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The involvement of calcium carriers and of the vacuole in the glucose-induced calcium signaling and activation of the plasma membrane H$^+$-ATPase in *Saccharomyces cerevisiae* cells

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ABSTRACT

Previous work from our laboratories demonstrated that the sugar-induced activation of plasma membrane H$^+$-ATPase in *Saccharomyces cerevisiae* is dependent on calcium metabolism with the contribution of calcium influx from external medium. Our results demonstrate that a glucose-induced calcium (GIC) transporter, a new and still unidentified calcium carrier, sensitive to nifedipine and gadolinium and activated by glucose addition, seems to be partially involved in the glucose-induced activation of the plasma membrane H$^+$-ATPase. On the other hand, the importance of calcium carriers that can release calcium from internal stores was analyzed in glucose-induced calcium signaling and activation of plasma membrane H$^+$-ATPase, in experimental conditions presenting very low external calcium concentrations. Therefore the aim was also to investigate how the vacuole, through the participation of both Ca^{2+}-ATPase Pmc1 and the TRP homologue calcium channel Yvc1 (respectively, encoded by the genes *PMC1* and *YVC1*) contributes to control the intracellular calcium availability and the plasma membrane H$^+$-ATPase activation in response to glucose. In strains presenting a single deletion in *YVC1* gene or a double deletion in *YVC1* and *PMC1* genes, both glucose-induced calcium signaling and activation of the H$^+$-ATPase are nearly abolished. These results suggest that Yvc1 calcium channel is an important component of this signal transduction pathway activated in response to glucose addition. We also found that by a still undefined mechanism Yvc1 activation seems to correlate with the changes in the intracellular level of IP_3. Taken together, these data demonstrate that glucose addition to yeast cells exposed to low external calcium concentrations affects calcium uptake and the activity of the vacuolar calcium channel Yvc1, contributing to the occurrence of calcium signaling connected to plasma membrane H$^+$-ATPase activation.

1. Introduction

It has been described that calcium entry in yeast cells is mediated by a complex located in the plasma membrane composed by Cch1, a protein homologous to α-1 subunit of mammalian voltage-dependent calcium channels [1] and Mid1 protein, a "stretch-activated" calcium channel [2]. In minimal media, these channels are activated under special conditions, such as membrane depolarization, depletion of calcium from internal stores, stimulation by pheromones or hypotonic shock; but in rich media their inhibition is triggered by calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase, probably by dephosphorylation [3,4]. There are also experimental evidences supporting the hypothesis of a different system of calcium influx with low affinity (mediated by Fig. 1), acting at the late stage of yeast mating process [5]. Besides, Pdr5p a member of the *Saccharomyces cerevisiae* PDR family of ABC proteins, seems to be involved in calcium uptake in yeast cells [6]. Other data suggest that in non-stimulated yeast cells grown in standard conditions, small amounts of calcium enter cells through not yet identified transporters (referred to as M and X), which would be located in the plasma membrane. These systems would present different affinities for calcium, being competitively inhibited by magnesium ions [7,8].

Recent data suggest the existence of another high affinity system that was called GIC (for glucose induced calcium) transporter, resistant to the presence of nickel ions and verapamil, being the major responsible for calcium influx upon addition of glucose in rich medium [9]. This hypothetical transporter is sensitive

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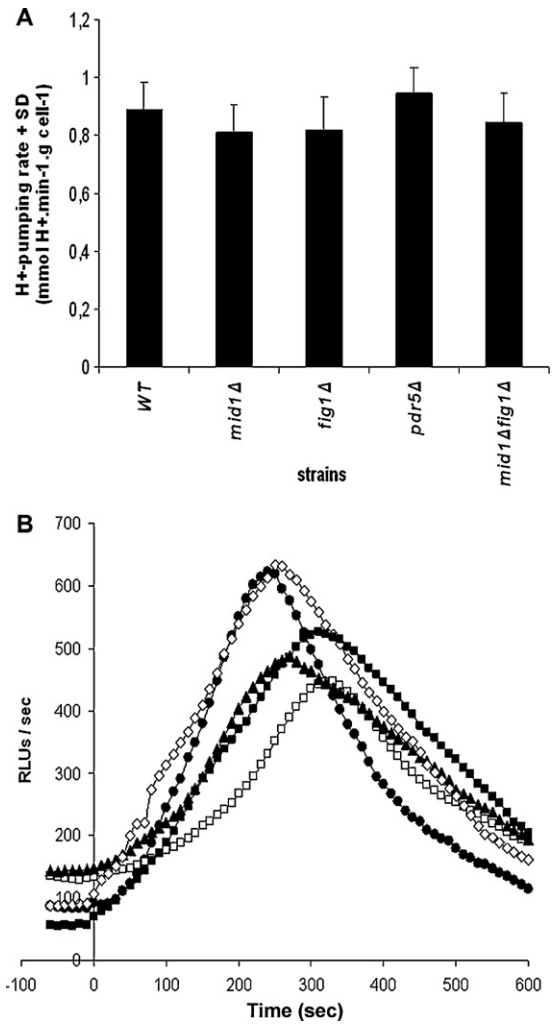

Fig. 1. Plasma membrane H^+-ATPase activation and calcium signaling are not affected in yeast strains carrying mutations in genes encoding known calcium carriers: effects on glucose-induced extracellular acidification (panel A) and calcium signaling (panel B) in *Saccharomyces cerevisiae* cells (genetic background BY4741). Panel B: wild-type strain BY4741 (●), *mid1Δ* (□), *fig1Δ* (▲), *pdr5Δ* (◊) and *mid1Δ fig1Δ* (■). RLUs/sec: Relative Luminescence Units per second.

to magnesium (apparent IC50 of 1.04 ± 0.05 mM), to gadolinium (apparent IC50 of 16.7 ± 1.7 μM) and to nifedipine (apparent IC50 of 0.36 ± 0.13 μM). This is a peculiar behavior, since both verapamil and nifedipine are L-type voltage-dependent calcium channel inhibitors, even with a slightly different specificity. This particular feature might suggest that this hypothetical carrier might belong to a new class of calcium channels, since *S. cerevisiae* genome does not encode any protein homologous to the known calcium channels in addition to those channels already characterized and described previously.

Besides, other systems are responsible for calcium transport into yeast organelles through either ATP-dependent pumps (Ca^{2+}-ATPases) or cationic exchangers (antiporter H^+/Ca^{2+}) calcium channels. In the Golgi apparatus and endoplasmic reticulum (ER), there is a Ca^{2+}-ATPase (Pmr1), which under normal physiological conditions, pumps Ca^{2+} (and Mn^{2+}) into the lumen ensuring a correct calcium and manganese concentrations, particularly in ER, which is very important for an appropriate folding and processing of proteins during their transport through the secretory pathway [10–12]. Nevertheless, the most important storage compartment for calcium accumulation in yeast is the vacuole, that presents at least three systems that cooperate to accumulate Ca^{2+}: a Ca^{2+}-ATPase (Pmc1), a Ca^{2+}/H^+ exchanger (antiport) (Vcx1) and a calcium channel homologous to transient receptor potential channels, called Yvc1. This calcium channel appears to mediate efflux of Ca^{2+} from the vacuole to the cytoplasm, under conditions of mechanical stress and in response to a hypertonic shock [13–18].

A less studied topic in yeast cells in comparison with mammalian cells is the involvement of the phosphatidylinositol (PI) metabolism in calcium signaling. In yeast cells, the phospholipids are synthesized in the endoplasmic reticulum [19] through specific mechanisms highly conserved in eukaryotic cells [20,21]. The PI is used to synthesize phosphatidylinositol phosphates (PIPs), inositol polyphosphates (IPs) and other substances. Both, phosphatidylinositol-4-phosphate (PI(4)P) and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) are synthesized from PI through the action of PI 4-kinases (Stt4 and Pik1) and PI(4)P5-kinase (Mss4), respectively [22,23]. When $PI(4,5)P_2$ is hydrolyzed by the phospholipase C (encoded by *PLC1* gene), two intracellular messengers are produced: diacylglycerol (DAG) and inositol-(1,4,5)-trisphosphate (IP_3) [24,25]. IP_3 is further phosphorylated by a dual inositol kinase, encoded by the *ARG82* gene, generating two types of inositol-tetrakisphosphate – $I(1,3,4,5)P_4$ and $I(1,4,5,6)P_4$, and an inositol-pentaphosphate $I(1,3,4,5,6)P_5$ [26,27]. Then, these inositol poliphosphates are phosphorylated by the Ipk1 enzyme generating inositol-(1,2,3,4,5,6)-hexakisphosphate [28]. Thus, IP_5 and IP_6 are considered precursors of the inositol pyrophosphates [27,29]. Though, the possible relationship between IP_3 and the control of Ca^{2+} levels is not well established, since the yeast genome presents no IP_3 receptors homologous to those found in mammals [23]. Evidence suggesting such relationship is limited to "in vitro" studies with isolated yeast vacuoles [30]. Nevertheless, it was previously demonstrated that glucose addition stimulates the phosphatidylinositol turnover in parallel to the activation of the plasma membrane H^+-ATPase [31]. Moreover, it was found that both glucose-induced increase of IP_3 and calcium signaling are more pronounced in *arg82*Δ mutant cells [32].

In the last few years, we have obtained many evidences indicating that there is a relationship between sugar-induced calcium signaling and the activation of the plasma membrane H^+-ATPase in *S. cerevisiae* cells [31,33,34]. Here, we have demonstrated that the calcium uptake system, sensitive to nifedipine and gadolinium, is essential to the glucose-induced activation of the ATPase, even when no external calcium is present in the medium. Moreover, our results also suggest that the combined action of the vacuolar proteins Ca^{2+}-ATPase Pmc1 and the calcium channel Yvc1 plays an essential role in the control of intracellular Ca^{2+} availability and activation of the plasma membrane H^+-ATPase. In addition, we present new data that point out for a possible relationship between IP_3 and the vacuolar Ca^{2+} channel, Yvc1, suggesting that this channel could participate to a mechanism involved in intracellular calcium level control in response to glucose addition.

2. Materials and methods

2.1. Strains and growth conditions

The *S. cerevisiae* strains shown in Table 1 were grown in YP medium supplemented with carbon sources. The cells were grown in a New Brunswick Model G25 (200 rpm) rotatory incubator at 30 °C until the end of logarithmic phase ($OD_{600nm} \sim 2.0$). Cells were harvested and washed by centrifugation (approximately $2000 \times g$) with 25 mM MES buffer pH 6.0.

Table 1
Saccharomyces cerevisiae strains used in this study.

Strain	Genotype	Source
K601	MATa *ade2-1can1-11his3-11,15leu2-3,112trp1-1ura3-1*	[7]
RT960	K601 *yvc1::Sphis5*$^+$	[9]
RT973	K601*mid1::LEU2cch1::TRP1fig1::Sphis5*$^+$	[9]
RT974	K601*mid1::LEU2cch1::TRP1fig1::Sphis5*$^+$*yvc1::kanMX4*	[9]
PJ69-2a (wild type)	MATa *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δgal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James Caffrey
PJ69-4a	PJ69-2A *arg82::KanMX2*	James Caffrey
LBCM 630	PJ69-2A *yvc1::KanMX2*	This work
LBCM 633	PJ69-4A *arg82::KanMX2 yvc1::URA3*	This work
BY4741 (wild type)	MATa *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*	Euroscarf
YDR315C	BY4741; *mid1:: KanMX4*	Euroscarf
YDR315C	BY4741; *mid1:: KanMX4*	Euroscarf
YOR153W	BY4741; *pdr5:: KanMX4*	Euroscarf
LBCM 514	BY4741; *fig1::KanMX4 mid1::URA3*	Euroscarf
YOR087W	BY4741; *yvc1:: KanMX4*	Euroscarf
YGL006W	BY4741; *pmc1:: KanMX4*	Euroscarf
LBCM 520	BY4741; *pmc1::KanMX4 yvc1::URA3*	This work

2.2. Measurement of H^+-ATPase activity

The cells were re-suspended in 100 mM MES/Tris buffer (pH 6.5) and incubated at a density of 150 mg ml^{-1} (wet mass) in a shaking water bath at 30 °C. After 20 min control samples were collected and glucose was added to a final concentration of 100 mM. After 10 min samples (4.5 g cells wet weight) were taken and the cells collected as quickly as possible on glass fiber filters by vacuum filtration. For time-course measurements, after sugar addition, samples (750 mg wet weight) were taken from the suspension at different times and immediately frozen in liquid nitrogen and stored until use. The procedures used to obtain plasma membranes and to determine ATPase activity were described previously [35].

2.3. Measurement of proton-pumping activity

Cells were re-suspended 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 [33].

2.4. Measurement of total cellular Ca^{2+} level and intracellular free calcium concentrations

The determination of total cellular calcium was performed essentially as described [36]. Briefly, yeast cells were grown in YPD to a density of about 1 OD_{600nm}/ml. Ten OD_{600nm} units were harvested and washed with YP by centrifugation. The pellets present in Eppendorf tubes were dried in a Savant SpeedVac system and then re-suspended in 1 M HCl. Calcium content of the samples was measured by atomic absorption in a Varian AA-2 spectrophotometer.

The intracellular free calcium concentration was measured by using the aequorin-based method [37]. Glucose-induced calcium uptake related 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. Results of representative experiments out of at least three repetitions are shown. Experimental results were corrected according to the level of actual apoaequorin expression, evaluated from total light yield obtained by disrupting cells with 0.5% Triton X-100.

2.5. Molecular biology methods

Preparation and manipulation of nucleic acids were done using standard procedures. The pVTU-AEQ plasmid was generated by inserting in *XhoI*/*PstI* sites of pVTU the *XhoI*/*PstI*-digested fragment obtained by PCR on pYX212-AEQ [37] using the oligonucleotides TTTCTCGAGAATCTATAACTACAAAAAA-CACATACAGGAA and TAACTGCAGGCCCTAGGATCCATGGTGAA. 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).

2.6. Reproducibility of results

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

3. Results

We have previously demonstrated that in the glucose-induced activation of the yeast plasma membrane H^+-ATPase the presence of extracellular calcium is required since the pre-incubation of the cells with EGTA severely affects the intensity of the calcium signaling and the subsequent activation of the enzyme [34], suggesting that glucose-induced calcium uptake could be essential. In our experimental conditions, the extracellular calcium concentration ranged between 35 and 75 μM as measured by atomic absorption (data not shown). Its presence results from contamination of the solutions and of yeast cell walls. However, Fig. 1 shows that the sugar-induced plasma membrane H^+-ATPase activation (panel A) and calcium signaling (panel B) are not affected at all in strains presenting single deletions in genes claimed to encode calcium carriers (*MID1*; *FIG1*) or affected in calcium transport (*PDR5*) as well as in the *mid1Δ fig1Δ* double mutant when compared to wild-type strain in the same conditions.

These results seem to suggest that, in these conditions (no addition of calcium in the working solutions), the glucose-induced calcium signaling and consequently the plasma membrane ATPase activation probably involve different calcium carriers and that the presence of at least one functional carrier could allow the occurrence, even partially, of both signaling processes.

Another possibility would be that other carriers might exist and they would also be involved in the transport of extracellular calcium and/or that, in such low external calcium concentrations,

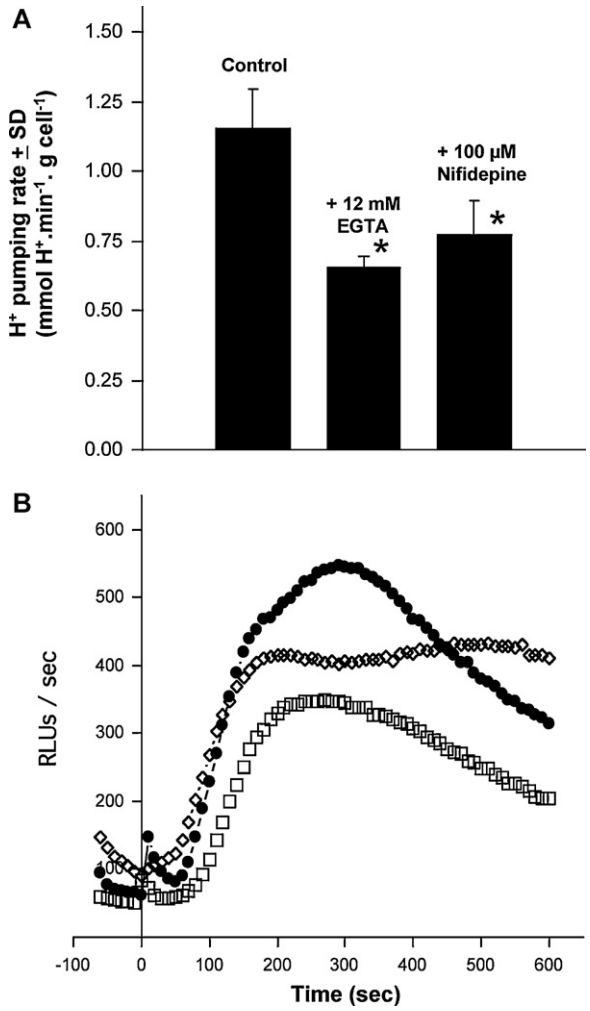

Fig. 2. Plasma membrane H^+-ATPase activation (panel A) and calcium signaling (panel B) are affected in yeast strains pre-incubated with EGTA or nifedipine. The wild type strain (genetic background BY4741) was pre-incubated or not with 12 mM EGTA or 100 μM nifedipine. Panel B: wild-type strain BY4741 – control (●) or pre-incubated with 12 mM EGTA (□) or 100 μM nifedipine (◊). *Mean values are statistically different from those seem in the wild type without pre-incubation with any substance ($p < 0.05$). RLUs/sec: Relative Luminescence Units per second.

the glucose-induced calcium signaling occurs in a more complex way. To address these questions, we first investigated the glucose-induced activation of the plasma membrane H^+-ATPase and calcium signaling by measuring the glucose-induced extracellular acidification in wild type cells either pre-incubated or not with EGTA and/or nifedipine (described to inhibit a still unknown glucose-responsive calcium carrier – [9]). Fig. 2 reveals that pre-incubation of a wild type strain with EGTA or nifedipine triggers the same level of inhibition in the glucose-induced activation of plasma membrane H^+-ATPase (panel A) and calcium signaling (panel B). Nevertheless, they also lead us to suggest that additional cellular mechanisms seem to contribute to the glucose-induced calcium signaling in yeast cells.

In order to clarify this situation, and considering recent data from our groups [9], we decided to investigate the glucose-induced activation of the plasma membrane H^+-ATPase in strains presenting combined mutations in the genes *MID1*, *CCH1* and *FIG1* either pre-incubated or not with nifedipine. Fig. 3 demonstrates that in these strains the sugar-induced activation of the plasma membrane H^+-ATPase is only partially affected when the cells are pre-incubated with nifedipine, suggesting that glucose-induced calcium signaling and ATPase activation be also depending on other sources.

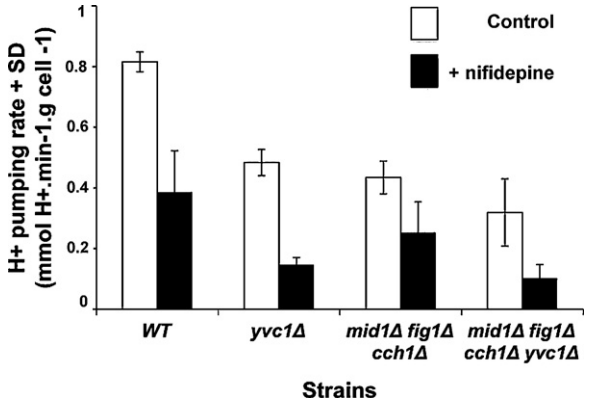

Fig. 3. Plasma membrane H^+-ATPase activation is more affected by nifedipinein mutants lacking a functional Yvc1 calcium channel. Glucose-induced extracellular acidification in *Saccharomyces cerevisiae* strains (genetic background K601) pre-incubated or not with 100 μM nifedipine.

Taking in account that the vacuole is the main organelle involved in calcium storage in yeast cells and that the vacuolar proteins Ca^{2+}-ATPase Pmc1 and the TRP channel homologue Yvc1 could contribute to regulate calcium signaling also in these conditions, we investigated in detail the involvement of these proteins in

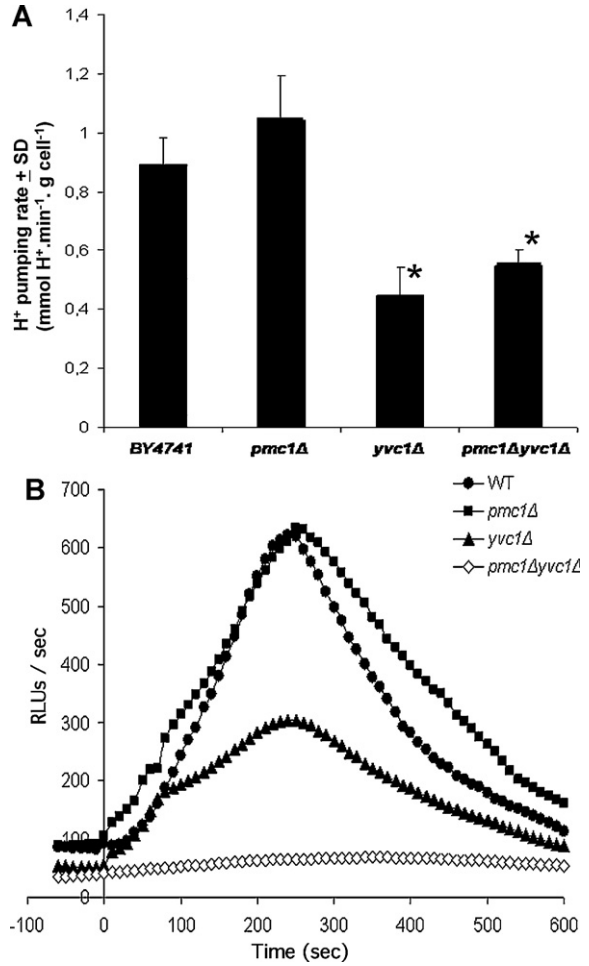

Fig. 4. Plasma membrane H^+-ATPase activation (panel A) and calcium signaling (panel B) are more strongly affected in *yvc1Δ* mutants. Panel B: wild-type strain BY4741 (●), *pmc1Δ* (■), *yvc1Δ* (▲), *yvc1Δ pmc1Δ* (◊). *The mean values are statistically different from those seem in the wild type ($p < 0.05$). RLUs/sec: Relative Luminescence Units per second.

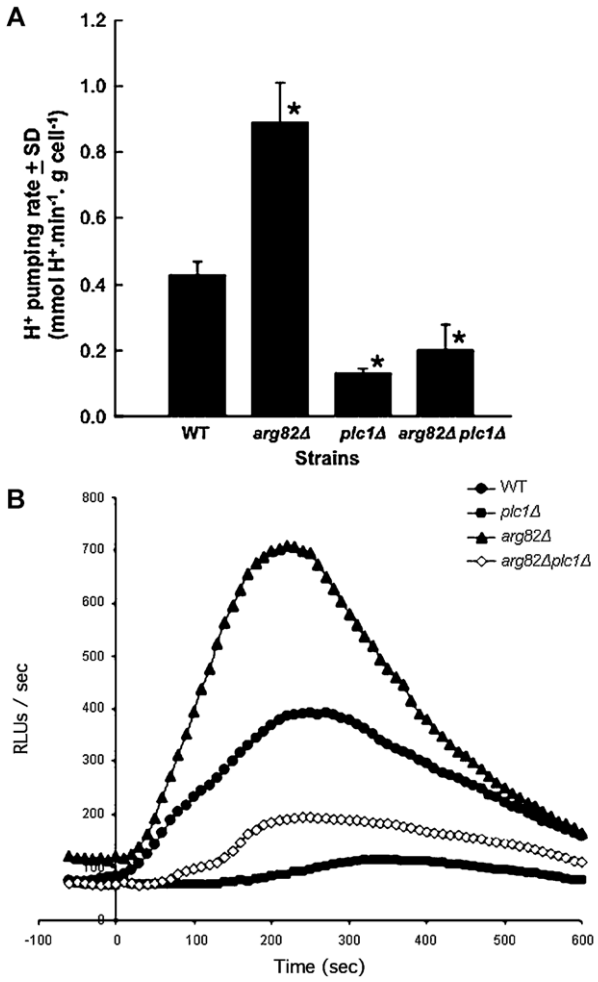

Fig. 5. Glucose-induced extracellular acidification (panel A) and calcium signaling (panel B) are dependent of the IP_3 turnover. Panel B: wild-type strain PJ69-4A1 (●), *arg82* (▲), *plc1Δ* (■) and *arg82Δ plc1Δ* (◊) mutants. RLUs/sec: Relative Luminescence Units per second.

glucose-induced plasma membrane H^+-ATPase activation and calcium signaling. Therefore, we studied the effects of the addition of glucose (in media presenting low external calcium concentrations) to yeast cells with single deletion in the genes *YVC1* and *PMC1* or in a strain with a double deletion in *YVC1* and *PMC1* genes. Fig. 4 (panels A and B) clearly demonstrated that, in shortage of external calcium, Yvc1 channel activity seems to be more important in order to sustain higher glucose-induced effects. In the *yvc1Δ* strain, as well as in the *pmc1Δ yvc1Δ* strain, glucose-induced responses (panel A – H^+-ATPase activation and panel B – calcium signaling) are quite similar, while in *pmc1Δ* are comparable to the wild type.

In order to strengthen this hypothesis, we measured the sugar-induced activation of plasma membrane H^+-ATPase in a mutant presenting combined mutations in *MID1*, *CCH1*, *FIG1* and *YVC1* genes, either pre-incubated or not with nifedipine. Fig. 3 demonstrates that in this strain sugar-induced activation of plasma membrane ATPase is more strongly affected when the cells are pre-incubated with nifedipine, suggesting that ATPase activation in very low external calcium concentrations are indeed dependent on both sources: calcium uptake and vacuolar release. In fact, similar results have already been demonstrated for the glucose-induced calcium uptake [9].

Interestingly, it was already demonstrated that IP_3 has a clear effect on glucose-induced calcium signaling and ATPase activation [32,34]. Consequently, we decided to investigate a possible relationship between Yvc1 and IP_3 in connection to the glucose-induced H^+-ATPase activation and calcium signaling. Nevertheless, to avoid any misinterpretation of our data, we first confirmed the connection between the activities of the phospholipase C (that releases IP_3 from PIP_2) with the inositol kinase, encoded by the *ARG82* gene (involved in the IP_3 turnover). Fig. 5 shows that glucose-induced activation of the plasma membrane ATPase (panel A) and calcium signaling (panel B), over-stimulated in a *arg82Δ* mutant, are nearly absent in a *plc1Δ* mutant as well as in a *plc1Δ arg82Δ* double mutant. Therefore, these results confirm again that the generation of IP_3 is connected to calcium signaling in yeast cells and that the activation of the H^+-ATPase is then clearly IP_3 dependent.

Moreover, Fig. 6 shows that both glucose and CCCP-induced calcium signaling (panels A and B, respectively) and plasma membrane H^+-ATPase activation (panels C and D, respectively) clearly increased in the *arg82Δ* strain [32,34] and were equally reduced in the *yvc1Δ* mutant and in the *arg82Δ yvc1Δ* double mutant. By its turn, Fig. 7 shows the effects observed when different cells were pre-incubated with 2-aminoethoxydiphenylborate (2-APB), an IP_3 receptor inhibitor in mammalian cells [38]. In panel A, we demonstrated that 2-APB has an inhibitory effect in glucose-induced calcium signaling in a wild-type strain. Similar results on glucose-induced activation of the plasma membrane H^+-ATPase were also obtained (panel B). Moreover, the pre-incubation of the *arg82Δ* mutant with 2-APB produced a reduction in both glucose-induced calcium signaling and plasma membrane H^+-ATPase activation (Fig. 7 – panels A and B). Very interestingly, in the *arg82Δ yvc1Δ*

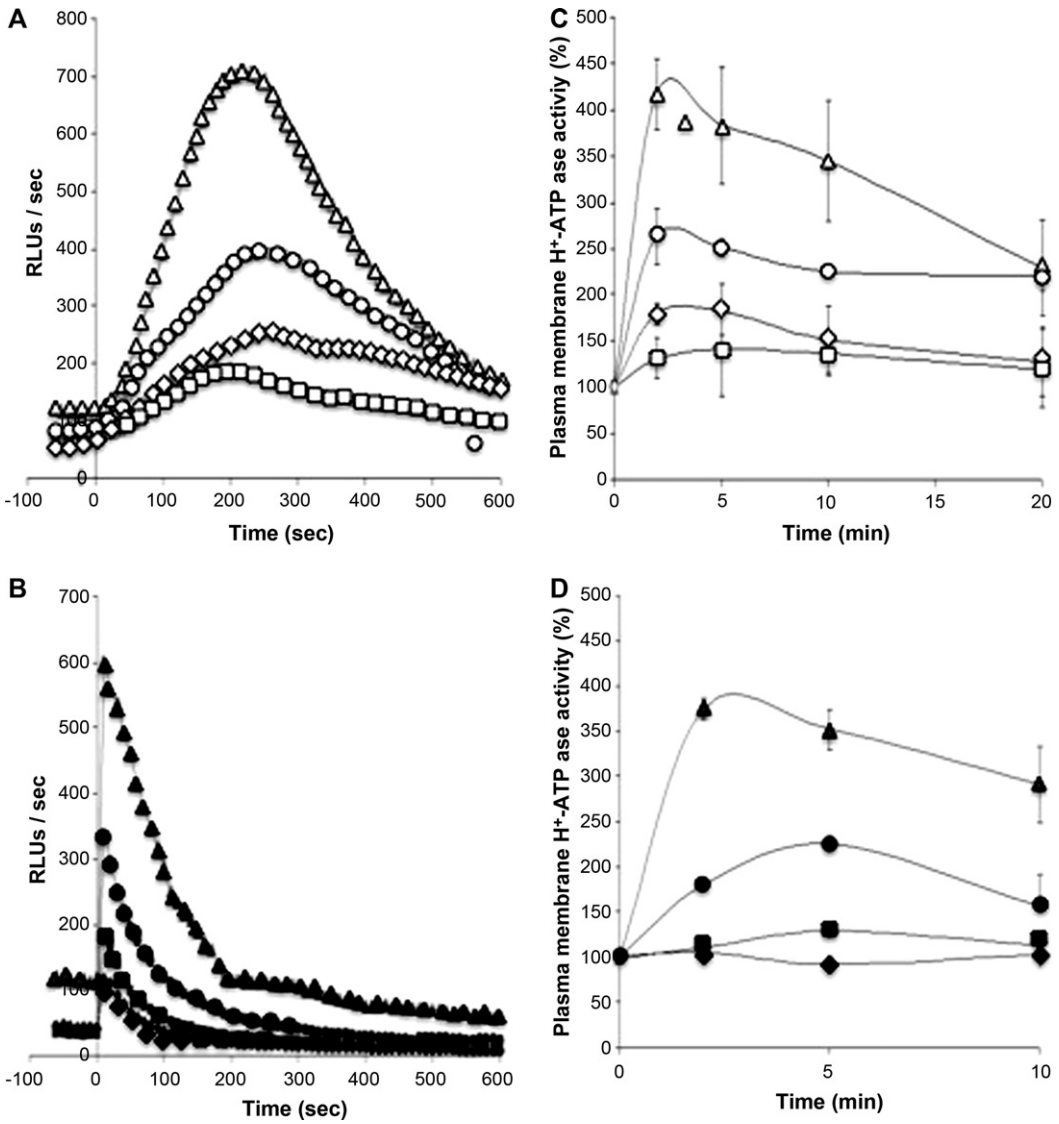

Fig. 6. Connection between IP_3 availability and Yvc1 function. Calcium signaling (panels A and B) and plasma membrane H^+-ATPase activation (panels C and D) in *Saccharomyces cerevisiae* strains: wild-type strain PJ69-4A1 (circles) and in corresponding *arg82Δ* (triangles), *yvc1Δ* (squares) and in *arg82Δ yvc1Δ* (diamonds) mutants. Glucose-induced (panels A and C, open symbols) and CCCP-induced (panels B and D, closed symbols). RLUs/sec: Relative Luminescence Units per second.

double mutant the addition of 2-APB did not show further decrease in glucose-induced calcium signaling and extracellular acidification.

4. Discussion

In our previous studies, additional external calcium was supplied in order to demonstrate its importance for the glucose-induced calcium signaling process [33,34,39]. Nevertheless, even in conditions of low external calcium concentration, the pre-incubation of the cells with EGTA revealed that external calcium, even if only present in traces as contaminant of the working solutions and in cell wall is very important for calcium signaling. Therefore, and also based on the apparent influence of external calcium on glucose-induced plasma membrane H^+-ATPase and intracellular calcium signaling process [34,37], the most obvious conclusion is that sugar would induce calcium uptake and as higher the external calcium concentration would be, as higher would be calcium signaling.

However, our results clearly demonstrated that, without supplementary addition of calcium, the presence of glucose still triggers both calcium signaling and activation of the H^+-ATPase, also in strains presenting mutations in genes encoding for known calcium transporters. Obviously, these data would not exclude the possibility that other and still unknown calcium transporters could be involved in this process. Indeed, there is some evidence for the existence of proteins responsible for Mg^{2+}-sensitive Ca^{2+} influx. In this connection, the proteins Alr1, Alr2, and Mnr2 have been identified as hetero-oligomeric proteins required for Mg^{2+} uptake that also promote sensitivity to high (but not low) environmental Ca^{2+} [40–42]. Very recently, evidence was found for the existence of glucose induce calcium (GIC) transporter that would contribute to the glucose-induced calcium signaling. The GIC transport is sensitive to inhibition by gadolinium and nifedipine, but is resistant to verapamil [9]. Gadolinium is a specific inhibitor for stretch-activated ion channels; nifedipine, a dihydropyridine inhibitor, is specific for blood vessels smooth muscle L-type calcium channel; verapamil, a phenylalkylamine inhibitor, is effective on both vascular and cardiac L-type calcium channels. These calcium channel

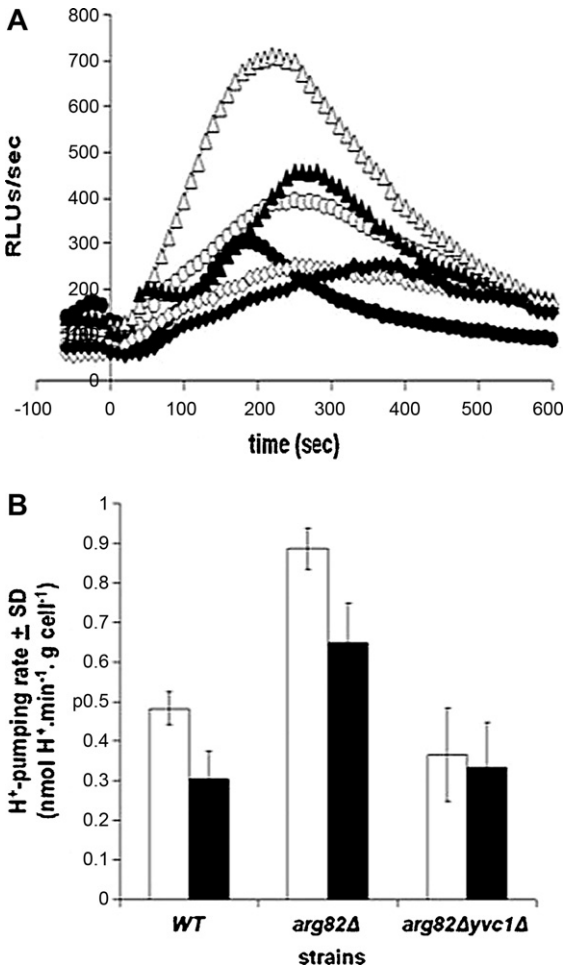

Fig. 7. Inhibitory effects of 2-APB are dependent of functional Arg82 and Yvc1 proteins. Glucose-induced calcium signaling (panel A) and extracellular acidification (panel B) in *Saccharomyces cerevisiae* strains in wild-type strain PJ69-4A1 (circles) and in corresponding *arg82Δ* (triangles), and in *arg82Δ yvc1Δ* (diamonds) mutants in absence (open symbols or bars) and in the presence of 50 μM 2-APB (closed symbols or bars). RLUs/sec: Relative Luminescence Units per second.

blockers were chosen because they were previously tested in yeast, and besides their effectiveness on GIC channel, they also partially affect a component of the high-affinity Ca^{2+} influx system (HACS) in yeast, Cch1 [43] that is similar to mammalian L-type voltage-gated Ca^{2+} channels (VGCCs). Even though the molecular identification of GIC channel is not yet achieved the results shown in this work confirm the role of such transporter not only in calcium signaling, but also for H^+-ATPase activation.

Moreover, in order to better understand the role of the vacuole in the glucose-induced ATPase activation and calcium signaling, we investigated how the activity of the Ca^{2+}-ATPase Pmc1 and the TRP homologue calcium channel Yvc1 contribute to control calcium availability in the cytosol. Our results strongly suggest that in low external calcium concentration, the vacuolar calcium channel Yvc1 activity seems to become more important for proper glucose-induced calcium signaling and plasma membrane H^+-ATPase activation. Although the vacuolar calcium channel Yvc1 had been firstly described more than ten years ago [16], and the effects of glucose on calcium signaling were known even earlier [44,45], the role of Yvc1 in glucose-induced calcium signaling only starts to be clarified [9]. Our results clearly demonstrate that the intensity of the intracellular calcium signal, in lower external calcium concentrations, is strongly dependent on the action of Yvc1.

Besides the role played by Yvc1, there is also more evidence that highlighted the importance of Pmc1 in the control of calcium homeostasis: first, deletion of *PGM2* gene, that encodes for one isoform of the enzyme phosphoglucomutase (involved in the conversion of glucose 6-phosphate in glucose 1-phosphate), was previously shown to cause perturbation in calcium homeostasis. One believes that an increase in the ratio of glucose 1-phosphate and glucose 6-phosphate causes a metabolic bottleneck intrinsically related to the control of calcium homeostasis [46,47]. However, if an additional deletion in the *PMC1* gene is introduced in the single mutant *pgm2Δ*, there is a restoration of the normal sugar-induced calcium signaling [46,47]. Second, the calcium signaling defects observed in a *snf3Δ* mutant are also overcome in a double mutant *snf3Δ pmc1Δ* [34].

Additionally, it is already known that *pmc1Δ* mutant grows as well as the corresponding wild type in standard culture media, but it grows very poorly when environmental Ca^{2+} is elevated, suggesting that high intracellular free Ca^{2+} can be toxic to yeast [14]. Therefore, calcium uptake and accumulation must be coordinated events due to the fact that the amount of calcium necessary to trigger a signaling transduction process is quite low (50–200 nM) [48] and because of calcium toxicity [14].

In spite of the fact that yeast genome presents no receptors for IP_3 homologous to those found in mammals [23], previous results have demonstrated that the occurrence of an IP_3 signal is connected to calcium signaling in yeast cells [32]. Moreover, both glucose-induced [34] and CCCP-induced [39] activation of plasma membrane H^+-ATPase seem to constitute physiological processes depending on IP_3 and consequently on calcium signaling in yeast cells. In this context, many questions arise: first, if calcium signaling occurs as a consequence of an increase in the intracellular level of IP_3, which would be the IP_3 – responsive organelle that accumulates calcium? Secondly, to be responsive to IP_3 effects, such organelle might present an IP_3 receptor. Nevertheless, since no IP_3 receptor-homologue has been identified in *S. cerevisiae* genome, the idea pointing out for the existence of such transduction pathway is a matter of controversy.

The involvement of IP_3 in calcium releasing from vacuolar membrane vesicles of *S. cerevisiae* was previously suggested [30], but no further work was done in this field. Moreover, an IP_3-activated calcium channel seems to regulate tip growth in *Neurospora crassa* [49]. In any case, neither the identity of the organelle nor the IP_3 receptor was confirmed. Since plasma membrane Ca^{2+}-ATPases (PMCA) have not been described in yeast cells and the TRP channel homologue Yvc1 seems to mediate Ca^{2+} efflux from the vacuole [13,16,17], the vacuole and the Yvc1 channel could be good candidates as components of a signaling transduction pathway responding to IP_3 action.

As previously demonstrated [32], the accumulation of IP_3 revealed to be quite difficult to detect particularly in yeast cells probably due the fact that IP_3 has a very low level (about 1 nmol g^{-1} wet weight) and a very short half-life. Thus, we studied the role of Yvc1 in a strain where the addition of sugar [32] or a depolarizing compound [39] triggers stronger IP_3 and calcium signaling. Therefore, our results show that both glucose-induced and CCCP-induced calcium signaling and plasma membrane H^+-ATPase activation are over-stimulated in a *arg82Δ* mutant in comparison to the corresponding wild-type strain, consistently with data reported in literature [32,34]. By contrast, they are significantly reduced in both *yvc1Δ* single and *arg82Δ yvc1Δ* double mutants.

On the other hand, the pre-incubation of yeast cells with 2-APB, an IP_3 receptor inhibitor in mammalian cells [38], shows that this drug has an evident inhibitory effect on both glucose-induced calcium signaling and plasma membrane H^+-ATPase activation. However, others works also have demonstrated that 2-APB could have other targets, including store-operated Ca^{2+} channels (SOCs)

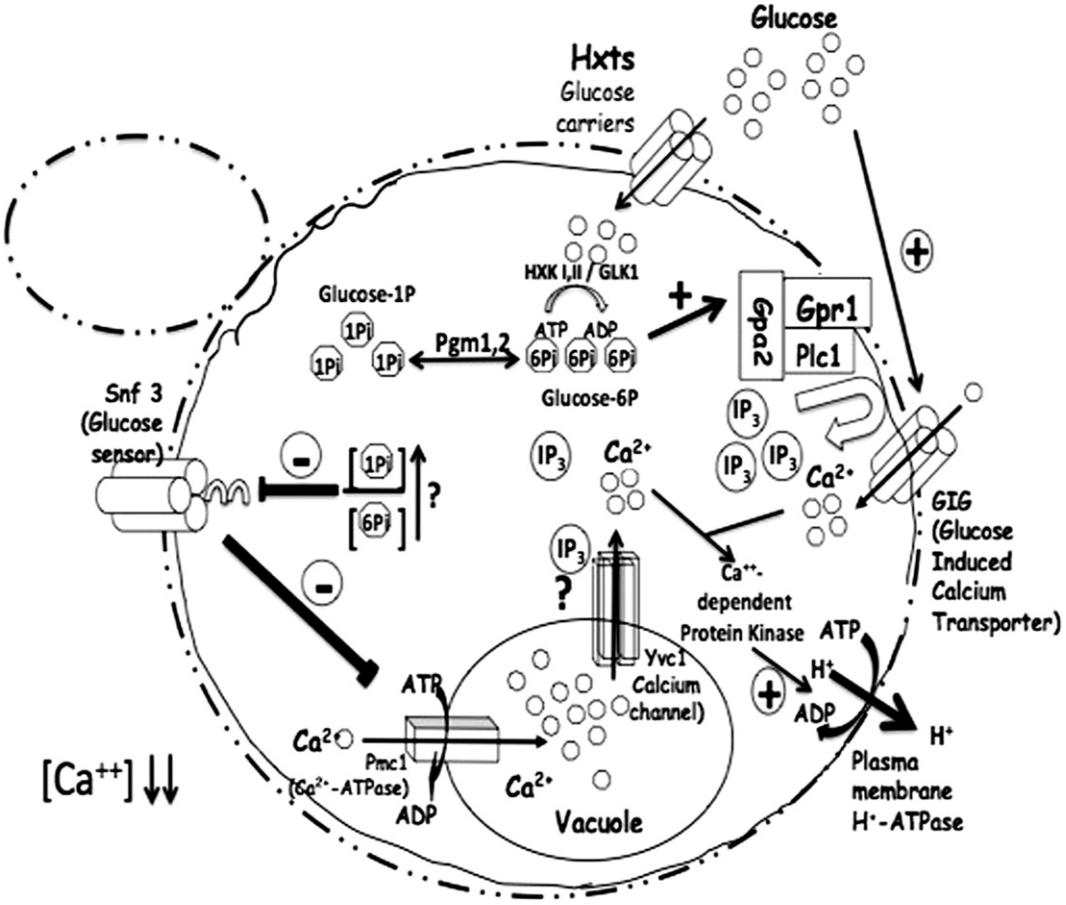

Fig. 8. Working model: the sugar-induced activation of plasma membrane H^+-ATPase in yeast cells. The internalization followed by phosphorylation of glucose or galactose generates a signal (relative amounts of glucose-6-P and/or glucose-1-P?) that would stimulate the complex presenting the G protein, Gpa2p, and phospholipase C eliciting the activation of the phospholipase C. Then the PIP_2 hydrolysis would generate DAG and IP_3. IP_3 would act directly or indirectly on the vacuolar calcium channel Yvc1p leading to an increase in the intracellular calcium signal. Besides, the C-terminal tail of the glucose sensor Snf3p controlling the activity of the vacuolar $Ca2^+$-ATPase, Pmc1p, would detect the signal. The final intensity of the calcium signal would be the result of the partial contribution of each branch of this system.

making its utilization to study IP_3-dependent calcium signaling from internal stores a matter of controversy [50,51]. Nevertheless, and in spite of the fact that there was not a classical dose dependence effect of 2-APB on both events (data not shown), the absence of further effect of 2-APB in the *arg82Δ yvc1Δ* double mutant suggests that the action of 2-APB seems to be related to the IP_3, at least in our works conditions, and that Yvc1 actually works in connection with IP_3 signaling.

Previous results obtained by our groups have already demonstrated that in absence of a functional phospholipase C there is no glucose-induced calcium signaling [37] as well as plasma membrane H^+-ATPase activation [31]. Moreover, in a *plc1Δ arg82Δ* double mutant, both CCCP- [39] and glucose-induced (this work) plasma membrane H^+-ATPase activation and calcium signaling are strongly reduced. Therefore, all these results seem to reinforce the hypothesis of a physiological role played by IP_3 in the control of calcium metabolism and plasma membrane H^+-ATPase activity, as well as they seem to indicate that Yvc1 could respond to IP_3 levels.

Nevertheless, these data do not allow us to conclude that IP_3 would act directly on Yvc1 leading to the opening of this channel and then increasing the calcium availability in the cytosol, particularly in this glucose-induced process. Interestingly, the effects of IP_3 on Yvc1 under a number of patch-clamp conditions were already tested, but no apparent evidence of modulation had been found yet [16].

It was demonstrated that Yvc1 is a transient receptor potential channel homolog in *S. cerevisiae* that forms an intracellular Ca^{2+}-permeable channel in the yeast vacuolar membrane [16]. Up to now, it has been shown that Yvc1 is activated by hypertonic shock [17], most probably through a mechanosensitive mechanism [18]. Interestingly, in animal cells Ca^{2+}-permeable transient receptor potential channels (TRPC1) forms heterotetramers with others TRPC polypeptides being a component of store-operated Ca^{2+} channels (SOCs); or participating of activation of SOCs; or still being activated by stretch or by interaction with IP_3 receptors [52]. Thus, and taking in account the animal model, an alternative possibility in yeast cells would be the involvement of another (or other) proteins forming a complex with Yvc1 that would be sensitive to the presence of IP_3 signaling in a glucose-induced process.

In conclusion, the data shown in this and in our previous papers [9,31,33–35,39,53] lead us to propose the existence of a pathway with two branches by which the addition of glucose control calcium availability in the cytosol having as a consequence the activation of the H^+-ATPase (Fig. 8). In the first branch, glucose (sugar) uptake and its subsequent phosphorylation generate a signal (sugar phosphates?) that would stimulate the G protein Gpa2 that by its turn would activate phospholipase C. Then, PIP_2 would be hydrolyzed generating diacylglycerol (DAG) and inositol tri-phosphate (IP_3). Our data suggest that IP_3 would interact directly or indirectly with Yvc1 regulating the intensity of calcium signaling in cytosol. In the second branch, devoted to the control of Pmc1 Ca^{2+}-ATPase activity, the glucose sensor Snf3 [54] would also detect the sugar phosphates [55], and in some way would transduce this signal leading to an increase in Pmc1 activity [33,34,39]. In such model, the participation of a calcium transporter, sensitive to gadolinium and nifedipine, would also connect the external calcium availability

with the activity of Pmc1. In any case, the balance of these two branches would be responsible for the transient nature of calcium signaling and would also avoid the accumulation of toxic calcium concentrations in the cytosol.

Of course, this working model is still incomplete and further studies must be performed in order to clarify many different aspects, for instances: (1) how the G protein Gpa2 and the glucose sensor Snf3 would detect fluctuations in the levels of sugar phosphates and then would trigger the respective effects on phospholipase C and the Ca^{2+}-ATPase Pmc1; (2) by which mechanism(s) IP_3 would control the opening of the vacuolar calcium channel Yvc1 to trigger a calcium signaling. These aspects are being studied now in our laboratories.

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