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Glucose-induced activation of plasma membrane H^+-ATPase in mutants of the yeast *Saccharomyces cerevisiae* affected in cAMP metabolism, cAMP-dependent protein phosphorylation and the initiation of glycolysis

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Addition of glucose-related fermentable sugars or protonophores to derepressed cells of the yeast *Saccharomyces cerevisiae* causes a 3- to 4-fold activation of the plasma membrane H^+-ATPase within a few minutes. These conditions are known to cause rapid increases in the cAMP level. In yeast strains carrying temperature-sensitive mutations in genes required for cAMP synthesis, incubation at the restrictive temperature reduced the extent of H^+-ATPase activation. Incubation of non-temperature-sensitive strains, however, at such temperatures also caused reduction of H^+-ATPase activation. Yeast strains which are specifically deficient in the glucose-induced cAMP increase (and not in basal cAMP synthesis) still showed plasma membrane H^+-ATPase activation. Yeast mutants with widely divergent activity levels of cAMP-dependent protein kinase displayed very similar levels of activation of the plasma membrane H^+-ATPase. This was also true for a yeast mutant carrying a deletion in the *CDC25* gene. These results show that the cAMP-protein kinase A signaling pathway is not required for glucose activation of the H^+-ATPase. They also contradict the specific requirement of the *CDC25* gene product. Experiments with yeast strains carrying point or deletion mutations in the genes coding for the sugar phosphorylating enzymes hexokinase PI and PII and glucokinase showed that activation of the H^+-ATPase with glucose or fructose was completely dependent on the presence of a kinase able to phosphorylate the sugar. These and other data concerning the role of initial sugar metabolism in triggering activation are consistent with the idea that the glucose-induced activation pathways of cAMP-synthesis and H^+-ATPase have a common initiation point.

Introduction

The addition of glucose or related fermentable sugars to yeast cells grown on nonfermentable carbon sources is known to trigger an extensive series of regulatory effects, both short-term effects and long-term effects. Short-term effects are independent of protein synthesis and include activation of trehalase [1], phosphofructokinase 2 [2], plasma membrane H^+-ATPase [3,4], inactivation of fructose-1,6-bisphosphatase [5], isocitrate lyase [6,7], cytoplasmic malate dehydrogenase [8,9], phosphoenolpyruvate carboxykinase [10], galactose and high-affinity glucose transport [11,12]. Long-term effects are manifested at the level of transcription and include glucose repression [13], induction of pyruvate decarboxylase [14] and induction of ribosomal RNA and ribosomal protein synthesis [15,16].

The earliest effect known to occur after addition of fermentable sugar to derepressed yeast cells is a transient increase in the cAMP level [17]. It appears to be triggered by a specific signaling pathway beginning somewhere at the level of sugar transport/sugar phosphorylation and involving several components, includ-

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; YPD, yeast extract-peptone-dextrose; Ile, isoleucine.

ing the CDC25 and RAS proteins (see recent review, Ref. 18). Evidence has been presented that cAMP-dependent protein phosphorylation might be the trigger for the glucose-induced effect on trehalase [2,19], phosphofructokinase 2 [2], fructose-1,6-bisphosphatase [20,21], isocitrate lyase [7], galactose and high-affinity glucose transport [12]. The glucose-induced effect on cAMP synthesis can be mimicked by addition of protonophores at low external pH [22–24]. This acidification-induced stimulation of cAMP synthesis appears to interfere at some point with the glucose-induced signaling pathway (see review, Ref. 18).

Although it is well established that glucose-induced activation of plasma membrane H^+-ATPase involves covalent modification [3,4], it is unclear whether it is triggered by cAMP-dependent protein phosphorylation. If it is, the effect must be indirect, since the gene encoding the H^+-ATPase does not show a consensus site for cAMP-dependent protein phosphorylation in the predicted amino acid sequence [25]. Recently, a putative protein phosphorylation site was identified which was shown to be important for glucose-induced activation [26]. Goffeau and colleagues [27] have shown that incubation at the restrictive temperature of temperature-sensitive mutants in cAMP synthesis leads to a drop in H^+-ATPase activity. Moreover, this drop could be suppressed by exogenous cAMP in strains containing an additional mutation, allowing cAMP uptake from the medium and, therefore, suppression of the temperature-sensitive mutation in cAMP synthesis by exogenous cAMP. Glucose-induced activation of plasma membrane H^+-ATPase was also observed in *Fusarium oxysporum* and a correlation between H^+-ATPase activity and cAMP levels was demonstrated (Brandão et al., unpublished data). On the other hand, Portillo and Mazon [28] did not find evidence for involvement of the RAS-cAMP-protein kinase A pathway in glucose-induced activation of H^+-ATPase, using a series of mutants affected at different points in the pathway, with the exception of the *cdc25*^{ts} mutant. In this mutant, glucose-induced activation of H^+ ATPase was deficient at the restrictive temperature. Based on this finding, they concluded that CDC25 had, in addition to its function in cAMP metabolism, an independent function in controlling glucose-induced H^+-ATPase activation. The identity of the mutant used in this study, however, has been questioned because the mutation mapped very close to the position of the *cdc28* mutation, rather than to the real position of the *CDC25* gene [29]. An opposite relationship between H^+-ATPase activity and cAMP levels during diauxic growth of yeast cultures has been demonstrated [30]. This also appears to point against positive regulation of H^+-ATPase activity by cAMP-dependent protein phosphorylation. Increased H^+ ATPase activity at the end of the exponential phase was not due to increased

TABLE I

Saccharomyces cerevisiae strains used in this study

Strain	Genotype	Source (+ Ref.)
SP1	*MAT***a** *his3 leu2 ura3 trp1 ade8 can1*	M. Wigler [58]
S18-1D	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk1*^{w1} *tpk2::HIS3 tpk3::TRP1*	M. Wigler [48]
S15-5B	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2*^{w1} *tpk3::TRP1*	M. Wigler [48]
S22-5D	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3 tpk3*^{w1}	M. Wigler [48]
S7-7A	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1*	M. Wigler [48]
T139-5A-6A^w/ pCI^{25}-8	*MAT***a** *his3 leu2 ura3 trp1 ade8 cdc25::HIS3* + *pTPK1 (LEU2)*	M. Wigler [59]
PD1224	*MAT***a** *his3 leu2 ura3 trp1 ade8 can1* + *pTPK1 (LEU2)*	P. Durnez (our lab.)
JT2117	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3*	Our laboratory [47]
JT2119	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk3::TRP1*	Our laboratory [47]
Be333	*MAT***a** *cdc35-10*	F. Hilger [38]
PD6517	*MAT***a** *leu2 trp1 ade8 cdc35-10*	This work
OL86	*MAT***a** *cdc25-5 ade2 leu2 trp1*	M. Jacquet [39]
OL97-1-11B + pRAS2^{Ile152}	*MAT***a** *cdc25-5 leu2 ura3 his3 his7* + *pRAS2*^{Ile152}	M. Jacquet [41]
A364A	*MAT***a** ade1 ade2 ura1 his7 lys2 tyr1 gal1	Yeast Genetic Stock Center
D308	*MAT***a** *hxk1 hxk2 ade1 trp1 his2 met14*	Yeast Genetic Stock Center
D308.3	*MAT***a** *hxk1 hxk2 glk1 ade1 trp1 his2 met14*	Yeast Genetic Stock Center
P1T8C	*MAT***a** *hxk2 glk1 ade1 his2*	Yeast Genetic Stock Center
P2T22D	*MAT***a** *hxk1 glk1 ade1*	Yeast Genetic Stock Center
WAY.14-1A	*MAT***a** *his301 ura3-52 leu2-3, 112 hxk2::LEU2 glk1::LEU2 MAL2-8*^{c} *MAL3 SUC3 lcr1*	K.D. Entian
WAY.glk1-1A	*MAT***a** *his301 ura3-52 leu2-3, 112 hxk1::HIS3 hxk2::LEU2 MAL2-8*^{c} *MAL3 SUC3 lcr1*	K.D. Entian
WAY.glk1-5C	*MAT***a** *his301 trp1-289 leu2-3, 112 hxk1::HIS3 glk1::LEU2 MAL2-8*^{c} *MAL3 SUC3 lcr1*	K.D. Entian

transcription and was, therefore, probably due to post-translational modification [31]. In the present paper we have made use of a series of mutants affected either in cAMP synthesis or in the activity of cAMP-dependent protein kinase and we provide evidence that glucose-induced activation of plasma membrane H^+-ATPase is not mediated by cAMP-dependent protein phosphorylation. Our results, however, do not exclude the possibility indicated by the results of Ulaszewski et al. [27], that in derepressed cells increased cAMP levels lead to higher H^+-ATPase activity.

Previous experiments have shown that sugar phosphorylation is required for sugar-induced activation of cAMP synthesis and that the concentration causing half-maximal activation (±15–20 mM) fits with the K_m of low-affinity sugar transport [32]. Similar experiments in the present paper, with mutants affected in sugar phosphorylation and with different sugars and sugar concentrations, indicate that the glucose-induced signaling pathways for activation of adenylate cyclase and H^+-ATPase might share a common initiation point.

Experimental procedures

Yeast strains and growth conditions

The strains used in this work are shown in Table I. They were grown in a gyrotory incubator (200 rpm) at 30°C or at 24°C (for temperature-sensitive mutants) in YPG medium (1% wt/vol yeast extract, 2% wt/vol bactopeptone and 3% vol/vol glycerol). For strains containing plasmids, SDglycerol (defined, minimal medium; for composition see Ref. 33) minus the amino acid specified by the marker on the plasmid was used. Cells were harvested by centrifugation at 3000 rpm for 5 min during the logarithmic phase of growth (A_{600nm} = ±1), washed twice with distilled water and resuspended in 100 mM Mes buffer adjusted to pH 6.5 with Tris. For cAMP measurements in strains with deletions

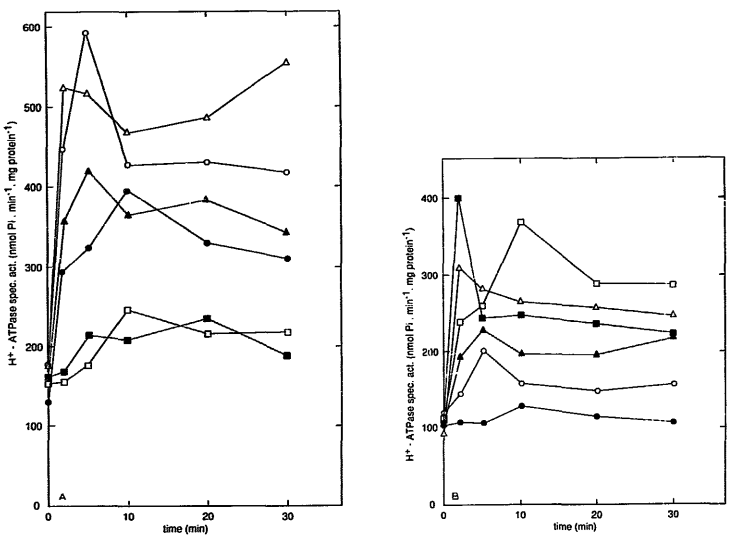

Fig. 1. Sugar-induced activation of plasma membrane H^+-ATPase. (A) Activation with different sugars (100 mM each). (●) Glucose; (○) fructose; (▲) mannose; (△) sucrose; (■) galactose; (□) lactose. (B) Activation with different concentrations of glucose. (●) 0 mM; (○) 5 mM; (▲) 20 mM; (△) 50 mM; (■) 100 mM; (□) 200 mM. Spec. act. is expressed in nmol phosphate liberated per min per mg protein.

in sugar kinase genes, the cells were also grown on YPD (1% w/v yeast extract, 2% w/v bactopeptone and 2% w/v glucose) until the glucose in the medium was exhausted (see legend of Fig. 7).

Incubation conditions

For time-course measurements of ATPase activity, the cells were incubated at a density of 35 mg (wet mass)/ml in a reciprocating water bath shaker at 30°C, or 22°C and 37°C for the temperature-sensitive mutants. Incubation was carried out in 100 mM Mes/Tris buffer (pH 6.5) for 20 min before addition of glucose or another carbon source (to a final concentration of 100 mM). For the protonophores 50 mM glycine/HCl (pH 4.5, 5.0 and 5.5) or 50 mM Mes/Tris (pH 6.0 and 6.5) was used. At different times, samples containing 175 mg cells (wet mass) were taken from the suspension and the cells collected as quickly as possible on glass fibre filters by vacuum filtration. The cells were quickly removed from the filters and immediately frozen in liquid nitrogen and stored until use.

Membrane preparation

Frozen samples were allowed to thaw in 0.5 ml 0.33 M sorbitol, 0.1 M Tris, 5 mM EDTA, 2 mM β-mercaptoethanol (pH 8) and homogenized by vortex-mixing with 1.5 g glass beads (0.5 mm diameter) for 3 min, with intermittent cooling on ice. Crude membranes were obtained by subsequent centrifugation of the homogenate first at $1000 \times g$ for 2 min and the resulting supernatant at $15000 \times g$ for 30 min. The pellet was resuspended in 2 ml Tris 10 mM, glycerol 26%, EDTA 1 mM, 1 mM β-mercaptoethanol (pH 7.5). Crude membranes were further fractionated by centrifugation for 90 min at $100000 \times g$ on a discontinuous sucrose gradient (43.5%, 53.5%) in a Beckman SW50 rotor (4°C). The band containing the ATPase was collected at the interface ($100000 \times g$ for 90 min) and washed with distilled water ($130000 \times g$ for 25 min). The membranes were resuspended in Tris-glycerol buffer and used for determination of ATPase activity.

ATPase assay

Plasma membrane ATPase activity was assayed using between 5 and 25 μg protein in 500 μl 50 mM Hepes buffer (pH 6.5), containing 2 mM ATP/$MgCl_2$, 10 mM NaN_3 to inhibit any remaining mitochondrial ATPase activity and 4 mM ammonium molybdate to inhibit phosphatase activity. The reaction was stopped by addition of 500 μl TCA 10%. Liberation of P_i was measured as described by Peterson [34].

Protein determination

The amount of protein was determined according to Lowry et al. [35], using bovine serum albumin as standard.

cAMP assay

Determination of cAMP levels was performed, as described previously [24].

Reproducibility of the results

All experiments were repeated at least twice with independent yeast cultures. Representative results are shown.

Results

Examination of different sugars for their activating effect on the plasma membrane H^+-ATPase showed that sucrose and fructose were most effective, followed by glucose and mannose, while lactose and galactose only had a small effect (Fig. 1A). This order of effectiveness is similar to the one described before for sugar-induced activation of cAMP synthesis and trehalase activity [19]. The concentration of glucose required for half-maximal stimulation was about 20 mM (Fig. 1B). This is very similar to the concentration of glucose required for half-maximal stimulation of cAMP synthe-

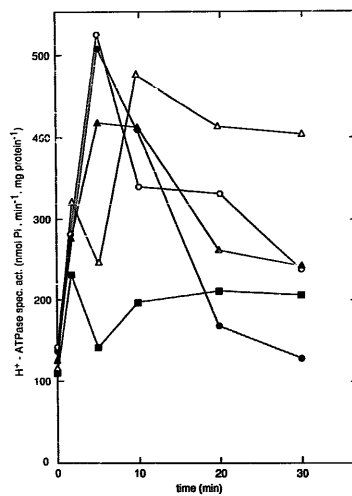

Fig. 2. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)-induced activation of plasma membrane H^+-ATPase at different pH values of the external medium. (●) pH 4.5; (○) pH 5.0; (▲) pH 5.5; (△) pH 6.0; (■) pH 6.5. Spec. act. is expressed in nmol phosphate liberated per min per mg protein.

sis [32] and fits with the K_m of low-affinity glucose transport [36].

Activation of H^+-ATPase by acid pH during growth has been described before [37]. We found that addition of the protonophores 2,4-dinitrophenol (results not shown) and CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) (Fig. 2) to derepressed cells causes rapid and pronounced increases in the activity of plasma membrane H^+-ATPase. The effect was strongly dependent on a low external pH, it disappeared nearly completely at pH 6.5 (Fig. 2 and unpublished results for 2,4-dinitrophenol). A similar dependence on the external pH was found for protonophore-induced stimulation of cAMP synthesis [24]. The optimum concentration for the protonophore effect was 1–2 mM with 2,4-dinitrophenol and 0.5 mM with CCCP (results not shown).

To check whether glucose-induced activation of plasma membrane H^+-ATPase is triggered by a cAMP increase, we made use of temperature sensitive mutants in cAMP synthesis. The *cdc35-10* mutant contains a temperature-sensitive mutation in adenylate cyclase itself [38]. The *cdc25-5* mutant contains a temperature-sensitive mutation in the *CDC25* gene product which is required for activation of the RAS proteins. Since the latter are required for adenylate cyclase activity, incubation of the *cdc25-5* mutant at the restrictive temperature leads to a rapid drop in the cAMP level [39,40]. This cAMP drop is largely suppressed in the presence of yeast equivalents of mammalian *ras* oncogenes, e.g., $RAS2^{Ile152}$. The latter allows adenylate cyclase activity without causing overstimulation of the cyclase. It also suppresses the temperature-sensitive phenotype caused by the *cdc25-5* mutation [41]. When the *cdc25-5* $pRAS2^{Ile152}$ strain is incubated at a temperature of 37°C, it has a normal cAMP level but it lacks glucose-induced stimulation of cAMP synthesis [42]. Incubation of the *cdc35-10* and *cdc25-5* strains at the temperature of 37°C nearly abolished glucose-induced activation of the plasma membrane H^+-ATPase while the *cdc25-5* $pRAS2^{Ile152}$ strain showed a partial reduction (Fig. 3). This tends to indicate that the glucose-induced cAMP signal has at least a stimulating effect on the activation of the H^+-ATPase. However, control experiments with two wild type strains incubated at the same temperature of 37°C also showed a reduction of plasma membrane H^+-ATPase activity (Fig. 3B). Even at 34°C there was already a significant effect (Fig. 3B). The effect was not always very large but was reproducibly observed in all

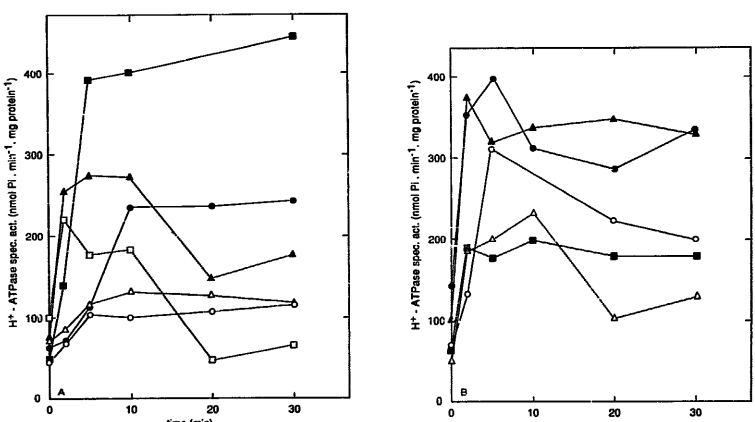

Fig. 3. Glucose-induced activation of plasma membrane H^+-ATPase at the permissive and restrictive temperature in yeast strains with temperature-sensitive mutations in cAMP synthesis, in a strain with a suppressor of the temperature-sensitive mutation and in wild type strains incubated at the same temperatures. (A) (●) *cdc35-10* at 22°C; (○) *cdc35-10* at 37°C; (▲) *cdc25-5* at 22°C; (△) *cdc25-5* at 37°C; (■) *cdc25-5* + $pRAS2^{Ile152}$ ($RAS2^{Ile152}$ largely restores basal cAMP level at restrictive temperature, but not glucose-induced cAMP signal; this strain is not temperature-sensitive) at 22°C; (□) *cdc25-5* + $pRAS2^{Ile152}$ at 37°C. (B) (●, ○) strain SP1; (▲, ■, △) strain A364A. (●, ▲) 22°C; (■) 34°C; (○, △) 37°C. Spec. act. is expressed in nmol phosphate liberated per min per mg protein.

experiments on glucose-induced activation (Fig. 3B) as well as protonophore-induced activation (results not shown). In addition, one would normally expect higher activities at this higher temperature, such as observed, for instance, for activation of trehalase at 37°C (unpublished observations). Such temperature-induced reductions in H^+-ATPase activation were also observed by Mazon et al. [43]. It is unclear, therefore, whether reduction of H^+-ATPase activation at the restrictive temperature in yeast mutants temperature-sensitive in cAMP synthesis is entirely due to the mutation or whether it is, at least partially, a temperature effect. Since it has been claimed that the *CDC25* gene product plays a specific role in H^+-ATPase activation [28], independent of its function in cAMP metabolism, we have also made use of a yeast mutant carrying a deletion in the *CDC25* gene (and rescued by overexpression of *TPK1*). This strain, however, also showed activation of the H^+-ATPase (both on minimal and rich medium), compared to a corresponding control strain with a wild type *CDC25* gene and overexpression of *TPK1* (only on minimal medium, to avoid plasmid loss) (Fig. 4). Hence CDC25 function is not required for glucose-induced activation of H^+-ATPase.

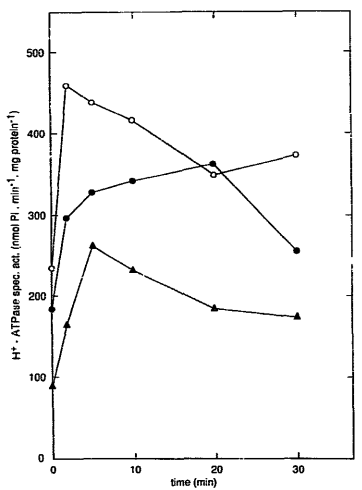

Fig. 4. Glucose-induced activation of plasma membrane H^+-ATPase in a *cdc25Δ* strain and a corresponding control strain. (●) *cdc25Δ pTPK1*, grown on minimal medium; (○) *CDC25 pTPK1*, grown on minimal medium; (▲) *cdc25Δ pTPK1*, grown on YPG. Spec. act. is expressed in nmol phosphate liberated per min per mg protein.

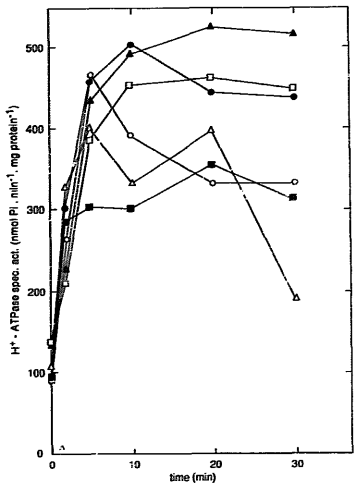

Fig. 5. Glucose-induced activation of plasma membrane H^+-ATPase in yeast strains with levels of activity of the catalytic subunit of protein kinase A. Strains with only one wild type *TPK* gene, coding for the catalytic subunit: (●) *TPK1*; (○) *TPK2*; (▲) *TPK3*. Strains with only one, partially inactivated, gene coding for the catalytic subunit (*tpk*^{w1}): (△) *tpk*^{w1}; (■) *tpk2*^{w1}; (□) *tpk3*^{w1}. Spec. act. is expressed in nmol phosphate liberated per min per mg protein.

In the yeast *Saccharomyces cerevisiae* three genes, *TPK1*, *TPK2* and *TPK3*, code for the catalytic subunit of cAMP-dependent protein kinase [44,45] and one gene, *BCY1*, for the regulatory subunit [44,46]. Yeast strains which contain only one wild type *TPK* gene, and the other two *TPK* genes deleted, display different degrees of protein kinase activity (TPK2 > TPK1 > TPK3), as measured by the heat-shock or nitrogen-starvation phenotype [45], by the extent of glucose-induced activation of trehalase or by the extent of protein kinase induced feedback-inhibition of cAMP synthesis [47]. No significant difference was seen, however, for the extent of glucose-induced activation of plasma membrane H^+-ATPase in strains containing only one wild type *TPK* gene (Fig. 5). We have also made use of strains containing an additional point mutation in the remaining *TPK* gene (*tpk*^{w1}) which results in very low protein kinase activity [48]. The extent of glucose-in-

duced activation of plasma membrane H^+-ATPase in these strains was similar to those in wild type strains (Fig. 5).

In yeast, three genes code for glucose phosphorylating enzymes. *HXK1* and *HXK2* code for hexokinase PI and PII, respectively, while *GLK1* codes for glucokinase [49]. Glucokinase is specific for glucose, while hexokinase phosphorylates both fructose and glucose. Experiments with yeast mutants containing inactivating point (Fig. 6A) or deletion (Fig. 7A, C) mutations in two of the three genes show that the presence of a kinase able to phosphoryiate glucose is necessary for

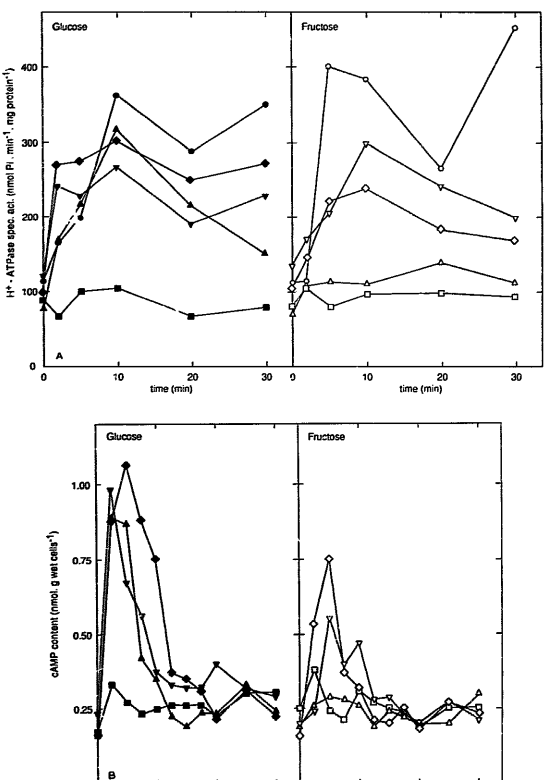

Fig. 6. Glucose- and fructose-induced activation of plasma-membrane H^+-ATPase and glucose- and fructose-induced cAMP signaling in yeast strains with combinations of point mutations in the genes coding for hexokinase PI (*HXK1*), hexokinase PII (*HXK2*) or glucokinase (*GLK1*). (A), H^+-ATPase; (B), cAMP. Full symbols: glucose; open symbols: fructose. (●, ○) wild type: *HXK1 HXK2 GLK1*; (▲, △) *hxk1 hxk2 GLK1*; (■, □) *hxk1 hxk2 glk1*; (▼, ▽) *HXK1 hxk2 glk1*; (◆, ◇) *hxk1 HXK2 glk1*.

glucose-induced activation of H^+-ATPase, while the presence of a kinase able to phosphorylate fructose is necessary for fructose-induced activation of H^+-ATPase. Although the point mutants show a clear correlation between sugar-induced activation of H^+-ATPase and sugar-induced cAMP signaling (Fig. 6B), this correlation is absent in the deletion mutants because these strains contain an additional mutation, *lcr1* (= lack of cyclic AMP responses), in their genetic background which prevents both sugar- (Fig. 7B, D) and protonophore-induced (M. Vanhalewyn and J.C. Argüelles, unpublished results) cAMP increases. Recent data indicate that *LCR1* is allelic to *CDC35* (M. Vannalewyn, unpublished results). Glycerol-grown cells of *lcr1* strains have relatively high basal cAMP levels (Fig. 7B). However, when the cells are grown in glucose-containing media till the glucose in the medium is exhausted, the basal cAMP level is similar to the basal

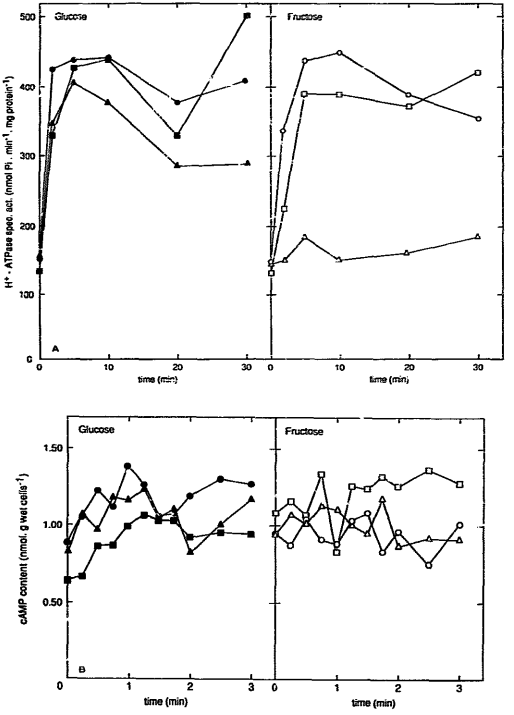

Fig. 7. Glucose- and fructose-induced activation of plasma-membrane H^+-ATPase and glucose- and fructose-induced cAMP signaling in yeast strains with combinations of deletion mutations in the genes coding for hexokinase PI (*HXK1*), hexokinase PII (*HXK2*) or glucokinase (*GLK1*). These strains contain an additional mutation (*lcr1*) in their genetic background, which causes absence of all cAMP responses. Glycerol-grown cells: (A), H^+-ATPase; (B), cAMP. Cells grown on glucose medium until the glucose in the medium was exhausted: (C), H^+-ATPase; (D), cAMP. Full symbols: glucose; open symbols: fructose. (●, ○) *HXK1 hxk2 glk1 lcr1*; (▲, △) *hxk1 hxk2 GLK1 lcr1*; (■, □) *hxk1 HXK2 glk1 lcr1*. Spec. act. is expressed in nmol phosphate liberated per min per mg protein.

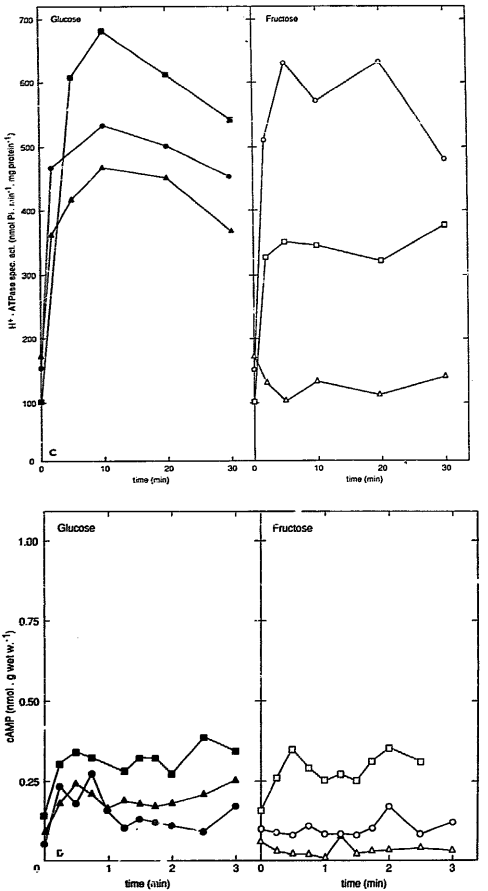

Fig. 7. (continued).

level in wild type strains (Fig. 7D). In both cases, however, there is no reliable glucose-induced increase in the cAMP level (Fig. 7B, D). The presence of sugar-induced H^+-ATPase activation in the deletion mutants (Fig. 7A, C), in spite of the absence of a sugar-induced cAMP increase (Fig. 7B, D), confirms

that the cAMP signal is not required for H^+-ATPase activation.

Discussion

With respect to the post-translational mechanisms regulating plasma membrane H^+-ATPase activity one should distinguish between the mechanism involved in glucose-induced activation of H^+-ATPase and mechanisms able to regulate basal H^+-ATPase activity. Our results with the protonophore effects, which are well known to cause pH-dependent increases in the cAMP level [24], are in agreement with those of the group of Goffeau that increased cAMP levels can activate plasma membrane H^+-ATPase [27]. However, it is also clear that such increased cAMP levels are not required for glucose-induced stimulation of H^+-ATPase activity (Figs. 3, 4, 7). Even incubation of temperature-sensitive mutants in cAMP synthesis at the restrictive temperature does not abolish glucose-induced activation completely (Fig. 3). One possible explanation is that a certain basal level of cAMP is required for the activation. The reduction observed at the restrictive temperature could (at least partially) be due to a negative temperature effect on the activation mechanism itself, since glucose-induced activation in wild type strains is also reduced at elevated temperatures (Fig. 3). This reduction cannot be due to an effect on the cAMP level, since in all strains examined up to now (with the obvious exception of temperature-sensitive mutants in cAMP synthesis) elevated temperature always resulted in higher cAMP levels [32,40,50 and unpublished observations]. In addition, a specific phenotype observed in point mutants does not necessarily imply involvement of the gene product in the expression of the phenotype in wild type cells. For example, *snf3* point mutants are affected in glucose repression while *snf3* deletion mutants are not [51]. Our results with the *cdc25Δ* strain (Fig. 4) clearly contradict the conclusion by Portillo and Mazon [28] that the *CDC25* gene product would play a specific role in glucose-induced activation of H^+-ATPase, independent of its function in cAMP metabolism.

Examination of glucose-induced activation of plasma membrane H^+-ATPase in mutants displaying a very wide range of protein kinase A activity confirmed that the cAMP-protein kinase A pathway is not required for the glucose effect and is, therefore, also probably not involved in mediating the glucose effect (Fig. 5). Since no reduction at all in the extent of activation was observed, it is also unlikely that protein kinase A would act together in a cumulative or synergistic way with another protein kinase. However, it cannot be excluded that multiple pathways exist which can fully and independently activate the H^+-ATPase. In addition, it cannot be excluded that in derepressed cells elevated activity of this pathway leads to enhanced H^+-ATPase activity. This must be an indirect effect (i.e., through another protein kinase), since the gene coding for plasma membrane H^+-ATPase does not contain consensus sequences for potential sites of cAMP-dependent protein phosphorylation [25]. Whether H^+-ATPase is phosphorylated itself during the glucose-induced activation process has been unclear until a very recent demonstration by Chang and Slayman [53] that phosphorylation of one or some specific sites in the protein correlates clearly with glucose-induced activation. Phosphorylation of the enzyme in vivo and in vitro had been demonstrated before [52] but most of this phosphorylation is now known to be present already before activation of the enzyme [53]. Portillo et al. [26] demonstrated that a putative calmodulin-dependent protein phosphorylation site in the deduced amino acid sequence was important for activation of the plasma membrane H^+-ATPase. Although we have been unable to confirm the claim by Portillo and Mazon [54] that phorbol esters activate the H^+-ATPase in vivo, we did find activation by diacylglycerol (unpublished results). Hence, the phosphatidylinositol–protein kinase C pathway might be a good candidate for the signaling pathway mediating glucose-induced activation of plasma membrane H^+-ATPase. Addition of glucose to glucose-starved yeast cells is known to stimulate phosphatidylinositol turnover [55,56].

Although glucose-induced activation of plasma membrane H^+-ATPase is not mediated by the glucose-induced cAMP signaling pathway both pathways might share a common initiation point. Little is known about the actual mechanisms involved upstream in triggering glucose-induced activation of the CDC25-RAS-adenylate cyclase system. A number of mutants, deficient in this part of the pathway however, have recently been identified [18,57]. Mutants deficient in sugar kinase activity are deficient in sugar-induced cAMP signaling [32] and the results in this paper show that this is also true for sugar-induced activation of plasma membrane H^+-ATPase (Figs. 6, 7). Moreover, the range of sugars able to activate H^+-ATPase (Fig. 1A) and the sugar concentration required for half-maximal stimulation (Fig. 1B) fit quite well with those for sugar-induced stimulation of the RAS-adenylate cyclase pathway [19,32]. These results tend to indicate that the signaling pathways for glucose induced activation of RAS-adenylate cyclase on the one hand and plasma membrane H^+-ATPase on the other hand share a common initiation point. Recently, additional evidence supporting this view has been obtained. The yeast *fdp1* mutant, which is deficient in glucose-induced activation of the RAS-adenylate cyclase pathway [57], is also deficient in glucose-induced activation of plasma membrane H^+-ATPase (Jomar Becher dos Passos, unpublished results).

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References

1 Thevelein, J.M. (1988) Exp. Mycol. 12, 1–12.
2 Francois, J., Van Schaftingen, E. and Hers, H.-G. (1984) Eur. J. Biochem. 145, 187–193.
3 Serrano, R. (1983) FEBS Lett. 156, 11–14.
4 Sychrová, H. and Kotyk, A. (1985) FEBS Lett. 183, 21–24.
5 Holzer, H. (1984) in Molecular aspects of cellular regulation. (Cohen, P., ed.), Vol. 3, pp. 143–154. Elsevier, Amsterdam.
6 Lopez-Boado, Y.S., Herrero, P., Gascon, S. and Moreno, F. (1987) Arch. Microbiol. 147, 231–234.
7 Lopez-Boado, Y.S., Herrero, P., Fernandez, T., Fernandez, R. and Moreno, F. (1988) J. Gen. Microbiol. 134, 2499–2505.
8 Witt, I., Kronau, R. and Holzer, H. (1966) Biochim. Biophys. Acta 118, 522–537.
9 Ferguson, J.J. Jr., Boll, M. and Holzer, H. (1967) Eur. J. Biochem. 1, 21–25.
10 Haarasilta, S. and Oura, E. (1975) Eur. J. Biochem. 52, 1–7.
11 Bisson, L.F. and Fraenkel, D.G. (1984) J. Bacteriol. 159, 1013–1017.
12 Ramos, J. and Cirillo, V.P. (1989) J. Bacteriol. 171, 3545–3548.
13 Entian, K.D. (1986) Microbiol. Sciences 3, 366–371.
14 Schmitt, H.D. and Zimmermann, F.K. (1982) J. Bacteriol. 151, 1146–1152.
15 Donovan, D.M. and Pearson, N.J. (1986) Mol. Cell. Biol. 6, 2429–2435.
16 Kief, D.R. and Warner, J.R. (1981) Mol. Cell. Biol. 1, 1007–1015.
17 Van der Plaat, J.B. (1974) Biochem. Biophys. Res. Commun. 56, 580–587.
18 Thevelein, J.M. (1991) Mol. Microbiol. 5, 1301–1307.
19 Thevelein, J.M. and Beullens, M. (1985) J. Gen. Microbiol. 131, 3199–3209.
20 Müller, D. and Holzer, H. (1981) Biochem. Biophys. Res. Commun. 103, 926–933.
21 Mazon, M.J., Gancedo, J.M. and Gancedo, C. (1982) J. Biol. Chem. 257, 1128–1130.
22 Caspani, G., Tortora, P., Hanozet, G.M. and Guerritore, A. (1985) FEBS Lett. 186, 75–79.
23 Purwin, C., Nicolay, K., Scheffers, W.A. and Holzer, H. (1986) J. Biol. Chem. 19, 8744–8749.
24 Thevelein, J.M., Beullens, M., Honshoven, F., Hoebeeck, G., Detremerie, K., den Hollander, J.A. and Jans, A.W.H. (1987) J. Gen. Microbiol. 133, 2191–2196.
25 Serrano, R. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 61–94.
26 Portillo, F., Eraso, P. and Serrano, R. (1991) FEBS Lett. 287, 71–74.
27 Ulaszewski, S., Hilger, F. and Goffeau, A. (1989) FEBS Lett. 245, 131–136.
28 Portillo, F. and Mazon, M.J. (1986) J. Bacteriol. 168, 1254–1257.
29 Johnson, D.I., Jacobs, C.W., Pringle, J.R., Robinson, L.C., Carle, G.F., Olson, M.V. (1987) Yeast 3, 243–253.
30 François, J., Eraso, P. and Gancedo, C. (1987) Eur. J. Biochem. 164, 369–373.
31 Eraso, P., Cid, A. and Serrano, R. (1987) FEBS Lett. 224, 193–197.
32 Beullens, M., Mbonyi, K., Geerts, L., Gladines, D., Detremerie, K., Jans, A.W.H. and Thevelein, J.M. (1988) Eur. J. Biochem. 172, 227–231.
33 Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
34 Peterson, G.L. (1978) Anal. Biochem. 84, 164–172.
35 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
36 Bisson, L.F. and Fraenkel, D.G. (1983) Proc. Natl. Acad. Sci. USA 80, 1730–1734.
37 Eraso, P. and Gancedo, C. (1987) FEBS Lett. 224, 187–192.
38 Boutelet, F, Petitjean, A. and Hilger, F. (1985) EMBO J. 4, 2635–2641.
39 Camonis, J.H., Kalékine, M., Gondré, B., Garreau, H., Boy-Marcotte, E. and Jacquet, M. (1986) EMBO J. 5, 375–380.
40 Van Aelst, L., Jans, A.W.H. and Thevelein, J.M. (1991) J. Gen. Microbiol. 137, 341–349.
41 Camonis, J.H. and Jacquet, M. (1988) Mol. Cell. Biol. 8, 2980–2983.
42 Van Aelst, L., Boy-Marcotte, E., Camonis, J.H., Thevelein, J.M. and Jacquet, M. (1990) Eur. J. Biochem. 193, 675–680.
43 Mazon, M.J., Behrens, M.M., Portillo, F. and Pinon, R. (1989) J. Gen. Microbiol. 135, 1453–1460.
44 Cannon, J.F. and Tatchell, K. (1987) Mol. Cell. Biol. 7, 2653–2663.
45 Toda, T., Cameron, S., Sass, P., Zoller, M. and Wigler, M. (1987) Cell 50, 277–287.
46 Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J.D., McBullen, B., Hurwitz, M., Krebs, E.G. and Wigler, M. (1987) Mol. Cell. Biol. 7, 1371–1377.
47 Mbonyi, K., Van Aelst, L., Argüelles, J.C., Jans, A.W.H. and Thevelein, J.M. (1990) Mol. Cell. Biol. 10, 4518–4523.
48 Nikawa, J., Cameron, S., Toda, T., Ferguson, K.W. and Wigler, M. (1987) Genes Dev. 1, 931–937.
49 Fraenkel, D.G. (1982) in The molecular biology of the yeast *Saccharomyces*. Metabolism and gene expression (Strathern, J.N., Jones, E.W. and Broach, J.R., eds.), pp. 1–37, Cold Spring Harbor Laboratory, Cold Spring Harbor.
50 Mbonyi, K., Beullens, M., Detremerie, K., Geerts, L. and Thevelein, J.M. (1988) Mol. Cell. Biol. 8, 3051–3057.
51 Neigeborn, L., Schwartzberg, P., Reid, R. and Carlson M. (1986) Mol. Cell. Biol. 6, 3569–3574.
52 McDonough, J.P. and Mahler, H.P. (1982) J. Biol. Chem. 257, 14579–14581.
53 Chang, A. and Slayman, C.W. (1991) J. Cell Biol. 115, 289–295.
54 Portillo, F. and Mazon, M.J. (1985) FEBS Lett. 192, 95–98.
55 Kaibuchi, K., Miyajima, A., Arai, K. and Matsumoto, K. (1986) Proc. Natl. Acad. Sci. USA 83, 8172–8176.
56 Frascotti, G., Baroni, D. and Martegani, E. (1990) FEBS Lett. 274, 19–22.
57 Van Aelst, L., Hohmann, S., Zimmermann, F.K., Jans, A.W.H. and Thevelein, J.M. (1991) EMBO J. 10, 2095–2104.
58 Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K. and Wigler, M. (1985) Cell 40, 27–36.
59 Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S. and Wigler, M. (1987) Cell 48, 789–799.