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Calcium signaling and sugar-induced activation of plasma membrane H^+-ATPase in *Saccharomyces cerevisiae* cells

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Received 23 February 2006
Available online 23 March 2006

Abstract

In this work, we show that glucose-induced activation of plasma membrane H^+-ATPase from *Saccharomyces cerevisiae* is strongly dependent on calcium metabolism and that the glucose sensor Snf3p works in a parallel way with the G protein Gpa2p in the control of the pathway. The role of Snf3p is played by the Snf3p C-terminal tail, since in a strain with the deletion of the SNF3 gene, but also expressing a chimera protein formed by Hxt1p (a glucose transporter) and the Snf3p C-terminal tail, a normal glucose-activation process can be observed. We present evidences indicating that Snf3p would be the sensor for the internal signal (phosphorylated sugars) of this pathway that would connect calcium signaling and activation of the plasma membrane ATPase. We also show that Snf3p could be involved in the control of Pmc1p activity that would regulate the calcium availability in the cytosol.
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Keywords: Plasma membrane ATPase; Calcium signaling; Sugar-induced activation

Many experimental evidences suggest that the regulatory mechanisms operating to control the plasma membrane ATPase activity in yeast are mainly post-translational [6,26,29]. The glucose-induced activation of the enzyme leads to a combined effect on the kinetic parameters provoking a reduction of K_m for ATP and an increase of the V_{max} of ATP hydrolysis, both provoked by the phosphorylation of two different sites found in the C-terminal regulatory domain of the enzyme [8,12,28]. The RAS-cAMP-protein kinase A pathway is not involved in glucose-induced activation of ATPase, but glucose phosphorylation is required in this activation process [4]. It seems that a phosphatidylinositol type-signaling pathway could be involved in the glucose-induced activation of the plasma membrane H^+-ATPase [7] corroborating the role played by phospholipids in the control of ATPase activity [25].

The involvement of phospholipase C in the glucose-induced phosphatidylinositol turnover and in the activation of the plasma membrane H^+-ATPase of *Saccharomyces cerevisiae* was also demonstrated, as well as the requirement of the cell wall integrity/remodeling MAPK cascade and/or protein kinase C, leading to the idea that, similar to mammalian cells, the glucose-induced activation of the plasma membrane H^+-ATPase could be related to a similar pathway [9,10,32]. Evidences were also found indicating the involvement of the G protein Gpa2 and the glucose sensor Snf3 in this activation process. Moreover, sugar phosphorylation seems to be the internal signal and it seems to be transduced via Gpa2p [32].

Interestingly, the pathway(s) participating in calcium homeostasis share many similarities with the mechanism that regulates plasma membrane H^+-ATPase activity in yeast cells. Both are stimulated by sugars that require sugar

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.03.078

transport and phosphorylation as well as the participation of phospholipase C and Gpa2 [34,35].

Here, we demonstrate that an appropriated calcium signaling is necessary for the glucose-induced activation of the H^+-ATPase. Snf3p seems to work in parallel to Gpa2p, and its C-terminal tail is apparently responsible for his role. Moreover, our data also suggest the involvement of Snf3p in the control of calcium accumulation, most probably through the control of the vacuolar Ca^{2+}-ATPase, Pmc1p.

Together, the data lead us to propose a pathway that establishes how calcium metabolism and regulation of plasma membrane H^+-ATPase activity would be connected in yeast cells.

Materials and methods

Strains and growth conditions. The yeast strains used in this study are shown in Table 1. The plasmids pRS316 and pHXT1/SNF3 were kindly provided by M. Johnston (Washington University School of Medicine—St. Louis). Yeast cells were grown in medium containing 2% peptone and 1% yeast extract (YP) supplemented with carbon sources and 1 M sorbitol (when indicated). In all experiments, the cells were grown in a rotatory incubator New Brunswick Model G25 (200 rpm) at 30 °C until the end of logarithmic phase ($OD_{600nm} \sim 2.0$). Cells were washed three times by centrifugation (approximately 2000*g*) with 25 mM Mes buffer, pH 6.0, with or without 1 M sorbitol (as required).

Measurement of H^+-ATPase activity. The cells were resuspended in 100 mM Mes/Tris buffer (pH 6.5) and incubated at a density of 150 mg/ml (wet mass) in a shaking water bath at 30 °C. After 20 min control samples were collected and sugars were added to a final concentration of 100 mM. After 10 min samples containing 4.5 g cells (wet weight) were taken from the suspension and the cells collected on glass fibre filters by vacuum filtration. For time-course measurements, after sugar addition, samples containing 750 mg (wet weight) were taken from the suspension at different times. The cells were immediately frozen in liquid nitrogen and stored until use. The procedures used to obtain plasma membranes and to determine ATPase activity were described previously [4]. Protein content was determined by using a standard method [20].

Measurement of proton-pumping activity. Five hundred milligrams (wet weight) of cells were resuspended in 100 mM Tris–HCl buffer, pH 4.5, containing 100 mM KCl and incubated in a vessel in a total volume of 5.0 ml. Changes in the pH of the suspension were recorded before and after the addition of 100 mM glucose. Calibration pulses of 100 nmol HCl were also added. The maximal rate of proton pumping was calculated from the slope of the line indicating the pH variation in the medium [32].

Measurement of total cellular Ca^{2+} levels and cytosolic free calcium concentration. The determination of total cellular calcium was performed essentially as previously described [21]. Briefly, yeast cells grown in YPD or YPGal were washed with fresh YP by centrifugation. The pellets present in Eppendorf tubes were dried in a Savant SpeedVac system and then resuspended in 1 M HCl. The calcium contents of the samples were measured by atomic absorption in a Varian AA-2 spectrophotometer.

The cytosolic free calcium concentration was measured by using the aequorin-based method [33]. Strains containing the apoaequorin-expressing plasmid pVTU-AEQ were grown in rich (YP) medium, while strains carrying the pRS316 or the pHXT1/SNF3 plasmid, and the corresponding wild type, were grown on minimal media (SD) supplemented either with 2% glucose (YPD or SDGlu) or galactose (YPGal), to exponential phase ($3–8 \times 10^6$ cells/ml). Then, the cells were washed by filtration and resuspended in Mes/Tris 0.1 M, pH 6.5. After a 2-h incubation at room temperature, cells were loaded with coelenterazine as described [33]. To measure the sugar-induced calcium uptake, aequorin luminescence was measured in a Berthold Lumat LB 9501/16 luminometer at intervals of 10 s for 1 min before and for at least 6 min after the addition of 100 mM glucose or galactose. Results of representative experiments out of at least three repetitions are shown.

The pVTU-AEQ plasmid was generated by inserting in *Xho*I/*Pst*I sites of pVTU the *Xho*I/*Pst*I-digested fragment obtained by PCR on pYX212-AEQ [33] using the oligonucleotides TTTCTCGAGAATCTATAACTA CAAAAAACACATACAGGAA and TAACTGCAGGCCCTAGGAT CCATGGTGAA. The pVTW-AEQ plasmid was generated with the same strategy using the pVTW plasmid, a pVTU version where the *URA3* marker was substituted with the *TRP1* marker, gently given by Marco Vanoni (Università di Milano-Bicocca, Milan, Italy).

Molecular biology methods. Preparation and manipulation of nucleic acids were done using standard procedures [27,30]. *Escherichia coli* cells were transformed by calcium chloride method. Yeast cells were transformed by using the lithium acetate protocol [15]. To verify the disruption made in different genes, standard Southern blot analysis was performed.

Reproducibility of results. The experiments were performed at least three times with consistent results. Statistics analysis were done by using the Student's *t* test. Differences were considered statistically significant when the *P* value was smaller than 0.05.

Table 1
Saccharomyces cerevisiae strains used in this study

Strain	Genotype	Source
YM 6217	MATa ura 3-52 his 3-200 ade 2-101 lys 2-801 trp 1-903 leu 2-3, 112 tyr 1-501 MET?	Mark Johnston
YM 6175	YM6217 *snf3::* HIS 3	Mark Johnston
LBCM430	YM6217 *gpa2::* LEU2	This work
LBCM431	YM6175 *snf3::* HIS 3 *gpa2::* LEU2	This work
LBCM432	YM6217 *snf3::* HIS 3 + *pBM3436* (*pHXT1-HXT1/SNF3*)	This work
LBCM433	YM6217 *snf3::* HIS 3 *gpa2::* LEU2 + *pRS316* (vector)	This work
BY4742	MATα his3 Δ1 leu2 Δ0 lis2 Δ0 ura3 Δ0	Euroscarf
YGL006w	BY4742 *pmc1::*KanMX2	Euroscarf
Y11153	BY4742 *mid1:: KanMX2*	Euroscarf
Y13177	BY4742 *fig1:: KanMX2*	Euroscarf
Y12409	BY4742 *pdr5:: KanMX2*	Euroscarf
Y17201	BY4742 *snf3:: KanMX2*	Euroscarf
LBCM438	BY4742 *pgm2:: URA3*	This work
LBCM439	BY4742 *pmc1:: sKanMX2 pgm2::URA3*	This work
LBCM440	BY4742 *pmc1:: KanMX2 snf3::HIS3*	This work
PJ69-4A	MATa trp1-901 leu2-3,112 ura3-52 his3-200 gsal4 Δ gal80 Δ LYS2::GAL1-HIS3s GAL2-ADE2 met2::GAL7-lacZ	James Caffrey
PJ69-4A1	PJ69-4A *arg82::KanMX2*	James Caffrey

Results

Calcium metabolism is essential for the glucose-induced activation of the H^+-ATPase

It has already been suggested as regards the existence of a pathway in which glucose (sugar) uptake and phosphorylation take place, the glucose sensor Snf3p, the G protein Gpa2, phospholipase C, and protein kinase C interacting with each other leading to the activation of the H^+-ATPase [7,9,32]. Since Gpa2 and phospholipase C are also involved in the glucose-induced calcium signaling, we further inquired on the involvement of calcium as an active messenger in this pathway.

Extracellular calcium seems to play a role in glucose-induced H^+-efflux, since pre-incubation of wild type cells with EGTA inhibits this process. This inhibitory effect of EGTA could also be caused by a possible interference with the measurement of proton pumping, with ATPase activity itself and/or to toxic effects on the yeast cells. However, the re-addition of increasing calcium concentrations, together with a fixed EGTA concentration, causes a clear recovery in the glucose-induced H^+-efflux (Table 2, Lines 1–5).

The pre-incubation of wild type cells with 12 mM EGTA also inhibits the glucose-induced activation of ATPase (Fig. 1); but, contrary to the glucose-induced H^+-efflux observed in vivo, the pre-incubation of wild type cells with EGTA plus calcium (both at 12 mM) did not restore the activation of the enzyme. This is probably due to differences in the pre-incubation conditions, since the addition of 12 mM of both EGTA and calcium at pH 4.5 (measurement of proton-pumping activity) turns out in around 9 mM of free calcium, while at pH 6.5 (measurement of ATPase activity), the concentration of free calcium is nearly 0.2 mM. Therefore, the concentration of calcium was calculated to assure that the free calcium available in these two different conditions were equivalent. The results demonstrate that excess of free calcium available is indeed important for the activation of the plasma membrane ATPase (Fig. 1).

The effects of EGTA in the sugar-induced calcium signaling were also verified by using the aequorin assay. Fig. 2A demonstrates that when free calcium availability is titrated by varying both the amount of EGTA and calcium in the incubation medium, the sugar-induced calcium signaling is affected in a similar way as observed for the measurement of ATPase activity.

Table 2
Glucose- or galactose-induced external acidification in different *Saccharomyces cerevisiae* strains grown and/or incubated in different conditions

Genetic background	Growth carbon source*	Inducer	Strain	H^+-pumping rate + SD (mmol H^+ min^{-1} $gcell^{-1}$)	Line
YM6217	Glucose	Glucose	Wild type	0.574 ± 0.084	1
			+12 mM EGTA	0.422 ± 0.051	2
			+12 mM EGTA + 9 mM Ca^{2+}	0.464 ± 0.007	3
			+12 mM EGTA + 17 mM Ca^{2+}	0.646 ± 0.008	4
			+12 mM EGTA + 22 mM Ca^{2+}	0.724 ± 0.108	5
		Galactose	Wild type	0.074 ± 0.018	6
	Galactose	Glucose	wild type	0.295 ± 0.024	7
		Galactose		0.191 ± 0.014	8
	Glucose	Glucose	*snf3* Δ	0.299 ± 0.065	9
		Galactose		0.093 ± 0.017	10
	Galactose	Glucose		0.274 ± 0.051	11
		Galactose		0.108 ± 0.028	12
	Glucose	Glucose	*snf3* Δ + *pHXT1-HXT1/SNF3*	0.515 ± 0.004	13
		Galactose		0.068 ± 0.011	14
	Galactose	Glucose		0.443 ± 0.010	15
		Galactose		0.192 ± 0.021	16
	Glucose	Glucose	*gpa2* Δ	0.400 ± 0.010	17
		Galactose		$0.042 + 0.007$	18
	Galactose	Glucose		0.398 ± 0.076	19
		Galactose		$0.029 + 0.017$	20
	Glucose	Glucose	*snf3* Δ *gpa2*Δ	0.169 ± 0.033	21
		Galactose		0.052 ± 0.013	22
	Galactose	Glucose		0.123 ± 0.008	23
		Galactose		0.097 ± 0.027	24

Important remark: the concentrations of calcium indicated in Lines 3–5 represent the free calcium availability in the incubation conditions (pH 4.5) originated by the addition of 12, 20, and 24 mM, respectively.

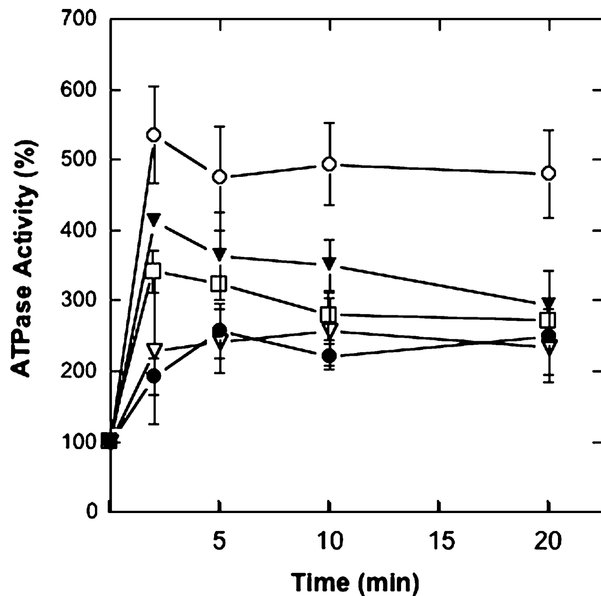

Fig. 1. Calcium metabolism and plasma membrane H^+-ATPase activation in *S. cerevisiae* strains. Glucose-induced activation in YM6217 wild type strain in the absence (○) or in the presence of 12 mM EGTA (●) or in the presence of 12 mM EGTA plus increasing free calcium concentrations*: 0.2 mM (▽), 17 mM (▼), and 22 mM (□). *These calcium concentrations represent the free calcium availability in the incubation conditions (pH 6.5) originated by the addition of 12, 28, and 33 mM, respectively.

Role of calcium transporters in the glucose-induced activation of the H^+-ATPase

It was already shown that the Mid1p is a plasma membrane protein involved in the high affinity calcium influx in yeast cells [14,35]. Nevertheless, the results obtained demonstrate that calcium uptake through Mid1p seems not to be required for the sugar-induced activation of plasma membrane H^+-ATPase. The H^+-efflux rate observed in the mutant is comparable to those of the corresponding wild type cells (Table 3, Lines 1–8). The same pattern was observed for the sugar-induced H^+-ATPase activation in the *mid1* Δ mutant and wild type cells grown on glucose or galactose (data not shown). These results seem to be in apparent contradiction with those suggesting the involvement of Mid1p in the sugar-induced transient elevation of cytosolic calcium; nevertheless, the results shown in Fig. 2B confirm that Mid1p is not required for the glucose-induced calcium signaling as demonstrated before.

Similar experiments performed with a mutant strain in the *FIG1* gene that encode for the low affinity Ca^{2+} transporter [22,23] demonstrated a normal activation comparable to the wild type strain (Table 3, Lines 9–12). Moreover, Pdr5p, a member of the *S. cerevisiae* PDR family of ABC proteins, seems to act in the control of calcium uptake in yeast cells [36]. However, the absence of a functional Pdr5p does not impair that the addition of sugars triggers normal activation of the ATPase (Table 3, Lines 13–16).

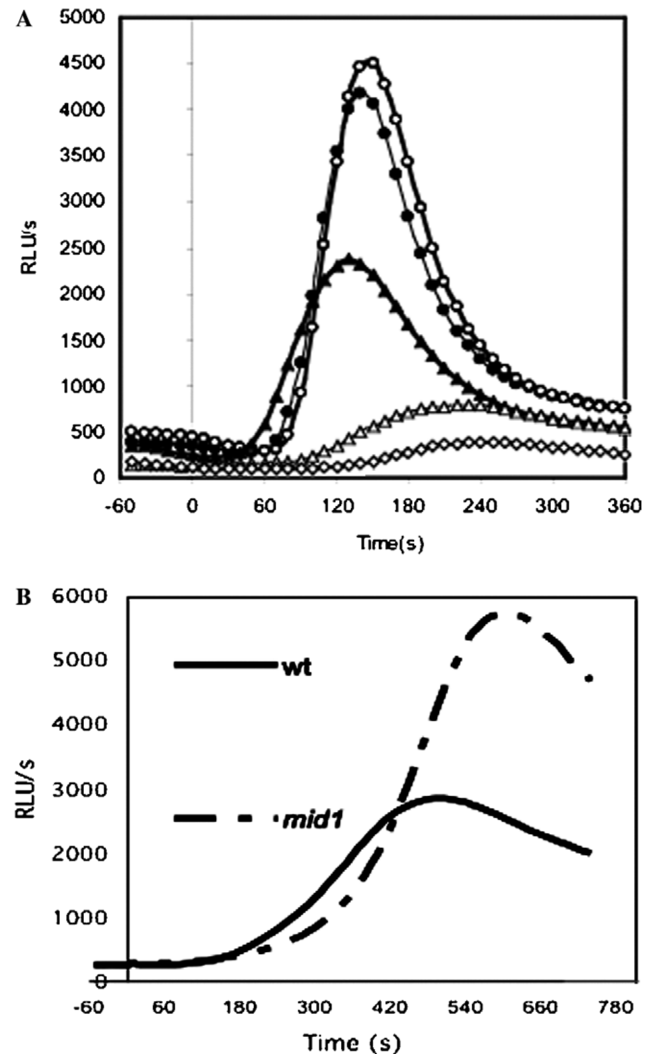

Fig. 2. The role of external calcium in the sugar-induced calcium signaling in *S. cerevisiae* strains. (A) Glucose-induced calcium signaling in glucose-grown YM6217 wild type cells in different conditions: in the presence of 12 mM $CaCl_2$ (●); in the presence of 12 mM EGTA (◊); or in the presence of 12 mM $CaCl_2$ and 1 mM (○), 12 mM (▲), or 30 mM (△) EGTA, giving, respectively, 11 mM, 0.2 mM or 0.002 mM free calcium concentration. (B) Glucose-induced calcium signaling in glucose-grown BY4742 wild type cells (solid line) and its correspondent *mid1* Δ mutant (dashed line).

Calcium signaling and glucose-induced activation of the H^+-ATPase

The involvement of calcium metabolism in the ATPase regulation was further investigated by using a strain containing a deletion in the *ARG82* gene that encodes for a dual kinase that phosphorylates IP_3 generating IP_4 and IP_5. This strain presents a wider glucose-induced calcium signal with a clear amplification of IP_3 accumulation inside the cell [34], leading to the suggestion that IP_3 could be a messenger connected to the calcium homeostasis, like in mammalian cells. The glucose-induced extracellular acidification observed

Table 3
Glucose- or galactose-induced external acidification in different *Saccharomyces cerevisiae* strains grown and/or incubated in different conditions

Genetic background	Growth carbon source*	Inducer	Strain	H^+-pumping rate + SD (mmol $H^+ min^{-1} g cell^{-1}$)	Line
BY4742	Glucose	Glucose	Wild type	0.892 ± 0.093	1
		Galactose		0.063 ± 0.023	2
	Galactose	Glucose		0.451 ± 0.031	3
		Galactose		0.259 ± 0.016	4
	Glucose	Glucose	*mid1* Δ	0.809 ± 0.099	5
		Galactose		ND	6
	Galactose	Glucose		0.532 ± 0.063	7
		Galactose		0.226 ± 0.011	8
	Glucose	Glucose	*fig1* Δ	0.820 ± 0.113	9
		Galactose		0.015 ± 0.004	10
	Galactose	Glucose		0.475 ± 0.050	11
		Galactose		0.199 ± 0.022	12
	Glucose	Glucose	*pdr5* Δ	0.946 ± 0.087	13
		Galactose		ND	14
	Galactose	Glucose		0.483 ± 0.036	15
		Galactose		0.213 ± 0.014	16
	Glucose	Glucose	*pgm2* Δ	0.553 ± 0.057	17
		Galactose		0.031 ± 0.010	18
	Galactose	Glucose		0.351 ± 0.024	19
		Galactose		0.030 ± 0.009	20
	Glucose	Glucose	*pmc1* Δ	1.143 ± 0.150	21
		Galactose		0.010 ± 0.001	22
	Galactose	Glucose		0.420 ± 0.003	23
		Galactose		0.301 ± 0.007	24
	Glucose	Glucose	*pgm2* Δ *pmc1* Δ	2.010 ± 0.332	25
		Galactose		0.065 ± 0.002	26
	Galactose	Glucose		0.500 ± 0.060	27
		Galactose		0.244 ± 0.036	28
	Glucose	Glucose	*Snf3* Δ	0.642 ± 0.046	29
		Galactose		0.017 ± 0.003	30
	Galactose	Glucose		0.212 ± 0.033	31
		Galactose		0.134 ± 0.021	32
	Glucose	Glucose	*snf3* Δ *pmc1* Δ	0.866 ± 0.038	33
		Galactose		ND	34
	Galactose	Glucose		0.520 ± 0.033	35
		Galactose		0.198 ± 0.008	36

ND, not detectable.

demonstrates that the pumping activity is higher in the *arg82* Δ mutant (0.601 ± 0.090 mmol $H^+ min^{-1} g cell^{-1}$) than in the correspondent PJ69-4A wild type strain (0.408 ± 0.037 mmol $H^+ min^{-1} g cell^{-1}$). The glucose-induced ATPase activation is more pronounced in this mutant suggesting that calcium signaling is indeed involved in the ATPase regulation (Fig. 3).

Calcium homeostasis and sugar-induced activation of the H^+-ATPase

Yeast strains presenting mutation in the gene *PGM2* (that encodes for the major isoform of phosphoglucomutase) exhibit a carbon source-dependent defect in both cellular calcium homeostasis and signaling [1,13,35]. For

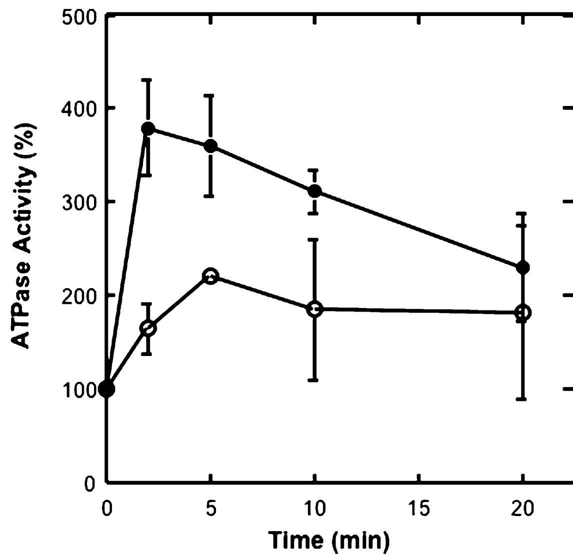

Fig. 3. IP3 metabolism and plasma membrane H^+-ATPase activation in *S. cerevisiae* strains. Glucose-induced enzyme activation in glucose-grown PJ69-4A wild type cells (○) and its correspondent *arg82* Δ mutant (●).

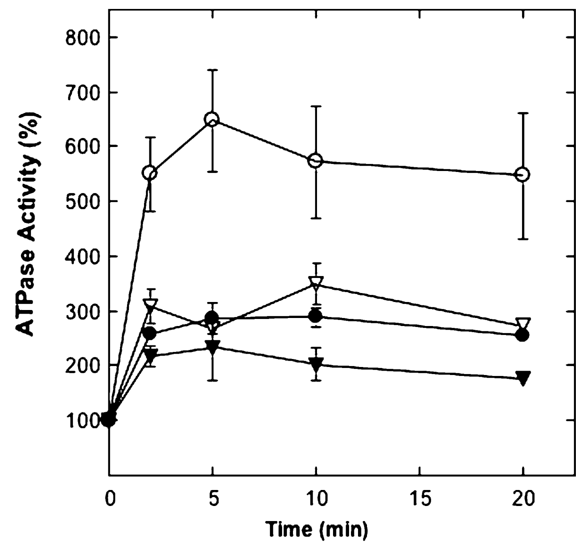

Fig. 4. The role of Snf3p in the sugar-induced activation of the plasma membrane H^+-ATPase in *S. cerevisiae* strains. Glucose-induced activation in the YM6217 wild type (○); in the *snf3* Δ mutant (●); in the *gpa2* Δ mutant (▽); in a *snf3* Δ *gpa2* Δ double mutant (▼) grown on glucose.

wild type cells the higher ATPase activity was measured when glucose-grown cells are re-exposed to glucose than when they are grown on galactose and glucose or galactose is re-added (Table 2, Lines 1, 7, and 8; Table 3, Lines 1, 3, and 4). However, in glucose-grown cells from the corresponding *pgm2* Δ mutant, the addition of glucose triggers a partial activation of the ATPase with a level of activation intermediary between those registered for wild type cells grown on glucose with re-exposition to glucose or grown on galactose and re-exposed to glucose (Table 3, Lines 1, 3, and 17). For the galactose-grown *pgm2* Δ cells, there is a partial activation of the enzyme upon addition of glucose and any ATPase activation after addition of galactose (Table 3, Lines 19 and 20), in agreement with the disturbs observed in the cytosolic calcium signaling in similar conditions [13].

The alteration in calcium homeostasis in *pgm2* Δ mutant cells was attributed to an 8-fold increase in the glucose-1-P level that would be related to a excessive vacuolar Ca^{2+} uptake by the Ca^{2+}-ATPase Pmc1p. Indeed, the disruption of *PMC1* gene in a strain presenting the *pgm2* Δ mutation suppresses the Ca^{2+} related phenotypes observed in this strain [2], and it also led to recovery of the sugar-induced activation of the ATPase (Table 3, Lines 1–4, 17–20, and 25–28).

The role of Snf3p and Gpa2p in the glucose-induced H^+-ATPase activation

Snf3p acts as extracellular glucose sensor, involved in the regulation of expression of specific hexose transporter genes [24]. By its turn, Gpa2p participates in a complex anchored in the plasma membrane with the Gpr1 receptor and phospholipase C [3,18]. Both Snf3p and Gpa2p are required for a normal glucose-induced H^+-ATPase activation [32]. Moreover, while strains carrying a single mutation in *SNF3* or *GPA2* genes present a partial reduction in the glucose-induced H^+-ATPase activation, in the double mutant *gpa2* Δ *snf3* Δ this activation is further affected (Table 2, Lines 1, 9, 17, and 21; Fig. 4). Interestingly, in galactose-grown cells the glucose-induced activation seems to be further affected in the double mutant (Table 2, Lines 7, 11, 19, and 23); however, the galactose-induced activation is absent in each one of the single mutants as well as in the double mutant (Lines 8, 12, 20, and 24). These data suggest that both proteins are acting in a parallel way to transduce the signal generated by the presence of sugars.

Moreover, in glucose-grown cells, the H^+-pumping rate of the wild type strain was statistically different ($P < 0.05$) from a *snf3* Δ mutant, but it was not different from a strain presenting the *snf3* Δ mutant transformed with the plasmid pBM3436 encoding a *HXT1*/ C-terminal SNF3 chimera, where the C-terminal tail of Snf3p is attached to Hxt1p (a glucose transporter) (Table 2, Lines 1, 9, and 13). Also for galactose-grown cells, the galactose-induced activation, strongly affected in *snf3* Δ mutant, was also reverted to the wild type level by the introduction of this chimeric construction (Table 2, Lines 8, 12, and 16). These results suggest that the activating signal also generated by galactose would be still weaker or even different from that generated by glucose, but in any case the C-terminal of Snf3p seems to be the internal sensor in this pathway.

Snf3p is also involved in the sugar-induced calcium signaling

The glucose or galactose-grown yeast cells present differences in the cytosolic calcium levels that were attributed to the differences in the internal level of glucose-6-P and

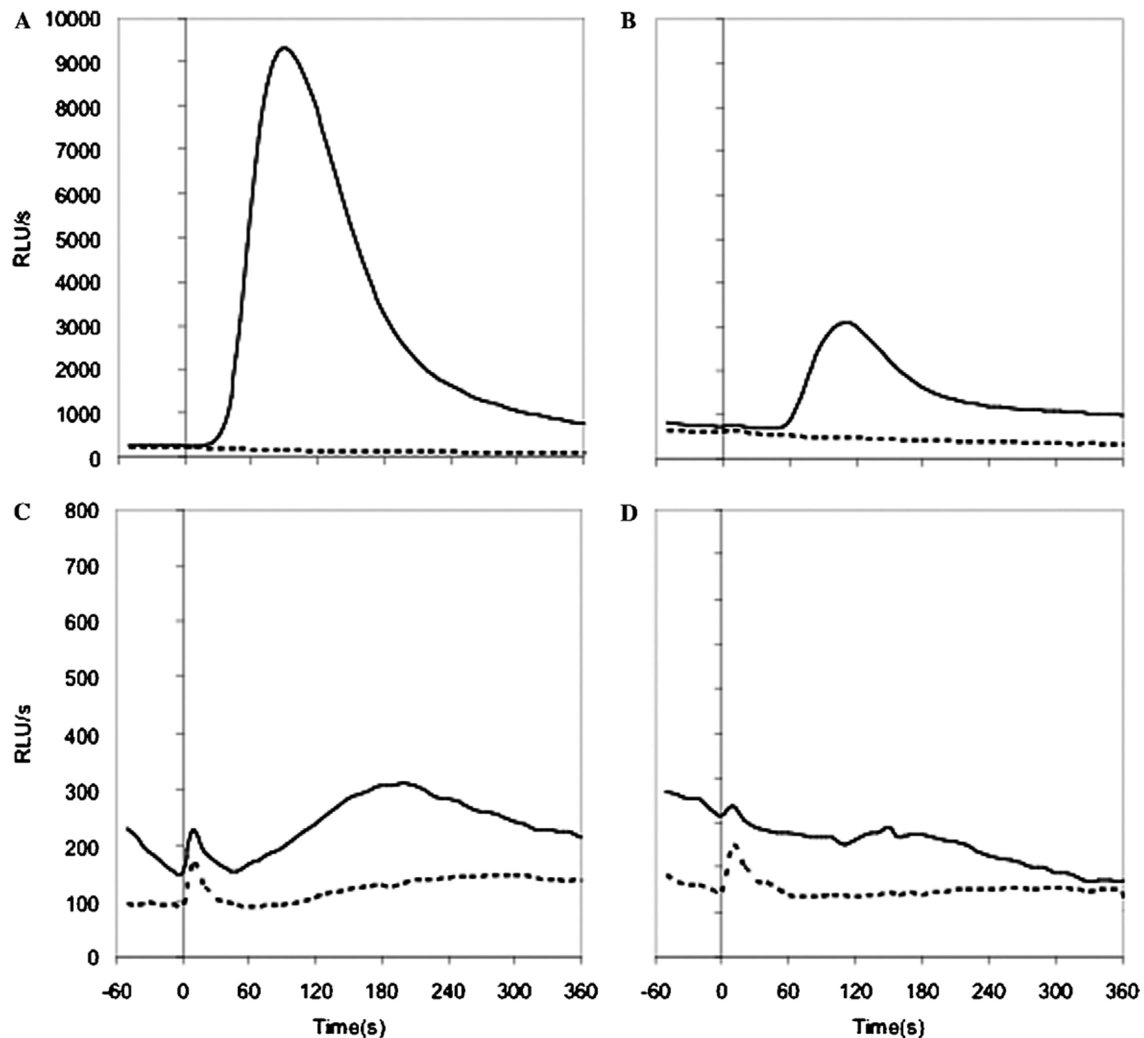

Fig. 5. The role of Snf3p in the sugar-induced calcium signaling in *S. cerevisiae* strains. Glucose- (solid line) and galactose- (dashed line) induced calcium signaling in the YM6217 wild type (A,C) or in the *snf3* Δ mutant (B,D) cells grown on glucose (A,B) or on galactose (C,D). Results shown are representative ones out of at least three consistent experiments.

glucose-1-P [1,13,35]. It was hypothesized that Snf3p may be responsive to intracellular glucose metabolites such as glucose-6-P [11]. The differences found for the ATPase activity in the glucose and galactose-grown *snf3* Δ mutant strain could be related to the inability of such mutant to detect properly the internal levels of glucose-6-P and/or glucose-1-P, and then to trigger an appropriated calcium signaling.

Fig. 5 shows that both glucose- and galactose-induced calcium intracellular signals are reduced or practically disappeared in glucose-grown (A and B) and in galactose-grown (C and D) *snf3* Δ strains. We also measured the glucose-induced calcium signaling in the *snf3* Δ strain presenting the chimera *HXT1/C-terminal SNF3*. Fig. 6 shows that the C-terminal tail of Snf3p is indeed required for sugar sensing in yeast cells, since the calcium signaling is also restored to the wild type level.

To try to understand how Snf3p could be interfering in the calcium homeostasis, we decided to measure the level of total cellular calcium in glucose- and galactose-grown cells. Fig. 7 demonstrates that the *snf3* Δ mutant also has a very high level of total cellular calcium in galactose-grown cells, very similar to the phenotype described in the *pgm2* Δ mutant [13]. Therefore, since the abnormal calcium homeostasis in *pgm2* Δ mutant cells is related to an excessive vacuolar Ca^{2+} uptake by the Ca^{2+}-ATPase Pmc1p [2,13], it is possible that Snf3p participates in the regulation of calcium accumulation in the vacuole, through the control of the Ca^{2+}-ATPase, Pmc1p. Indeed, our results suggest that Snf3p affects the activation of the plasma membrane ATPase (Table 2, Lines 1–4 and 29–35) and in Fig. 7, because it interferes with the capacity of yeast cells to accumulate calcium inside the vacuole.

Discussion

All our data [7,9,32, this work] led us to propose that calcium metabolism would be connected to the sugar-induced activation of the plasma membrane ATPase. Three different findings strengthen this hypothesis: first, it was

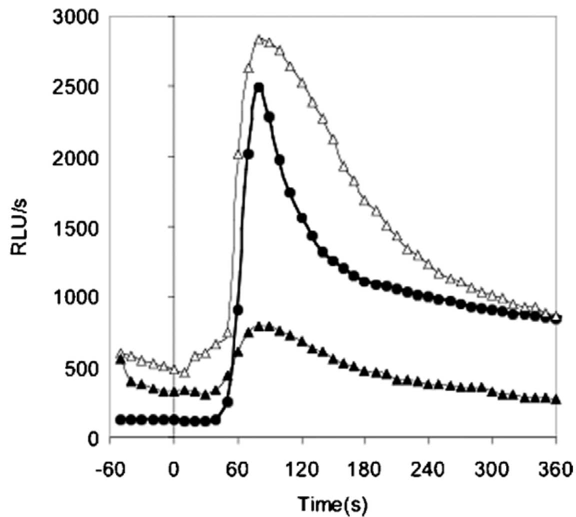

Fig. 6. The role of the C-terminal tail of Snf3p in the sugar-induced calcium signaling in *S. cerevisiae* strains. Glucose-induced calcium signaling in the YM6217 wild type (●); in the *snf3* Δ mutant (▲); and in the *snf3* Δ strain presenting the chimera *HXT1/C-terminal SNF3* cells (△) grown on SD-glucose. Results shown are representative ones out of at least three consistent experiments.

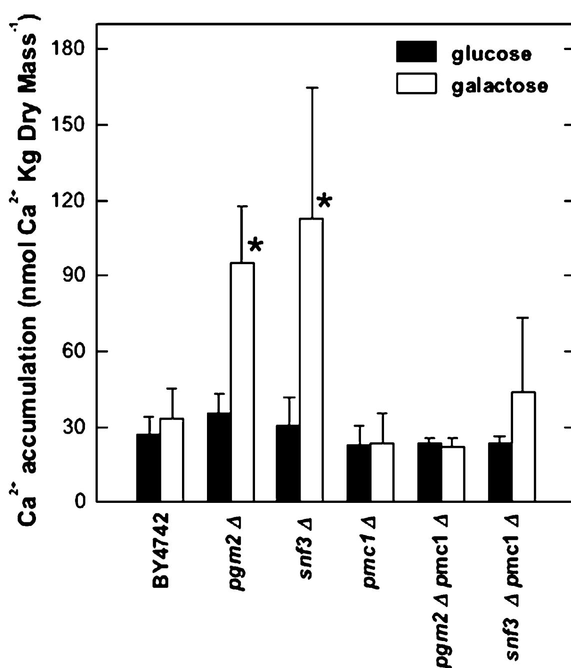

Fig. 7. Total cellular calcium concentrations in *S. cerevisiae* strains (BY4742 background) grown in YPglucose (black columns) or YPgalactose (white columns) media. The indicated strains were grown to a density of ±1 OD_{600nm}/ml. Ten OD_{600nm} units were harvested by centrifugation and washed with fresh YP. Total Ca^{2+} was then measured by atomic absorption spectrophotometry. *The mean values shown are statistically similar to each other and different from that found for the wild type ($p < 0.05$).

demonstrated that G protein Gpa2 seems to bind to the phospholipase C enzyme in yeast [3]; second, it was also shown that Gpr1 receptor was coupled with Gpa2p giving support to the idea of the existence of a trimeric complex [18], and third, evidences were obtained indicating the involvement of such complex in the glucose-induced calcium uptake from extracellular medium [33].

Our results demonstrate that changes in the external calcium availability affect the level of the sugar-induced ATPase activation; nevertheless, calcium uptake through transport systems seems not to be required. This apparent contradiction can be explained in different ways: in many experiments performed on calcium uptake and/or signaling in yeast, the external calcium concentrations are at millimolar range. Nevertheless, in all our experiments [this paper, 32], the calcium concentrations are still lower (micromolar range—data not shown). Moreover, in our experiments the yeast cells were grown in rich media, which inhibits the high affinity calcium influx system mediated by Cch1p/Mid1p complex, most probably as function of the calcineurin activation also observed in these conditions [22].

Interestingly, the glucose-induced increase of cytosolic calcium does not happen in a *mid1* Δ strain [35]. Nevertheless, in our work condition, the sugar-induced calcium signaling measured in a *mid1* Δ mutant is comparable to that of the wild type strain. Nevertheless, it was already demonstrated [19] that in very low external calcium concentrations the Mid1p-mediated CCE-like mechanism could only be clearly detected in a *pmr1* Δ strain (*PMR1* gene encode for the Ca^{2+}-ATPase, present predominantly in Golgi complex). Moreover, the results with *fig1* Δ and *pdr5* Δ mutants also point out for a more complex mechanism. Of course, it is still possible to speculate that the right channel has not been identified or that a combination of different uptake pathways would be involved.

Interestingly, it was already proposed as regards the existence of other calcium signaling mechanisms [16]; thus, the results with the *arg82* Δ strain open a new field of research, since they suggest that the internal levels of IP_3 and/or calcium signaling would participate in the regulation of the H^+-ATPase activity in yeast cells, even considering that IP_3 receptor-homologues have been not identified in *S. cerevisiae* [37]. However, it was suggested that IP_3 is involved in calcium releasing from vacuolar membrane vesicles of *S. cerevisiae* [5] and it seems to regulate tip growth in *Neurospora crassa* [31]. Nevertheless, the real target or receptor of IP_3 and the mechanism by which calcium uptake and/or mobilization would be regulated in response to the addition of sugars to yeast cells remains to be identified.

Moreover, when 100 mM glucose is added to glucose-grown wild type cells, a higher level of calcium signaling is observed; but, the addition of 100 mM galactose does not produce any increase in the cytosolic calcium level due the repression of the galactose metabolism by glucose. For galactose-grown cells, the addition of glucose as well as galactose triggers calcium signaling and the differences in intensity of the calcium signaling were attributed to different intracellular levels of glucose-6-phosphate and glucose-1-phosphate [1,35] suggesting a strong correlation between

the amplitude of the pools of these glucose metabolites and the magnitude of calcium signaling.

In glucose-grown yeast strains presenting mutation in the gene *PGM2* (that encodes for the major isoform of phosphoglucomutase), the intracellular levels of glucose-6-P and glucose-1-P are comparable to those found in the isogenic wild type. However, for galactose-grown cells, there is an eight-fold increase of glucose-1-P level and a corresponding rate of calcium uptake five-fold higher than in an isogenic wild type. Moreover, while a partial calcium signaling could be observed in the glucose-grown *pgm2* Δ cells after glucose re-addition, for galactose-grown cells the re-addition of glucose or galactose triggers a reduced (glucose) or any (galactose) increase in the cytosolic calcium levels. Thus, the loss of Pgm2 p activity results in a "metabolic bottleneck" suggesting that the increase in the intracellular level of glucose-1-P would stimulate the accumulation of calcium in the *pgm2* Δ mutant grown on galactose; by inducing its rapid sequester into intracellular compartments. Therefore, this mutant exhibits a carbon source-dependent defect in both cellular calcium homeostasis and signaling. Interestingly, the results obtained with such strain also suggest that H^{+}-ATPase activation correlates with the intensity of calcium signaling as previously observed [1,13,35].

Moreover, the disruption of *PMC1* gene in a *pgm2* Δ mutant restores a normal galactose-induced calcium homeostasis suggesting that the accumulation related to the increase in the intracellular glucose-1-P levels would stimulate the Pmc1p activity [2]. Our results also seem to confirm that Pmc1p is in fact very important in the control of the intracellular calcium availability whose activity would influence the magnitude of sugar-induced H^{+}-ATPase activation. They also indicate that the phosphorylated sugars could be intracellular signals for both ATPase activation and calcium mobilization [32,35].

If sugar phosphates are internal signals involved in both the modulation of sugar-induced calcium signaling and H^{+}-ATPase activation, there might exist an internal sensor that would detect the oscillations of sugar phosphates (glucose-1-P) levels. Interestingly, a possible involvement of Snf3p in the detection of the intracellular glucose metabolites has already been suggested [11]. In this sense, our results lead us to propose a role for Snf3p (through its C-terminal tail) as detector of the internal levels of glucose-6-P and/or glucose-1-P, and responsible for triggering an appropriated calcium signaling that, by its turn, would lead to the ATPase activation. In spite of this, it might be stressed that the ATPase inhibition found in strains lacking a functional Snf3p seems to be dependent on their genetic background [17]. Therefore, the precise mechanism by which Snf3p would detect and transduce the oscillations in the sugar phosphate levels is a matter of investigation.

Nevertheless, in galactose-grown *snf3* Δ cells occurs a very high level of calcium accumulation very similar to the phenotype described in the *pgm2* Δ mutant [13]. Moreover, in the double mutant *snf3* Δ *pmc1* Δ there is also a recovery of the sugar-induced activation of the plasma membrane H^{+}-ATPase, as it was also observed in the strain *pgm2* Δ *pmc1* Δ. Therefore, we hypothesize that Snf3p would transduce this signal by producing an inhibition of the Ca^{2+}-ATPase vacuolar, Pmc1p.

By conclusion, the internalization followed by phosphorylation of sugars seem to generate a signal that would stimulate the complex formed by Gpa2 and phospholipase C. Once activated, phospholipase C hydrolyze PIP_2 generating DAG and IP_3. IP_3 by acting on calcium channel receptors present at level of the membranes of the internal organelles and/or plasma membrane would increase the cytosolic calcium. The signal (relative amounts of glucose-6-P and/or glucose-1-P) would be also detected by the C-terminal tail of Snf3 that would control the activity of the vacuolar Ca^{2+}-ATPase, Pmc1p. The balance of activities of these two branches would control the availability of appropriated cytosolic calcium concentrations able to trigger the activation of calcium-dependent signaling pathways leading to the activation of plasma membrane H^{+}-ATPase.

Acknowledgments

This work was granted by Fundação de Capacitação de Pessoal Docente (Ministry of Education), by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil) Process 300998/89-9 to R.L.B., and by a FAR (ex 60%) to E.M. We are grateful to Dr. Mark Johnston from Washington University, USA, Dr. James Caffrey from National Institute of Environmental Health Sciences, USA, and Dr. David M. Bedwell from University of Alabama, USA, for the strains used in this work. We also thank Ms. Luciana Brandão for technical assistance.

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