methods, the estimation of $K_M$ in the YPD grown wild-type strain was 56.2 ± 4.2 $\mu$M (Fig. 1B), suggesting that the systems involved in this response are high affinity calcium transport systems.

3.2. Different transporters are involved in glucose-induced calcium influx

In order to identify the transporters involved in GIC response, $FGI1$ gene, encoding the only component of the LACS system identified up to now, was deleted either alone or together with $MID1$ and $CCH1$ genes.

Glucose-induced calcium response was then analyzed in $mid1\Delta$, $cch1\Delta$, $mid1\Delta cch1\Delta$, $fig1\Delta$ and $mid1\Delta cch1\Delta fig1\Delta$ mutants. As can be observed in Fig. 2A, none of the mutants showed a completely impaired response to glucose when they were grown in rich medium, suggesting that the contributions of the HACS and LACS systems to this calcium influx are not essential in rich media: deletion of HACS subunits affected the signal more seriously than FGI1 deletion, which has a negligible effect, suggesting a role for HACS but not for LACS in this calcium influx. Actually, HACS system was reported to be repressed by calcineurin in rich media [5], while Fig. 1 is poorly expressed during exponential growth [13].

Anyway, the deletion of HACS subunit/s or of LACS component didn’t affect the biphasic signature of the response, that was affected only by co-deletion of HACS and LACS components in the $mid1\Delta cch1\Delta fig1\Delta$ mutant: in this mutant, in fact, the later peak at 180s becomes the major component of the response.

A different transport system is thus expected to drive the main part of glucose-induced calcium response in such conditions, which we will refer to as GIC (Glucose-Induced Calcium) transporter hereafter.

As it was found for the wild-type strain, in $mid1\Delta cch1\Delta fig1\Delta$ mutant GIC mediated response is still completely dependent on phospholipase C activity, since it is abolished by addition of the phospholipase C inhibitor, 3-nitrocoumarin [31] (data not shown); moreover, in this mutant glucose-induced calcium influx is still appreciable even in the presence of external sub-millimolar calcium concentrations (Fig. 2B), with an intensity comparable to the wild-type strain, indicating that the unknown transporter is not a low affinity transporter. In fact, apparent $K_M$ for calcium in the $mid1\Delta cch1\Delta fig1\Delta$ mutant GIC response was calculated as 43.8 ± 10.3 $\mu$M (Fig. 2C), not far from the $K_M$ calculated for the wild-type strain in the same conditions.

The involvement of an intracellular, vacuolar calcium channel, Yvc1, was also investigated. In yeast, vacuole serves as a major store for Ca$^{2+}$, for the purposes of both detoxification and signalling. The Yeast Vacuolar Channel Yvc1, a homolog of the constitutively active inwardly rectifying calcium channels in mammals known as TRP (Transient Receptor Potential) channels [32], likely representing a calcium-activated calcium channel, has been shown to release Ca$^{2+}$ from the vacuole into the cytosol in response to hyperosmotic shock both by mechanical activation and Ca$^{2+}$-induced calcium release [33]; thus, Yvc1 channel is a calcium-responsive

Fig. 2. Deletion of known transporters only marginally affects glucose-induced calcium response in YPD medium. (A) Glucose-induced calcium response was analyzed as previously described, in presence of 1 mM extracellular calcium chloride, in K601, $mid1\Delta cch1\Delta$, $yvc1\Delta$, $fig1\Delta$, $mid1\Delta cch1\Delta fig1\Delta$ strains grown in YPD medium (panel A). (B) Glucose-induced calcium response was analyzed as previously described, in presence of the indicated extracellular free calcium concentrations, in YPD-grown and then glucose-starved $mid1\Delta cch1\Delta fig1\Delta$ strain. (C) The apparent $K_M$ for calcium of the GIC response in YPD media grown $mid1\Delta cch1\Delta fig1\Delta$ cells, evaluated as described in Section 2, is 43.8 ± 10.3 $\mu$M (panel C).
channel, and it is involved in the amplification of several calcium signals [34].

The yvc1Δ mutant showed only a partial reduction in glucose response: our results suggest that Yvc1 could be involved in glucose-triggered calcium signal amplification also (Fig. 2A).

3.3. Glucose-induced calcium signalling is sensitive to magnesium

In order to better characterize the still unknown GIC transporter, the sensitivity to magnesium of the glucose-induced calcium response was tested. In fact, Mg2+ is the most abundant divalent cation in cells, where it acts as a counter-ion for solutes, as a cofactor in catalytic processes and as a stabilizer for membranes, physiological structures and proteins conformation. Cellular Mg2+ concentration is in the millimolar range, 3 orders of magnitude higher than Ca2+. The two ions appear to affect each other in a competitive way: the impaired growth of yeast cells in high calcium environments is ameliorated by Mg2+ salts in the medium. Recently, it was reported that Ca2+ influx pathways in yeast become (re)activated upon withdrawal of extracellular Mg2+ [35].

Exposure to different concentrations of Mg2+ revealed that glucose-induced calcium response is very sensitive to low concentrations of magnesium, and is already significantly reduced by [Mg2+] lower than 1 mM (Fig. 3A), showing an apparent IC50 of 0.52 ± 0.03 mM. The sensitivity to magnesium is only partially relieved by deletion of HACS components, and is still very high in mid1Δ cch1Δ fig1Δ mutant, where the total response shows an apparent IC50 of 1.04 ± 0.05 mM (Fig. 3B). The difference in sensitivity is reliable, and confirms that the target of inhibition is calcium transport and not some other component of the signalling pathway. Fitting of the curves of inhibition to Hill function gave n values not far from 1, suggesting a simple competitive inhibition to the same ligand site. In order to verify this issue, the initial slope of GIC responses was determined in the mid1Δ cch1Δ fig1Δ mutant while co-varying the calcium and magnesium concentrations: the kinetics are typical of a competitive inhibition (Fig. 3C), giving a Ki of 0.33 ± 0.05 mM.

3.4. The unknown channel is nickel insensitive

GIC response sensitivity to several bivalent cations was tested in the wild-type strain and in the mid1Δ cch1Δ fig1Δ mutant, in order to characterise the unknown channel sensitivity to metal ions. No differences were found in the sensitivity of GIC response to Zn2+ or Mn2+ in the wild-type and in the mutant strain, showing only half inhibition of the response in presence of millimolar levels of metals (data not shown), while the sensitivity to Ni2+ was different. GIC response is not very sensitive to Ni2+ in YPD grown wild-type strain (Fig. 4A), but it is completely resistant to submillimolar concentrations of Ni2+ in the mid1Δ cch1Δ fig1Δ mutant cells (Fig. 4B and C). Furthermore, 30 mM NiCl2 seems even to stimulate GIC response (Fig. 4B): in order to verify if this effect could be related on an agonist effect of Ni2+ on Yvc1-dependent signal amplification, sensitivity of calcium response to Ni2+ was also tested on a mid1Δ cch1Δ fig1Δ yvc1Δ mutant (Fig. 4D), revealing that Yvc1 is actually responsible for signal stimulation at higher concentrations of Ni2+. Anyway, even in the absence of Yvc1, Ni2+ is not able to completely abolish GIC transport, giving a maximal inhibition of less than 30%.

3.5. GIC transport is sensitive to inhibition by gadolinium and nifedipine, but is resistant to verapamil

Several different subtypes of voltage-gated calcium channels have been identified, depending on their electrochemical and biophysical properties. The calcium channel blockers in current use all act inhibiting calcium entry through L-type channels. Among them

Fig. 3. Glucose-induced calcium response is strongly inhibited by Mg2+. Glucose-induced calcium response was analyzed as previously described, in presence of 1 mM CaCl2 and the indicated concentration of MgCl2, in K601 (panel A) and mid1Δ cch1Δ fig1Δ (panel B) strains grown in YPD medium. The Lineweaver–Burk plot of calcium response initial slope (v0) was reported in panel C in the presence of the following magnesium concentration: circles and solid line, 0.3 mM; diamonds and dashed line, 0.5 mM; triangles and dotted line, 1.5 mM.
Fig. 4. Glucose-responsive unknown transporter is not sensitive to Ni^{2+} ions. Glucose-induced calcium (GIC) response was analyzed as previously described, in presence of 1 mM CaCl₂ and the indicated concentration of NiCl₂: K601 (panel A), mid1/cch1/fig1 (panel B) and mid1/cch1/fig1/yvc1 (panel D) strains were grown in YPD medium, and the calcium response to glucose was detected as described after addition of different concentrations of NiCl₂. The percentage of inhibition of total calcium response was reported for a comparison between wild-type (squares and solid line) and mid1/cch1/fig1 (triangles and dashed line) in panel C.

we chose the compounds that were reported to affect HACS channel in yeast [30]: gadolinium, which is specific for stretch-activated ion channels; nifedipine, a dihydropyridine inhibitor, specific for blood vessels smooth muscle L-type calcium channel; verapamil, a phenylalkylamine inhibitor, effective on both vascular and cardiac L-type calcium channels [36].

Glucose-induced calcium response was investigated in mid1Δ cch1Δ fig1Δ yvc1Δ mutant in order to get rid of any interference by intracellular calcium release from the vacuole. GIC transport was found to be extremely sensitive to gadolinium (apparent IC₅₀ 16.7 ± 1.7 μM) (Fig. 5A and D) and to nifedipine (apparent IC₅₀ 0.36 ± 0.13 μM) (Fig. 5B and D), while it is only slightly affected by 20 μM verapamil. Surprisingly, an agonist effect was observed for higher concentrations of verapamil (Fig. 5C), inducing the response up to the double than in the absence of the drug.

The inhibitory effect of nifedipine is likely to be specific for calcium transport, since any other target would show no difference in sensitivity in the mutant strain when compared to the wild-type: in contrast, glucose-induced calcium transport in the wild-type show an IC₅₀ for nifedipine of 1.8 ± 0.005 μM, six-times higher than in the mutant strain (data not shown). Inhibition mechanism was investigated by co-varying nifedipine and calcium (data not shown), which suggested a mixed competition affecting both Kₘ and Vₘₐₓ.

3.6. Glucose-induced calcium signalling is modulated by the cultural medium

Mid1 HACS subunit was reported in literature as essential for glucose-induced calcium peak [18], but those experiments were performed in minimal medium, where the signal is much lower than when yeast cells are grown in rich media (Fig. 6A). This discrepancy could be explained by the calcineurin dependent regulation of calcium transporters, that was already found for HACS system and could be important for other calcium transport systems involved in glucose response. In detail, HACS system was reported to be repressed by calcineurin in rich media [5].

In minimal medium, the effect of the deletion either of CCH1 alone and MID1 or both is more serious (Fig. 6B), consistently with previously observed role of HACS system in minimal medium. Surprisingly, an increase in the response was observed in minimal media-grown mid1Δ cch1Δ fig1Δ mutant: this mutant makes no more difference in calcium transport either if it is grown in minimal or in rich medium (Figs. 6B and 2A). The unknown transporter involved in GIC response, sustaining calcium influx in mid1Δ cch1Δ fig1Δ mutant, seems to be inhibited by Fig. 1 protein when cells are grown in minimal medium, since it becomes fully active in this condition only in Fig. 1 absence.

3.7. Glucose addition to minimal medium grown glucose–deprived cells activates calcineurin dependent transcription

The main effector of calcium availability in the extracellular medium is the Ca/calmodulin/calcineurin pathway, consequently the activity of calcineurin after glucose addition to nutrient deprived cells was investigated. The activation of calcineurin was detected taking advantage of a Crz1-responsive element (CDRE) in the promoter of a LacZ reporter gene [27]: once activated, calcineurin dephosphorylates the Zn-finger transcription factor Crz1/Tcn1, causing it to accumulate in the nucleus where it activates gene expression, thus promoting adaptation to stress.

Calcineurin activity was reported to be higher in rich media than in minimal media, and our results confirmed these data (Fig. 7A and B). After starvation, there was no change in the activity of β-galactosidase. In the wild-type strain, glucose-induced transcriptional activation was observed in minimal medium, but not
GIC transporter is sensitive to gadolinium and to nifedipine, but not to verapamil. GIC response was analyzed as previously described, in presence of 1 mM CaCl₂ and the indicated concentration of inhibitor:

- mid1Δ
- cch1Δ
- fig1Δ
- yvc1Δ

The percentage of inhibition of total calcium response was reported for a comparison between gadolinium (squares and solid line) and nifedipine (triangles and dashed line) in panel D.

Deletion of known transporters seriously affects glucose-induced calcium response only in minimal medium. Glucose-induced calcium response was analyzed as previously described, in presence of the indicated concentration of free extracellular calcium, in K601 (panel A), and in the presence of 1 mM extracellular calcium chloride in K601, mid1Δ cch1Δ, yvc1Δ, fig1Δ, mid1Δ cch1Δ fig1Δ strains (panel B), grown in minimal medium.

Both the effects, higher basal level in rich medium-grown cells and glucose-responsive Crz1-dependent transcription, are calcineurin dependent since none of them is observed in a calcineurin-deficient cnb1Δ strain (Fig. 7A and B).

The reporter gene is activated by glucose when calcium is available in the external medium, but it is not activated by 1 mM extracellular calcium alone (data not shown) or by glucose when calcium is not available in the external medium, indicating that glucose-induced calcium influx is required (Fig. 7A and B). In contrast, the reporter was equally activated in minimal medium in all the mutants in known calcium channels mentioned above (data not shown), showing that residual calcium signal is in any case sufficient to sustain the response.

There are many differences between rich and minimal medium, both in nutrients availability and quality, and in ion composition. The effect of nutrients addition in complete supplemented medium was previously described for pheromone-induced calcium entry.
Fig. 7. Calcineurin-dependent transcription is activated by glucose re-addition after starvation to cells grown in minimal medium, but not to cells grown in rich medium. Crz1-responsive promoter activity was assayed as described in Section 2 in wild-type strain grown in minimal (panel A) or in rich medium (panel B), with (open bars) or without (closed bars) the addition of either 1 mM CaCl₂ to minimal medium or 1 mM EGTA to YPD medium during cell growth. Grey bars represent the same results obtained in cnb1/Delta1 strain in minimal (panel A) or in rich medium (panel B). GIC response was analyzed as previously described, in presence of the indicated concentrations of free extracellular calcium, in K601 strain grown in minimal medium with 1 mM CaCl₂ added (panel C) or in YPD medium with 1 mM EGTA (panel D).

[5]. Nevertheless, among the differences between rich and minimal medium, there is also calcium availability: calcium concentration is far higher in rich media than in minimal medium, and this could be sufficient to impinge on basal Crz1-dependent response in differently cultivated cells.

In order to assess if what observed in glucose-induced calcineurin signals was related to the different level of calcium availability and consequent calcineurin activity during cell growth, calcineurin activity was assayed in wild-type cells either grown in YPD medium or in minimal medium, either in exponential growth, after nutrient-starvation or after glucose stimulation in presence or in absence of extracellular calcium.

Calcineurin activity dependence on calcium availability in the medium was investigated adding 1 mM CaCl₂ to minimal medium, in order to raise the free calcium concentration in this medium to levels comparable to YPD medium, or 1 mM EGTA to YPD medium, in order to low down the concentration of free calcium in this medium: these modifications were aimed to abolish the difference in calcium content in the two cultural media. Cells exponentially growing in these modified media were collected, starved for nutrients for 2 h and then tested for calcineurin dependent transcriptional activity. As can be observed in Fig. 7 A and Table 2, calcium availability during growth in minimal medium caused a rise in basal Crz1-dependent transcriptional activity, as expected, but most of all strongly inhibited stimulation upon glucose addition: this indicates that high basal calcineurin activity counteracts its glucose-responsiveness. Conversely, EGTA addition to YPD medium only marginally rescued calcineurin susceptibility to glucose, and had almost no effect on Crz1 basal activity level (Fig. 7B and Table 2). This could be due to inefficient calcium chelating by EGTA, but this is unlikely since the same treatment showed an evident effect on calcium signalling (see later), and actually a partial relief in glucose-triggered calcineurin-dependent transcriptional activation is observed in YPD/EGTA grown cells (Fig. 7B).

The effect of calcium content in the medium on glucose-induced calcium response was also analyzed, to reveal if modifications in calcium flux could justify what observed in glucose-induced Crz1-dependent transcription. As above, wild-type cells were grown either in minimal medium with the addition of 1 mM CaCl₂, or in YPD medium in the presence of 1 mM EGTA, then starved for nutrients and exposed to glucose in the presence of extracellular calcium. As Crz1-dependent transcription, GIC response was also affected by external calcium availability during cell growth: EGTA added to YPD medium exposed cells to low calcium availability during growth, and GIC response became lower, even if the shape of peaks and their sensitivity to extracellular calcium did not change (Fig. 7D). In contrast, GIC response was only slightly enhanced in wild-type cells grown in minimal medium with calcium added (Fig. 7C).

Consequently, extracellular calcium level seems to be important in the regulation of glucose-induced Crz1-dependent transcription, but other factors are expected to influence Crz1 responsiveness to nutrients in rich medium growing cells. Previous work suggested that PKA and calcineurin signalling pathways function antagonis-

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Table 2
Calcineurin activation stimulation-fold in wild-type K601 strain. Crz1-responsive promoter activity was assayed as described in Section 2 in wild-type strain grown in the indicated media supplemented either with 1 mM EGTA or 1 mM CaCl₂ when indicated. Stimulation-fold upon addition of 100 mM glucose in the presence of 1 mM CaCl₂ was calculated on at least three independent experiments.

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<th>EGTA</th>
<th>CaCl₂</th>
<th>Stimulation-fold</th>
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<tr>
<td>Minimal medium</td>
<td>–</td>
<td>+</td>
<td>7.1 ± 2.4</td>
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<tr>
<td>YPD medium</td>
<td>–</td>
<td>–</td>
<td>1.2 ± 0.2</td>
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<td></td>
<td>+</td>
<td>–</td>
<td>2.3 ± 0.2</td>
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PKA activity is involved in the inhibition of glucose-induced Crz1-dependent transcription in cells grown in YPD medium. Crz1-responsive promoter activity was assayed as described in Section 2 in \( \text{cyr1}^{\Delta} \text{pde2}^{\Delta} \text{yak1}^{\Delta} \) strain grown in minimal medium (panel A) or in rich medium (panel B), with (open bars) or without (closed bars) the addition of 3 mM cAMP in the cultural medium.

Table 3
Calcineurin activation stimulation-fold in \( \text{cyr1}^{\Delta} \text{pde2}^{\Delta} \text{yak1}^{\Delta} \) strain. Crz1-responsive promoter activity was assayed as described in Section 2 in \( \text{cyr1}^{\Delta} \text{pde2}^{\Delta} \text{yak1}^{\Delta} \) strain grown in the indicated media supplemented or not with 3 mM cAMP when indicated. Stimulation-fold upon addition of 100 mM glucose in the presence of 1 mM CaCl₂ was calculated on at least three independent experiments.

<table>
<thead>
<tr>
<th>Media</th>
<th>3 mM cAMP</th>
<th>Stimulation-fold</th>
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<tr>
<td>Minimal</td>
<td>–</td>
<td>13.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.6 ± 1.0</td>
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<tr>
<td>YPD</td>
<td>–</td>
<td>2.1 ± 0.2</td>
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<tr>
<td></td>
<td>+</td>
<td>1.4 ± 0.3</td>
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Fig. 8. PKA activity is involved in the inhibition of glucose-induced Crz1-dependent transcription in cells grown in YPD medium. Crz1-responsive promoter activity was assayed as described in Section 2 in \( \text{cyr1}^{\Delta} \text{pde2}^{\Delta} \text{yak1}^{\Delta} \) strain grown in minimal medium (panel A) or in rich medium (panel B), with (open bars) or without (closed bars) the addition of 3 mM cAMP in the cultural medium.

Calcineurin deficiency affects GIC transport functionality. GIC response was analyzed as described in Section 2 in the \( \text{cnb1}^{\Delta} \) (A) and in the \( \text{mid1}^{\Delta} \text{cch1}^{\Delta} \text{fig1}^{\Delta} \text{cnb1}^{\Delta} \) (B) mutant strains grown in YPD medium.

Taken together, these results suggest that both factors, calcium levels in external medium and PKA activity, seem to act on calcineurin-dependent transcriptional activity regulating its responsiveness to nutrient signals.

3.8. Calcineurin is required for nutrient availability dependent regulation of functionality of calcium transporters

In order to assess calcineurin role in adapting calcium transporters functionality to nutrient availability, the gene \( \text{CNB1} \), encoding for calcineurin activating subunit, was deleted both in the wild-type strain and in the \( \text{mid1}^{\Delta} \text{cch1}^{\Delta} \text{fig1}^{\Delta} \text{cnb1}^{\Delta} \) strain and GIC response was analyzed in the mutant strains grown in YPD medium (Fig. 9). In the \( \text{cnb1}^{\Delta} \) strain, GIC response was similar to the wild-type response, except for its sensitivity to calcium concentrations, which revealed a higher response at very low calcium concentrations, and an inhibitory effect at higher concentrations. In contrast, in the \( \text{mid1}^{\Delta} \text{cch1}^{\Delta} \text{fig1}^{\Delta} \text{cnb1}^{\Delta} \) strain, the response was seriously impaired, suggesting that calcineurin deficiency impinges on GIC transport system functionality. This last result also suggests that the peculiar response in the \( \text{cnb1}^{\Delta} \) strain could be due to the...
major contribution of the HACS transport, characterized by higher affinity than GIC system itself.

4. Discussion

Several yeast proteins show homology with human calcium channels that have been implicated in channelopathies, allowing for study of the genotype/phenotype correlation in several diseases [38].

Recently, conservation between yeast calcium system and mammalian cardiac myocytes was described. This suggests that knowledge on calcium transport in yeast can be used to help understanding of cardiac disease [39] or to create a model for channelopathies research. Thus, insights in calcium homeostasis/signalling in yeast can be very useful to understand homologous systems in other eukaryotic cells.

Electrophysiological recordings of ion channel activity in the plasma membrane of live yeast cells require non-physiological modification of the cells in order to remove the cell wall, but genetic and cellular biology tools available in yeast have allowed to dissect the different calcium transporters as they are mainly involved in responses induced by different stimuli: like pheromone-induced calcium influx, glucose-induced calcium influx involves several transporters on the plasma membrane. Two proteins, Mid1 and Cch1, composing a high affinity calcium transport system (HACS), are required for GIC response in cells growing in minimal medium (Fig. 2). This is not true for cells growing in rich medium, which again sustains the hypothesis of a nutrient-dependent regulation of calcium transporters, relying on different calcineurin activity related to nutrient and calcium availability in the medium [5]. Consistently, glucose-induced calcium influx response is very sensitive to cultural conditions, being much higher inYPD growing cells than in minimal medium cultured cells (Figs. 1 and 2). This is probably related to a differential expression or functionality of calcium transporters: HACS system was already reported to be negatively regulated by calcineurin in rich media [5]. Another calcium specific, high affinity ($K_M \sim 50 \mu M$) calcium transport has been identified in this work, physiologically fully working in rich medium but not in minimal medium. This putative calcium flux was defined as Glucose Induced Calcium flux (GIC). Differently from known calcium transporters in yeast it is rather resistant to nickel (Fig. 5); GIC transport is also resistant to verapamil, which acts as an agonist at high concentration. Conversely, GIC channel is sensitive to magnesium, with an apparent $IC_{50}$ of $1.04 \pm 0.05 \mu M$ (Fig. 4), to gadolinium (apparent $IC_{50}$ $16.7 \pm 1.7 \mu M$) and to nifedipine (apparent $IC_{50}$ $0.36 \pm 0.13 \mu M$) (Fig. 5). This is peculiar since both verapamil and nifedipine are inhibitors of L-type voltage-gated calcium channels, even if with a slightly different specificity. Anyway, these two classes of inhibitors act on different target sites in L-type voltage-gated channels [36], consequently there could be no relation between their effect on a non-conventional calcium channel. This peculiar pharmacological trait in fact could suggest that GIC transport would rely on a novel class of calcium channels; consistently, S. cerevisiae genome does not encode for any protein homolog to known calcium channels, besides the already characterized channels described above.

Wild-type yeast growing in rich medium was previously reported to accumulate calcium primarily by a low-affinity $Ca^{2+}$ uptake system. Raising extracellular $Mg^{2+}$ from 1 to 10 mM strongly inhibited a low affinity calcium transport, exposing a $Mg^{2+}$-resistant high affinity $Ca^{2+}$ uptake system [9]. More recently, a mathematical model was constructed to reproduce calcium transients in yeast [40] which suggested the existence of two $Mg^{2+}$-sensitive influx pathways (indicated as transporter X and transporter M), both targets of rapid $Ca^{2+}$-dependent feedback inhibition. Computational analysis revealed the existence of a transporter, called X, which should be inhibited by magnesium at very low concentrations and characterized by high affinity for calcium, consistently with the characteristic revealed for the transporter involved in glucose response.

GIC transport seems to be fully functional in rich medium, and almost completely inactivated in minimal medium. Glucose-induced calcium signal seems to be enhanced by calcineurin activity, since calcium addition to minimal media activates calcineurin-dependent transcription and can rise the faint calcium influx which is typical in this medium: it is possible that calcineurin is responsible for the responsiveness of GIC channel, which is in fact fully functional only in rich medium (Fig. 2), where calcineurin activity is higher (Fig. 7). In fact, calcineurin activity deficiency implies the severe inhibition of GIC transport in a mid1Δ cch1Δ fig1Δ cnb1Δ strain (Fig. 9B).

Molecular identification of GIC channel is not a trivial challenge, since no genes encoding mammalian calcium channels homologs were identified in S. cerevisiae genome, besides CCH1 and YVC1, either in literature or by bioinformatic researches we have performed (data not shown). Anyway, Mid1 itself, which was reported to generate a stretch-activated calcium channel when expressed in mammalian cells, doesn’t show any evident similarity to mammalian calcium channels. Further work on genetic screenings will take advantage of GIC channel pharmacological properties here identified.

Activation of calcineurin has been recognized as being essential for survival under diverse stress conditions, such as pheromones or ion-induced stress [23], which is in contrast with the effect of nutrient availability on calcineurin-responsive Crz1 transcription factor: here it is reported for the first time that calcineurin can be responsive to nutrients. A relation between calcineurin and carbon sources was already suggested [41], since many genes encoding carbohydrate-metabolizing enzymes were reported to be regulated by calcium/calcineurin pathway. Consistently, PKA and calcineurin signalling converge on regulation of Crz1 transcription factor. The effect on glucose-induced Crz1-dependent transcriptional response of these two main regulators of yeast metabolism was considered, in order to clarify their role in regulating calcium influx effect in these two nutritional conditions, revealing that the effect of glucose-induced calcium influx on calcineurin-dependent transcription is counteracted not only by PKA, as expected, but also by calcium availability in the medium during exponential growth. The phosphoinositide specific phospholipase C Pcl1 acts together with the membrane receptor Gpr1 and the associated Go protein Gpa2 in a pathway separated from Ras1/Ras2 and converging on activation of adenylate cyclase and PKA [42]: pcl1Δ strain displays phenotypes characteristic of cells with decreased PKA activity, such as increased expression of stress-responsive genes mediated by decreased PKA-mediated inhibitory phosphorylation of Msn2 stress-responsive transcription factor [42], while calcium stress seems to act both on Msn2/Msn4 transcription factors and Snf1 protein kinase regulatory phosphorylation [43]. However, the complex interaction of glucose-induced calcineurin activation and classical glucose activated pathways (Snf3/Rgt2-1, Snf1/Mig1, and PKA pathways) is far from being elucidated and deserves further study.

Conflict of interest statement

There are no conflicts of interest concerning this work.

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