RESEARCH ARTICLE

# Carbonyl cyanide *m*-chlorophenylhydrazone induced calcium signaling and activation of plasma membrane $H^+$-ATPase in the yeast *Saccharomyces cerevisiae*

Michele B.P. Pereira[1], Renata Tisi[2], Luciano G. Fietto[1], Anamaria S. Cardoso[1], Mônica M. França[1], Fernanda M. Carvalho[1], Maria José M. Trópia[1], Enzo Martegani[2], Ieso M. Castro[1] & Rogelio L. Brandão[1]

[1]Laboratório de Biologia Celular e Molecular, Núcleo de Pesquisas em Ciências Biológicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Campus do Morro do Cruzeiro, Ouro Preto, MG – Brazil; and [2]Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Milan, Italy

**Correspondence:** Rogelio Lopes Brandão, Laboratório de Biologia Celular e Molecular, Núcleo de Pesquisas em Ciências Biológicas, Universidade Federal de Ouro Preto, Campus do Morro do Cruzeiro – 35.400-000 Ouro Preto, MG – Brazil. Tel.: +55 31 3559 1723; fax: +55 31 3559 1680; e-mail: rlbrand@nupeb.ufop.br

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Plasma membrane $H^+$-ATPase; depolarizing compounds; *Saccharomyces cerevisiae*.

## Abstract

The plasma membrane $H^+$-ATPase from *Saccharomyces cerevisiae* is an enzyme that plays a very important role in the yeast physiology. The addition of protonophores, such as 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), also triggers a clear *in vivo* activation of this enzyme. Here, we demonstrate that CCCP-induced activation of the plasma membrane $H^+$-ATPase shares some similarities with the sugar-induced activation of the enzyme. Phospholipase C and protein kinase C activities are essential for this activation process while Gpa2p, a G protein involved in the glucose-induced activation of the ATPase, is not required. CCCP also induces a phospholipase C-dependent increase in intracellular calcium. Moreover, we show that the availability of extracellular calcium is required for CCCP stimulation of $H^+$-ATPase, suggesting a possible connection between calcium signaling and activation of ATPase.

## Introduction

The plasma membrane $H^+$-ATPase of *Saccharomyces cerevisiae* is very important for the function of different cellular systems: by pumping protons out of the cell, it not only contributes to intracellular pH regulation but it also creates an electrochemical gradient that is essential for nutrient uptake. The activity of the enzyme is regulated at the post-translational level by two main factors: sugars and acidity (Portillo, 2000). Glucose-activation of the $H^+$-ATPase results from a combined effect on the kinetic parameters of the enzyme (Serrano, 1983), leading to a rapid increase in the $V_{max}$ of ATP hydrolysis and a decrease in the $K_m$ for ATP. Acidification of the medium during cellular growth triggers a decrease in cytosolic pH, also leading to ATPase activation. However, this activation is different because there is a change in the $K_m$ for ATP, but no increase in the $V_{max}$ for ATP hydrolysis (Eraso & Gancedo, 1987; Carmelo *et al.*, 1997). This difference in the activation process leads us to propose that this enzyme could be regulated through different mechanisms.

Because it was demonstrated that the glucose-induced activation is the result of a phosphorylation process, many groups have tried to identify the protein kinase(s) and the respective mechanism(s) by which this activation process occurs. Considering that addition of either glucose or depolarizing compounds [carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or 2,4-dinitrophenol (DNP)] triggers an increase in the intracellular cAMP, and the subsequent activation of protein kinase A (Trevillyan & Pall, 1979; Thevelein, 1984; Purwin *et al.*, 1986; Portillo & Mazon, 1987; Thevelein *et al.*, 1987), it was first hypothesized that the cAMP-PKA pathway could be involved in the activation of the plasma membrane ATPase (Portillo & Mazon, 1987; Ulaszewski *et al.*, 1989; Brandão *et al.*, 1992). Conversely, many pieces of evidence indicated that the cAMP-protein kinase A pathway

is not involved in the glucose-induced $H^+$-ATPase activation (Mazon *et al.*, 1989; Becker dos Passos *et al.*, 1992). In addition, we have demonstrated that the glucose-induced activation seems to be dependent upon a phosphatidylinositol-type metabolism (Brandão *et al.*, 1994; Coccetti *et al.*, 1998; Souza *et al.*, 2001) and remarkable similarities were observed between the glucose-induced activation of the plasma membrane ATPase and the glucose-induced calcium signaling in yeast cells (Souza *et al.*, 2001; Tïsi *et al.*, 2002). More recently, we also found evidence for a clear relationship between calcium metabolism and sugar-induced activation of plasma membrane ATPase in *S. cerevisiae* (Trópia *et al.*, 2006).

The addition of a protonophore like CCCP also induced activation of the plasma membrane $H^+$ATPase that is dependent on the extracellular pH, being stronger at lower pH values such as 4.0–5.0 U (Becker dos Passos *et al.*, 1992; Brandão *et al.*, 1992), and it is well known that CCCP causes an acidification of the cytoplasm in cells incubated in medium of acidic pH (Eilam & Othman, 1990; Eilam *et al.*, 1990). More interestingly, the addition of CCCP also induced a transient increase of calcium influx (Eilam *et al.*, 1990).

The addition of protonophores leads to both an increase in intracellular cAMP and to a transient calcium signal, but considering that plasma membrane $H^+$-ATPase activation by glucose is apparently mediated by calcium availability and not by cAMP metabolism, we decided to examine the involvement of calcium and of the phosphatidylinositol metabolism in the CCCP-induced activation of yeast plasma membrane ATPase. In this paper, we demonstrate that, similarly to the results found for the sugar-induced activation of plasma membrane $H^+$-ATPase (Trópia *et al.*, 2006), the calcium metabolism seems to be also involved in the control of the depolarizing-induced activation of this enzyme.

## Materials and methods

### Strains and growth conditions

The *S. cerevisiae* strains used in this study are shown in Table 1. Yeast cells were grown in medium containing 2% peptone and 1% yeast extract (YP) supplemented with carbon sources (glucose or galactose) and 1 M sorbitol (used for *pkc1*Δ strain). In all experiments the cells were grown in a rotatory incubator New Brunswick Model G25 (200 r.p.m.) at 30 °C until the middle of logarithmic phase ($OD_{600\,nm}$ 1.0–1.5). Cells were harvested and washed three times by centrifugation (*c.* 2000 **g**) in 25 mM MES buffer pH 5.0 with or without 1 M sorbitol (depending of the strain).

### Measurement of $H^+$-ATPase activity

The cells were resuspended in 100 mM MES/Tris buffer (pH 5.0) 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 containing 750 mg of cells (wet weight) were collected and CCCP was added to make a final concentration of 0.5 mM. At different times, other samples also containing 750 mg of cells were taken from the suspension; the cells were collected as quickly as possible on glass fibre filters by vacuum filtration, immediately frozen in liquid nitrogen and stored until use. Because ethanol can also trigger an *in vivo* activation of yeast plasma membrane ATPase (Monteiro & Sá-Correia, 1998), we also tested the effect of the addition of an equivalent volume of ethanol, because this solvent was used to prepare concentrated CCCP solutions.

The procedures used to obtain plasma membranes and to determine ATPase activity were described previously (Becker dos Passos *et al.*, 1992). The reactions were started with concentrated ATP solutions to obtain the desired final concentration. Protein content was determined using the classical method (Lowry *et al.*, 1951).

**Table 1.** *Saccharomyces cerevisiae* strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| W303 | *MATa leu 2-3,112ura3-1 trip1-1, his3-11,15 ade2-1 can1-100 GAL SUC* | Johan Thevelein |
| YSH 850 | W303-1A MATa *pkc1*Δ::HIS 3 | Stefan Hohmaan |
| LBCM394 | W303 *gpa2*::LEU2 | This work |
| 3700 | *Matα ura3-52, his3-11,15 trip1-Δ901ade2-101* | Johan Thevelein |
| 3703 | 3700 *plc1*::*URA* | Johan Thevelein |
| PJ69-4A | *MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80ΔLYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ* | James Caffrey |
| PJ69-4A1 | PJ69-4A *arg82*::*KanMX2* | James Caffrey |
| LBCM506 | PJ69-4A1 *arg82*::*KanMX2 plc1*::*URA* | This work |
| BY4742 | *MATα his3Δ1 leu2Δ0 lis2Δ0 ura3Δ0* | Euroscarf |
| YDL194w | BY4742 *snf3*::*KanMX2* | Euroscarf |
| YGL006w | BY4742 *pmc1*::*KanMX2* | Euroscarf |
| Y11153 | BY4742 *mid1*::*KanMX2* | Euroscarf |
| Y13177 | BY4742 *fig1*::*KanMX2* | Euroscarf |
| BY4741 | *MATa his3Δ1 leu2Δ0 lis2Δ0 ura3Δ0* | Euroscarf |
| YGL167c | BY4741 *pmr1*::*KanMX2* | Euroscarf |

### Measurement of cytosolic free calcium concentration

The cytosolic free calcium concentration was measured using the aequorin-based method (Tïsi *et al.*, 2002). Strains containing the apoaequorin-expressing plasmid pVTU-AEQ were grown in YP supplemented with 2% glucose until the exponential phase ($3–8 \times 10^6$ cells $mL^{-1}$). The cells were harvested, washed by filtration and resuspended in Mes/Tris 0.1 M pH 5.0. After 30 min of incubation at room temperature, cells were loaded with coelenterazine as described. To measure the CCCP-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 0.5 mM CCCP. Results of representative experiments 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 lysing cells with 0.5% Triton X-100.

### Molecular biology methods

Preparation and manipulation of nucleic acids were done using standard procedures (Sherman *et al.*, 1986; Sambrook *et al.*, 1989). *Escherichia coli* cells were transformed by the calcium chloride method. Yeast cells were transformed using the lithium acetate protocol (Ito *et al.*, 1983). A 2.9-kb fragment *plc1*::*URA* was amplified from 3703 strain genomic DNA and used to transform the PJ69-4A strain to generate the PJ69-4A1 strain. Correct integration of the construction was confirmed by PCR using the forward primer sequence: AGAAGATTCCAAAACCGAAATC and reverse primer sequence: AATTAATTATTAGACGCTAACTGTG. To construct the *gpa2*Δ mutant in the W303 genetic background, we used a pUC18 plasmid containing the *gpa2*::*LEU* construction previously described (Trópia *et al.*, 2006) and the integration was confirmed by Southern blot analysis.

### Reproducibility of results

The experiments were performed at least three times with consistent results. SDs are indicated in each figure or table. Statistics analyses were done using the Student's *t*-test. Differences were considered statistically significant when the *P*-value was $< 0.05$.

## Results

### Protonophore-induced $H^+$-ATPase activation and intracellular calcium increase

The addition of protonophore (depolarizing) compounds, like CCCP, triggers activation of the plasma membrane ATPase (Becker dos Passos *et al.*, 1992); moreover, it also stimulates calcium uptake in *S. cerevisiae* (Eilam & Chernichovsky, 1987; Eilam *et al.*, 1990). Therefore, we decided to investigate if this activation process was dependent on calcium metabolism like the glucose-induced activation of this enzyme (Trópia *et al.*, 2006).

At first, we checked if the CCCP-induced and the glucose-induced $H^+$-ATPase activation were comparable. As shown in Fig. 1a the intensity of the effect triggered by CCCP was always less pronounced than that observed upon glucose addition. We also observed that the dinitrophenol (DNP)-induced effect was almost identical to that observed with

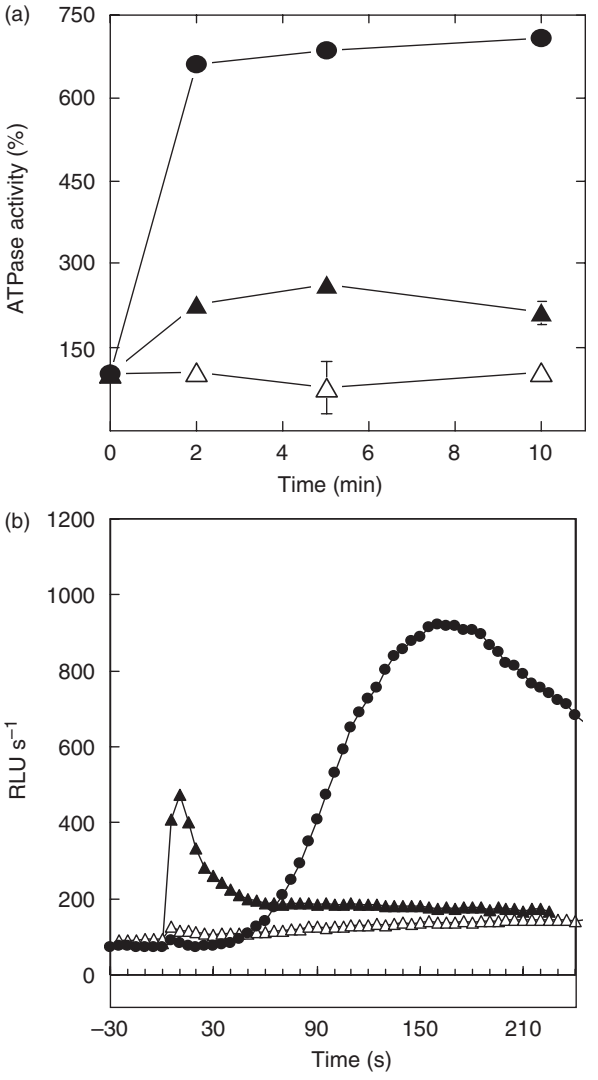


**Fig. 1.** Glucose- and CCCP-induced effects in *Saccharomyces cerevisiae* cells. (a) Plasma membrane ATPase activation in 3700 wild-type cells triggered by glucose or CCCP. (b) Calcium signaling in 3700 wild-type cells triggered by addition of glucose or CCCP. Glucose: circles; CCCP: triangles. Closed symbols: 100 mM glucose in water; 0.5 mM CCCP in ethanol (a) or DMSO (b); controls in open symbols: corresponding volume of ethanol or DMSO used to solubilize the CCCP.

CCCP (data not shown). Furthermore, CCCP was able to trigger an increase in intracellular calcium; however, while the CCCP-triggered signal was faster and transient, the glucose-induced signal was wider and sustained (Fig. 1b).

These results indicate that there could be a relationship between calcium metabolism and the regulation of the plasma membrane ATPase activity for the CCCP-mediated effects also, as previously demonstrated for the glucose-induced phenomena (Trópia *et al.*, 2006). Therefore, we decided to investigate if the proteins involved in the sugar-induced activation could also play a role in the CCCP-induced activation.

## Involvement of components of the glucose-induced activation of $H^+$-ATPase in the depolarizing-induced effects

CCCP-dependent activation of the ATPase was clearly dependent on protein kinase C activity (Fig. 2a) as already demonstrated for the glucose-induced activation (Souza *et al.*, 2001). Nevertheless, in contrast to what was observed for glucose-induced activation, the G protein Gpa2p was not required for the CCCP-induced activation of the ATPase (Fig. 2b).

Another similarity with the glucose-induced activation process is the involvement of phospholipase C; however, while for the glucose-induced activation Plc1p was reported to be only partially required (Coccetti *et al.*, 1998), its activity was essential for CCCP-induced activation (Fig. 3a). Because in yeast cells the phospholipase C is also involved in the glucose-induced calcium signaling (Tisi *et al.*, 2002), we measured the CCCP-induced calcium signaling in a *plc1*Δ strain. Fig. 3b demonstrates that Plc1 p activity was required also for CCCP-triggered calcium signaling.

In a strain bearing a deletion of the *ARG82* encoding a dual kinase that phosphorylates $IP_3$ generating $IP_4$ and $IP_5$, the glucose-induced calcium signal is greater than in the corresponding wild-type strain and $IP_3$ accumulation inside the cell is amplified (Tisi *et al.*, 2004). The results in Fig. 3c show that CCCP-induced ATPase activation is also higher in this mutant. These results indicate that when activated the phospholipase C would generate an $IP_3$ signal, that generates an increase in the internal calcium concentration possibly by acting on receptors located at the plasma membrane or at internal storages membranes. To confirm this idea, a deletion in the *PLC1* gene was also introduced in the *arg82*Δ strain and the CCCP-induced activation of the enzyme was investigated: the results indicate that the generation of $IP_3$ is essential for the activation of plasma membrane ATPase (Fig. 3c). Thus, our data indicate that in yeast acidification (or membrane depolarization) would lead to an activation of phospholipase C that in turn would promote an increase in the intracellular calcium.

It was suggested that the glucose sensor Snf3p as well as the $Ca^{2+}$-ATPase, Pmc1p, present at the vacuolar membrane, are important members of the cellular system contributing to keep cytosolic calcium availability in the appropriated concentration range. Moreover, sugar induced-activation of the plasma membrane $H^+$-ATPase involves both these proteins (Trópia *et al.*, 2006); in particular, the *snf3*Δ strain showed an inhibition while a *pmc1*Δ mutant presented an increase in glucose-induced activation of the ATPase.

CCCP addition to a *pmc1*Δ mutant triggered a greater $H^+$-ATPase activation, while the absence of a functional Snf3p caused only a partial inhibition of the CCCP-induced activation of the plasma membrane ATPase (Fig. 4a).

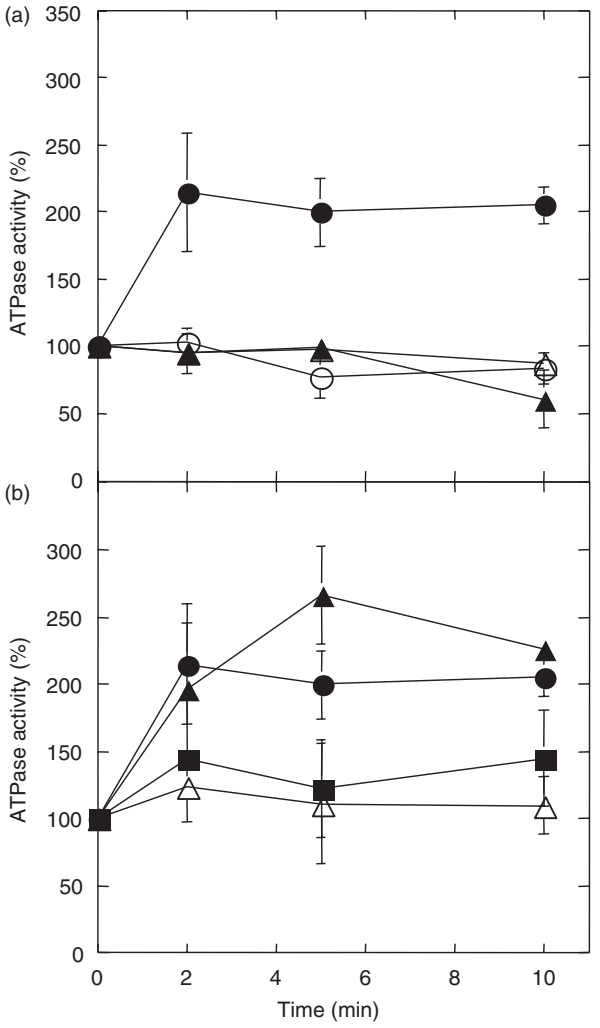


**Fig. 2.** Role of different components of the sugar-induced plasma membrane $H^+$-ATPase pathway in the CCCP-induced activation of this enzyme in *Saccharomyces cerevisiae* cells. (a) CCCP-induced effects in W303 wild-type (circles) and *pkc1*Δ (triangles) strains. (b) CCCP-induced effects in W303 wild type (circles) and *gpa2*Δ (triangles). Closed symbols: 0.5 mM CCCP in ethanol; open symbols: corresponding volume of ethanol used with the CCCP solution.

Although the vacuole is the principal calcium internal store in yeast cells, and Pmc1p is the main pump involved in its internalization into the vacuole, we also investigated the CCCP-induced effects in a strain lacking a functional Pmr1p, a $Ca^{2+}$-ATPase involved in calcium pumping inside the Golgi apparatus. Surprisingly, the results shown in Fig. 4b demonstrate that while the glucose-induced activation of the enzyme in the *pmr1*Δ mutant was only partially inhibited, the CCCP-induced activation of the pump was completely absent in this mutant.

Considering that CCCP also acts as an uncoupler inhibiting the synthesis of ATP, it would not trigger the activation

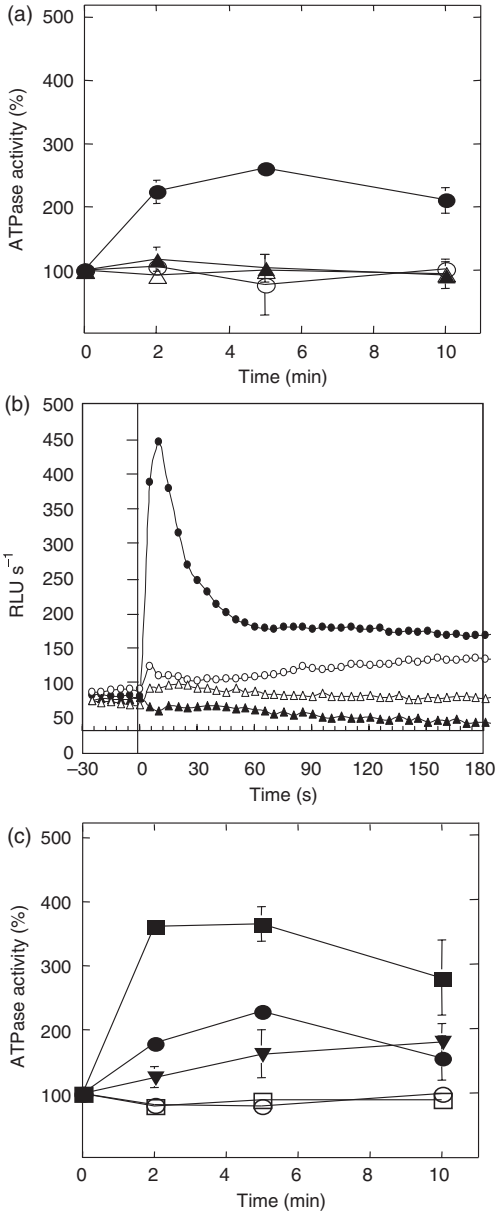


**Fig. 3.** Relationship between CCCP-induced calcium signaling and activation of the plasma membrane $H^+$-ATPase. CCCP-induced activation of plasma membrane ATPase (a) and CCCP-induced calcium signaling (b) in the 3700 wild-type and the corresponding *plc1*Δstrains. CCCP-induced plasma membrane $H^+$-ATPase activation in strains presenting a single deletion in the *ARG82* gene or double deletions in *PLC1* and *ARG82* genes (c). Wild-type (circles); *plc1*Δ (triangles); *arg82*Δ (squares) and *plc1*Δ*arg82*Δ (inverted triangles) strains. Closed symbols: 0.5 mM CCCP in ethanol (a and c) or in DMSO (b); open symbols: corresponding volume of ethanol or DMSO used with the CCCP solution.

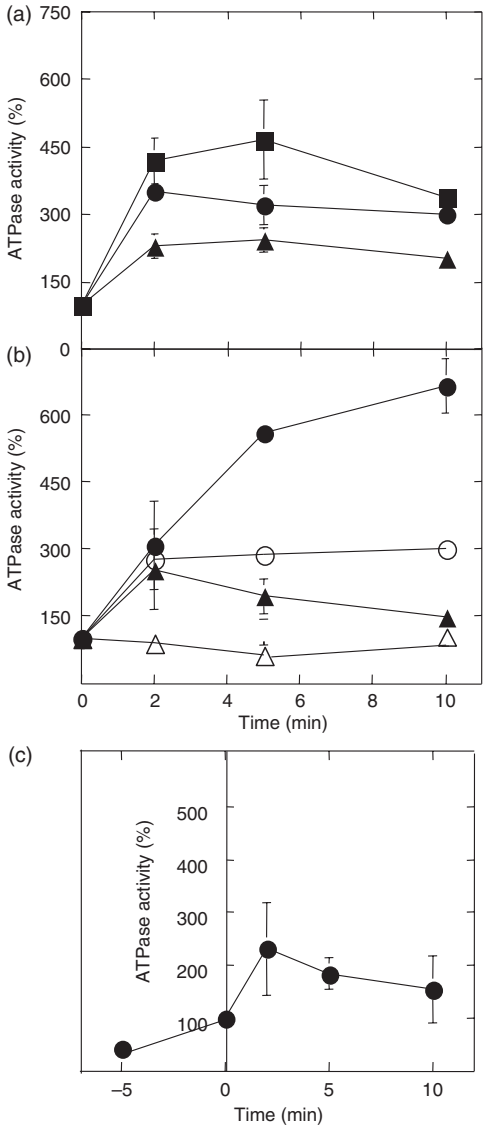


**Fig. 4.** The involvement of different proteins and metabolic conditions in the control of CCCP-induced activation of plasma membrane $H^+$-ATPase in *Saccharomyces cerevisiae* strains. (a) CCCP-induced effects in BY4742 wild type (●), *snf3*Δ (▲) and *pmc1*Δ (■) strains; (b) Glucose-induced effects in BY4741 wild type (●) and *pmr1*Δ mutant (○) and CCCP-induced effects in BY4741 wild type (▲) and *pmr1*Δ mutant (△) strains; (c) Effect of the addition of 5 mM glucose five minutes before the addition of CCCP at zero time to BY4741 wild type.

of the plasma membrane $H^+$-ATPase, because its presence would affect ATP availability. Therefore, we also performed an experiment by adding 5 mM glucose 5 min before the addition of CCCP to increase the cellular ATP levels. The results shown in Fig. 4a and c demonstrate that, in spite of its uncoupling effect, the addition of CCCP to yeast cells can still activate the $H^+$-ATPase, suggesting the existence of a specific effect that leads to this activation process.

## Influence of external calcium in the CCCP-induced effects

Considering the above results, we investigated if external calcium would also influence the magnitude of

the CCCP-induced phenomena. Therefore, we measured the effect of calcium deprivation, using the calcium chelator EGTA. As shown in Fig. 5, extracellular calcium appeared to be essential for the CCCP-induced activation of the ATPase, because preincubation of wild-type cells with EGTA completely inhibited this activation process. On the other hand, when an equimolecular calcium concentration was added together with EGTA, the CCCP-induced activation of the ATPase was nearly normal (Fig. 5).

Finally, we measured the CCCP-induced activation of the plasma membrane ATPase in a strain lacking Mid1p activity. This protein is functionally associated with Cch1p, a homologue of the mammalian high-affinity voltage-gated calcium channel component (Iida *et al.*, 1994; Locke *et al.*, 2000; Tokés-Fuzesi *et al.*, 2002). Surprisingly, and as previously observed in the glucose-induced activation of the plasma membrane ATPase (Trópia *et al.*, 2006), Mid1p seems not to be essential for the CCCP-induced activation of the ATPase, because the mutant showed a nearly normal activation under these circumstances. Similar results were also observed with a strain presenting a deletion in the gene *FIG1* that encodes for a low affinity calcium transport system (Muller *et al.*, 2001) (data not shown).

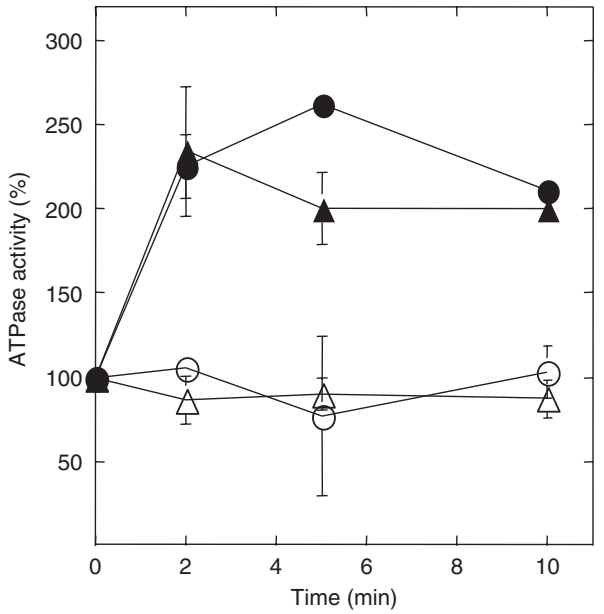


**Fig. 5.** The role of external calcium on the CCCP-induced activation of plasma membrane $H^+$-ATPase in *Saccharomyces cerevisiae* strains. CCCP-induced ATPase activation in glucose-grown 3700 wild-type cells in different conditions: in the absence of additional concentration of $CaCl_2$ (●); in the presence of 12 mM EGTA (△); or in the presence of 12 mM $CaCl_2$ and 12 mM EGTA (▲); control: corresponding volume of ethanol used with the CCCP solution (○).

## Discussion

### Comparison between CCCP- and glucose-induced effects

It was previously shown that the addition of protonophores, such as DNP and CCCP, triggers an *in vivo* activation of the plasma membrane $H^+$-ATPase (Becker dos Passos *et al.*, 1992; Brandão *et al.*, 1992). Although the addition of these compounds also stimulates the cAMP-PKA pathway (Thevelein & Beullens, 1985; Thevelein, 1991) it was demonstrated that the cAMP-PKA pathway was not involved in $H^+$-ATPase activation (Becker dos Passos *et al.*, 1992).

Nevertheless, the mechanism by which protonophores/depolarizing compounds would activate this enzyme has not been further investigated during the last 15 years. Curiously, the unique related effect reported for these compounds on yeast was an increase of calcium uptake triggered by CCCP (Eilam *et al.*, 1990).

A more consistent hypothesis about the mechanism by which the plasma membrane ATPase is regulated at post-translational level has been proposed on the basis of data showing a relationship between the glucose-induced activation of plasma membrane $H^+$-ATPase and the phosphatidylinositol metabolism, in connection with the availability of cytosolic calcium (Trópia *et al.*, 2006). Considering all these data, it seemed logical to propose an involvement of calcium metabolism in the CCCP-induced activation of the plasma membrane ATPase in yeast cells.

### Involvement of calcium in the signaling process

In spite of the fact that there are clear differences between the intensities of the CCCP- and glucose-induced activation of the $H^+$-ATPase, the results presented here indicate that calcium metabolism is a common factor in both cases. Support for this hypothesis came from the experiments demonstrating that proteins directly involved in both the sugar-induced activation of the plasma membrane $H^+$-ATPase and calcium signaling (phospholipase C and Arg82p) were also involved in the CCCP-induced activation of the enzyme as well as in calcium signaling.

The Gpa2p G protein, that is partially required for the glucose-induced activation of the plasma membrane $H^+$-ATPase (Souza *et al.*, 2001) and calcium signaling (Tïsi *et al.*, 2002), was not involved in the CCCP-induced activation of the enzyme. This was not surprising if one considers that Gpa2p is normally activated by the glucose receptor Gpr1p and it is well known that this system normally responds directly to glucose. The nature of the internal signal generated by addition of sugar (sugar phosphates?) or CCCP (drop in the internal pH) (Thevelein *et al.*, 1987) would be different: while glucose-induced activation requires Gpa2p, the decrease in the intracellular pH triggered by CCCP

would be enough to directly activate the phospholipase C generating $IP_3$. Interestingly, it was already demonstrated that Ras (a G protein) is specifically involved in the intracellular acidification-induced cAMP signaling in the yeast *S. cerevisiae* (Colombo *et al.*, 1998). Thus, if intracellular calcium is the internal signal involved in the CCCP-induced effects, Ras protein could also be involved in the Plc1p activation leading to a $IP_3$ signal and an increase of calcium availability in the cytosol.

Another set of results supporting the involvement of calcium in the CCCP-induced effects came from the experiments with a *pmr1*Δ mutant. Deletion of the *PMR1* gene provokes an overexpression of *PMC1* gene, leading to a stronger accumulation of calcium into the vacuole (Kellermayer *et al.*, 2003). As demonstrated here, in this mutant both the glucose- and the CCCP-induced activation of the $H^+$-ATPase were clearly affected. However, the fact that in the *pmr1*Δ mutant the plasma membrane $H^+$-ATPase was only partially activated by glucose and nearly not activated by CCCP suggests that the protonophore-induced effects are indeed weaker or more confined regarding calcium signaling.

Finally, the requirement of extracellular calcium in order to get a CCCP-induced activation of plasma membrane ATPase is also a clear indication that external calcium is involved in this activation process equally suggesting that calcium metabolism is indeed important for the enzyme activation.

### Framework of a signaling pathway for plasma membrane $H^+$-ATPase activation

The idea emerging from our results (Souza *et al.*, 2001; Trópia *et al.*, 2006; this paper) is that a pathway with two branches regulates activation of the $H^+$-ATPase. In the first, glucose (sugar) uptake, followed by phosphorylation, generates a signal, probably mediated by phosphorylated sugars. Besides, this signal would stimulate the G protein Gpa2p, affecting phospholipase C activity and leading to the hydrolysis of $PIP_2$ with subsequent generation of DAG and $IP_3$. The increase in the intracellular calcium concentration would activate a calcium-dependent protein kinase that, in turn, would phosphorylate and activate plasma membrane ATPase.

According to our results (this paper, Trópia *et al.*, 2006), protein kinase C would be involved in this activation process. Therefore, we searched for the existence of possible Pkc1 p phosphorylation sites on the plasma membrane ATPase using the available tools in the website NETPHOSK (Blom *et al.*, 2004). At least 30 putative sites with scores ranging between 0.5 and 0.92 were found. Two sites localized in the C-terminal domain were previously suggested as those involved in the enzyme activation (Eraso & Portillo, 1994). Very recently Lecchi *et al.* (2007) demonstrated that tandem phosphorylations of Ser-911 and Thr-912 at the carboxy terminus of yeast plasma membrane $H^+$-ATPase are responsible for the glucose-induced activation of the enzyme. Interestingly, these two amino acids can be considered as potential targets of Pkc1 p according the website NETPHOSK (data not shown).

In the second branch of this model, the glucose sensor Snf3p would also detect the sugar phosphates, and in some way would transduce a signal controlling the Pmc1p activity. The balance of these two branches would control the actual availability of calcium in the cytosol (Trópia *et al.*, 2006).

The CCCP-induced $H^+$-ATPase activation would be weaker by the fact that it does not use the G protein Gpa2p for this activation process and it is also difficult to imagine a mechanism controlling the Snf3p activity as suggested in the glucose-induced activation process. Thus, only one branch of this mechanism would be directly affected by CCCP or DNP.

Although the results shown in this paper provide a better understanding of the mechanism by which the activity of the plasma membrane ATPase is regulated, there are many questions that deserve further investigation. First, and as already observed in the glucose-induced activation, the apparent dependence on external calcium seems to be very complex: in both cases (Trópia *et al.*, 2006, this paper), the activation of the enzyme seems not to be affected in strains lacking proteins (Mid1p and Fig1p) involved in calcium uptake in yeast cells (Iida *et al.*, 1994; Locke *et al.*, 2000; Tokés-Fuzesi *et al.*, 2002). Otherwise, as we already mentioned before (Trópia *et al.*, 2006), it is possible that the right channel has not been identified or that a combination of different uptake systems may be involved.

Secondly, which is the mechanism responsible for the Plc1p activation by CCCP? Is this protein sensitive to membrane depolarization or to pH decrease? If so, how can one combine this mechanism with that of the glucose-induced phosphatidylinositol turnover already observed in yeast cells (Coccetti *et al.*, 1998)? The G protein, Ras, could be involved in this specific process? These and other aspects are currently under investigation in our laboratories.

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