Phospholipase C is required for glucose-induced calcium influx in budding yeast

Renata Tisi[a], Simona Baldassa[b], Fiorella Belotti[a], Enzo Martegani[a,*]

[a] *Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy*
[b] *Dipartimento di Fisiologia e Biochimica Generali, Università degli Studi di Milano, via Celoria 26, 20134 Milan, Italy*

Received 21 March 2002; revised 26 April 2002; accepted 28 April 2002

First published online 13 May 2002

Edited by Horst Feldmann

Abstract Intracellular calcium is a second messenger involved in several processes in yeast, such as mating, nutrient sensing, stress response and cell cycle events. It was reported that glucose addition stimulates a rapid increase in free calcium level in yeast. To investigate the calcium level variations induced by different stimuli we used a reporter system based on the photoprotein aequorin. Glucose addition (50 mM) to nutrient-starved cells induced an increase in free intracellular calcium concentration, mainly due to an influx from external medium. The increase of calcium reached its maximum 100–120 s after the stimulus. A concentration of about 20 mM glucose was required for a 50% increase in intracellular calcium. This response was completely abolished in strain *plc1*Δ and in the isogenic wild-type strain treated with 3-nitrocoumarin, a phosphatidylinositol-specific phospholipase C inhibitor, suggesting that Plc1p is essential for glucose-induced calcium increase. This suggests that Plc1p should have a significant role in transducing glucose signal. The calcium influx induced by addition of high glucose on cells previously stimulated with low glucose levels was inhibited in strains with a deletion in the *GPR1* or *GPA2* genes, which suggests that glucose would be detected through the Gpr1p/Gpa2p receptor/G protein-coupled (GPCR) complex. Moreover, the signal was completely abolished in a strain unable to phosphorylate glucose, which is consistent with the reported requirement of glucose phosphorylation for GPCR complex activation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Polyphosphoinositide turnover; *PLC1*; Aequorin; Glucose signalling

1. Introduction

Eukaryotic cells use calcium as a second messenger in several relevant signal transduction pathways, e.g. exocytosis, response to growth factors, synaptic activity, etc., and in many of these processes intracellular calcium increases as a consequence of the activation of polyphosphoinositide turnover (PI turnover), which in turn is regulated by the activity of phosphoinositide-specific phospholipases C (PLC) [1,2].

There is increasing evidence that the intracellular calcium level plays a regulatory role also in the budding yeast *Saccharomyces cerevisiae*. In fact, variations of intracellular calcium have been observed during mating [3,4], hypotonic stress [5,6], after addition of glucose to glucose-starved cells [7–10], and during some cell cycle events [11–14]. The inhibition of calcium-dependent processes (for example through the addition of calmodulin inhibitors) always causes an immediate growth inhibition [11], suggesting that calcium plays a role as a relevant second messenger also in yeast. Moreover, the intracellular calcium level appears to be tightly controlled; yeast cells contain huge amounts of calcium that is confined in cellular compartments (vacuoles, mitochondria, etc.) while the free cytoplasmic calcium concentration is very low, around 200–300 nM [3,9,15,16].

S. cerevisiae Plc1p is a phosphatidylinositol (PI)-specific PLC with high homology to the mammalian PLC δ isoform [17–19] that was shown to be essential for glucose-stimulated PI turnover and subsequent activation of plasma membrane H^+-ATPase [20]. Deletion of the Plc1p-encoding gene (*PLC1*) causes a pleiotropic phenotype including defects in the utilisation of carbon sources different from glucose, as well as sensitivity to osmotic stress and high temperature. The severity of this phenotype is strictly dependent on the genetic background [17–19].

The PI turnover pathway is well known in mammalian cells, where it leads to activation of protein kinase C directly by diacylglycerol and by inositol triphosphate through calcium release from intracellular stores [2]. A protein kinase C also exists in yeast, where it is involved in maintenance of cell integrity both during stress response to hypotonic downshift, heat shock or nutrient starvation, and during bud generation or cellular differentiation, through activation of a mitogen-activated protein (MAP) kinase cascade [21]. Despite the similarity with mammalian PI turnover, there is no direct evidence of Plc1p involvement in Pkc1p activation. Stress sensitivity phenotypes observed in both *plc1*Δ and *pkc1* mutants suggest a possible link, but no genetic interactions have been reported which support this model. Up to now, the only identified role of Plc1p is in transducing glucose signalling, which is consistent with reported data about the physical interaction of Plc1p with a G protein-coupled receptor, Gpr1p, involved in glucose sensing [22].

It was initially suggested that the Gpr1p/Gpa2p G protein-coupled receptor (GPCR) system was involved in nitrogen sensing [23], but recently it was shown that this system is also required for activation of cAMP synthesis by glucose [24]. In addition to Gpr1p and Gpa2p, glucose activation of cAMP synthesis also requires uptake and phosphorylation of the sugar [25,26].

It was reported that both hypotonic shock and glucose addition stimulate a rapid increase in free calcium level [5]. In order to clarify if Plc1p is required for these responses, we

*Corresponding author. Fax: (39)-2-64483565.
E-mail address: enzo.martegani@unimib.it (E. Martegani).

0014-5793/02/$22.00
PII: S0014-5793(02)02806-5

investigated the effect of deprivation of Plc1p activity, either by a *PLC1* gene deletion or by protein inactivation through a PI-PLC-specific inhibitor, 3-nitrocoumarin, which was previously proved to be very efficient and selective for the yeast enzyme [27].

2. Materials and methods

2.1. Yeast strains, plasmids, growth conditions and transformation

The wild-type strain WPY260 (*MATα ura3-52 his3-11,15 trp1-Δ901 ade2-101*) and the isogenic mutant WPY300 (*plc1::URA3*) were kindly provided by M. Fitzgerald-Hayes (Amherst). The W303-1B (*MATα leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal*) and its derivatives *gpr1*Δ::*LEU2*, *gpa2*Δ::*LEU2* and YSH758(a) (*hxk1*:*HIS3*, *hxk2*::*LEU2*, *glk1*::*LEU2*) were from the Laboratory of J.M. Thevelein (KU Leuven, Belgium). The W303 strain carrying the multicopy plasmid pWP101, containing the *PLC1* coding sequence, was previously described [20]. The W303-1A strain containing the pG2CT-112.2 plasmid [23], a multicopy plasmid carrying the constitutive $GPA2^{R273A}$ allele, was kindly provided by J. Winderickx (KU Leuven, Belgium).

The integrative expression vector pYX012 (Ingenius) was modified to generate the pYX012-$G418^R$ plasmid by inserting in *Sma*I site the *Sma*I–*Eco*RV KanMX4 cassette from plasmid pFA6a [28]. The aequorin coding sequence was excised from plasmid cytAEQ/pCDNAI (Molecular Probes) by cutting with *Eco*RI and insertion in the *Eco*RI site in pYX012, in pYX012-$G418^R$ or in pYX212 to generate respectively pYX012-AEQ, pYX012-cytAEQ-$G418^R$ or pYX212-AEQ.

Strains WPY260 and WPY300 were transformed with the integrative expression vector pYX012-cytAEQ-$G418^R$, while W303 and its derivatives were transformed with the multicopy vector pYX212-cytAEQ.

Yeast transformation was performed by a modified lithium acetate method [29] and Ura^+ transformants were selected for growth on synthetic complete medium lacking uracil, that is 2% glucose, 2% agar, 0.67% yeast nitrogen base (Difco), 0.77 g/l CSM−ura (Complete Synthetic Medium supplied by Bio-101, CA, USA, containing every amino acid and adenine). For selection for G418 resistance transformed cells were first incubated in liquid rich medium (YPD, containing 2% glucose, 2% tryptone and 1% yeast extract by Biolife, USA, supplemented with 50 mg/l adenine and tryptophan) at 24°C for about 16 h and then plated on solid YPD medium supplemented with 100 μg/ml G418 (Geneticin, Gibco BRL). For luminescence assays yeast cells were grown in flasks on rich medium (YPD) in a Dubnoff shaking incubator at 24°C.

2.2. In vivo monitoring of cytosolic free calcium concentration after nutrient starvation

Exponentially growing cells ($5–6\times10^6$ cells/ml) in liquid rich medium (YPD), or in YPGal (containing 2% galactose, 2% tryptone and 1% yeast extract) if indicated, at 24°C were harvested by filtration on nitrocellulose filters (Millipore; pore size 0.45 μm), washed three times with water (1 volume/wash) and resuspended in 0.1 M 2-(*N*-morpholino)ethanesulphonic acid (MES)/Tris, pH 6.5 (MES/Tris buffer) at a density of about 1×10^8 cells/ml. Cells were incubated for 1.5 h at 24°C, collected by centrifugation at 3500 rpm for 2 min at room temperature and resuspended in the same nutrient-free buffer (2.5×10^9 cells/ml). To reconstitute functional aequorin, 50 μM coelenterazine (Molecular Probes; stock solution 1 μg/μl dissolved in methanol) was added to 30 μl of the cell suspension and the cells were incubated for 20 min at 24°C, in the dark. Excess coelenterazine was removed by washing cells three times with 0.1 M MES/Tris buffer (pH 6.5) (200 μl/wash) by centrifugation at 6000 rpm for 2 min at room temperature. 500 μl of the cell suspension (5×10^7 cells/ml in 0.1 M MES/Tris buffer) was transferred to a luminometer tube and 10 mM $CaCl_2$ (final concentration) was added (or omitted where indicated). Glucose or other carbon sources were added to give the final concentrations indicated in the text and light emission was monitored with a Berthold Lumat LB 9501/16 luminometer for at least 6 min after the stimulus, at 6-s intervals and reported in relative luminescence units/s (RLU/s). The figures shown represent typical lines that were reproduced in at least three different experiments.

The effect of 3-nitrocoumarin on glucose-induced calcium pulse was tested by further incubating the coelenterazine-loaded cell suspension with the inhibitor (final concentration 50 μg/ml; stock solution dissolved in dimethylsulphoxide (DMSO) and stored at −20°C) for 15 min at room temperature, in the dark, before adding glucose. Cells treated with the same amount (% w/v) of DMSO were used as a control. The 3-nitrocoumarin was synthesised as described previously [27].

The calcium chelator BAPTA (tetrapotassium salt, Sigma; stock solution 10 mM dissolved in 0.1 M MES/Tris pH 6.5) was added to the cell suspension at a final concentration of 1 or 0.5 mM 3 min before the addition of glucose.

At the end of each experiment aequorin expression and activity were tested by lysing cells with 0.5% Triton X-100 or by adding the calcium ionophore A23187 (10 μM) (Calcimycin, free acid; Sigma).

2.3. In vivo monitoring of hypotonic shock-induced calcium pulse

Aequorin-expressing cells were grown in rich medium (YPD) at 24°C until the mid-exponential phase ($6–7\times10^6$ cells/ml) and then collected by centrifugation at 3500 rpm for 5 min, at room temperature. The cell pellet was resuspended in fresh rich medium (YPD) at a density of 2.5×10^9 cells/ml. 30 μl of the cell suspension was incubated with coelenterazine as described above. After three washes with fresh YPD medium (200 μl/wash) by centrifugation at 6000 rpm for 2 min at room temperature, cells (7.5×10^7 for each treatment) were resuspended in 100 μl of the same medium and incubated for 5 min at room temperature before being shocked with 400 μl distilled water. Aequorin-dependent light emission was monitored over a 3-min period following the stimulus, at 3-s intervals. 3-Nitrocoumarin (50 μg/ml) or DMSO was added to coelenterazine-loaded cells as described above, before hypotonic shock.

2.4. Calibration of aequorin-dependent light signal

The raw data obtained from the luminometer were recorded and transferred to a personal computer for analysis. $[Ca^{2+}]_i$ (cytosolic free calcium concentration) was evaluated from the ratio of aequorin light intensity of the yeast cells to maximum light intensity, calculated from total light yield obtained by lysing cells with 0.5% Triton X-100 at the end of the experiment, using a standard curve reported in the literature [30]. The Ca^{2+}-independent luminescence of cells carrying the corresponding empty vector was subtracted as blank value from all of the other data.

3. Results

3.1. The glucose-induced calcium increase is dependent on Plc1p

A reporter system was used to investigate the calcium level fluctuations, based on the calcium-dependent photoprotein aequorin [9]. Apoaequorin expression was obtained by cloning the corresponding cDNA under the TPI constitutive promoter in different yeast vectors as described in Section 2. The functional protein was reconstituted by adding its cofactor, coelenterazine, in the medium. Aequorin expression and activity were detected by bioluminescent assays, either testing its responsiveness to the cytosolic calcium level variation induced by a calcium ionophore or verifying its abundance by the total light emission obtained after lysing cells with a detergent, which exposes the aequorin protein to saturating calcium concentrations.

Glucose addition to nutrient-starved cells induced a calcium increase that reached its maximum within 100–120 s after the stimulus (Fig. 1), consistent with previously reported data [7–9]. According to the described calibration method, the light emission obtained indicates a calcium basal level of 300–500 nM, while the calcium increase reaches a concentration of 2–3 μM.

This peak was reproducible with the aequorin system in both wild-type strains utilised, WPY260 (Fig. 1) and W303 (Fig. 4). In contrast, the response was completely abolished in the *plc1*Δ strain (WPY300) (Fig. 1A). Additionally, glucose

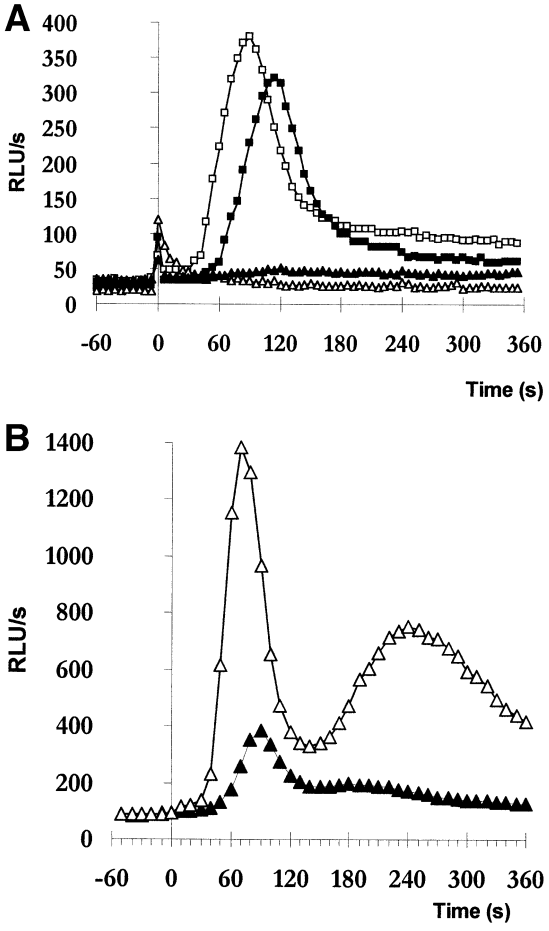

Fig. 1. A: Coelenterazine-treated apoaequorin-expressing cells were starved for all nutrients for 2 h, then 25 mM glucose was added at time zero and bioluminescence was monitored for 6 min. ■, WPY260 wild-type strain; □, WPY260 exposed to 0.001% DMSO; △, WPY260 treated with 50 mg/l 3-nitrocoumarin; ▲, WPY300 (*plc1*Δ strain). B: Coelenterazine-treated apoaequorin-expressing cells were starved for all nutrients for 2 h, then glucose 5 mM was added and bioluminescence was monitored for 6 min. ▲, W303 (wild-type strain); △, W303 carrying the multicopy plasmid pWP101, encoding Plc1p.

could not induce a calcium influx in the WPY260 wild-type strain when it was previously treated with 50 mg/l 3-nitrocoumarin, a concentration that is enough to completely inhibit the Plc1p-dependent glucose-induced PI turnover stimulation in vivo [27]. Moreover, in a strain carrying a multicopy plasmid containing the *PLC1* gene the calcium increase was higher than in the corresponding wild-type cells, which is particularly evident when cells are stimulated with low concentrations of glucose (Fig. 1B). These observations indicate that Plc1p is essential for glucose-induced calcium increase.

In order to characterise this Plc1p-dependent response to glucose, we detected cytoplasmic calcium levels after addition of different concentrations of glucose. We found that a concentration of 15–20 mM glucose was required for a 50% increase in glucose-induced calcium pulse (apparent K_m) (Fig. 2). Interestingly, this apparent K_m value is in the same range of that reported for cAMP signalling [25].

Total absence of calcium in the medium greatly inhibits the glucose-induced calcium peak (Fig. 3). Exposure to BAPTA, an extracellular Ca^{2+} chelator, gives the same result, apart from a slightly higher light emission probably due to an aspecific luminescence increase. These data suggest a major contribution of calcium influx from the external medium, though they cannot exclude a requirement for the signal elicited by calcium uptake to trigger calcium release from intracellular sources, i.e. through opening of a calcium-sensitive channel in the internal compartments.

The addition of other fermentable sugars different from glucose, such as fructose or mannose, induced a lower increase in cytoplasmic calcium concentration (Fig. 4A), while other carbon sources, such as galactose, raffinose, maltose and glycerol, have no effect (data not shown).

Unlike for glucose-induced calcium flux, both fructose (Fig. 4B) and mannose (data not shown) can induce a slight calcium rise also in the *plc1*::*URA3* strain. Therefore, Plc1p requirement is highly specific for glucose signalling, and not for detection of general fermentable carbon sources.

3.2. The high-glucose-induced calcium influx requires Gpr1p and Gpa2p

In order to identify other components of the Plc1p-mediated signalling pathway, we investigated the glucose-induced calcium response in mutant strains lacking some proteins involved in nutrient-triggered signal transduction systems.

The Gpr1p/Gpa2p GPCR system is required for full activation of cAMP synthesis by high glucose concentrations. Pre-addition of 5 mM glucose can abolish high-glucose-induced cAMP production in the *gpr1*Δ or *gpa2*Δ mutants. In fact, the initial low glucose addition does not activate the GPCR complex but is able to stimulate production of glucose 6-phosphate via the hexokinase system, which is a prerequisite for Gpr1p/Gpa2p activation. The addition of a high glucose concentration elicits the response transduced by the GPCR complex, which is abolished by deletion of either *GPR1* or *GPA2* [26]. In order to find out if the calcium signal was GPCR-dependent, we performed similar experiments measuring cytosolic calcium levels.

High glucose concentrations can induce a second calcium influx in a wild-type strain previously treated with low glucose (Fig. 5), even if the second signal is lower probably due to the activity of the recovery systems (calcium transporters are activated to reduce calcium concentration in the cytosol) [31].

As observed for the cAMP spike, also the calcium peak is induced by low glucose concentrations either in the wild-type or in both mutants, but pre-addition of 5 mM glucose completely inhibited the 100 mM glucose-induced calcium signal in the *gpr1*Δ and *gpa2*Δ strains (Fig. 5). This suggests that the GPCR complex is also involved in the calcium signal related to glucose sensing; this observation is consistent with the data reporting a physical and genetic interaction between Plc1p and Gpr1p receptor [22].

It is known that Gpr1p/Gpa2p activation is not the only requirement for cAMP signalling. In fact, it is necessary that glucose must be phosphorylated to allow activation of the adenylate cyclase [25,26]. To establish if glucose phosphorylation is also required for calcium signalling, we tested a strain, YSH758(a), which lacks both of the two hexokinases (*hxk1*Δ and *hxk2*Δ) and the glucokinase (*glk1*Δ). Addition of glucose on this strain does not induce any increase in cytoplasmic calcium levels (Fig. 6). This indicates that glucose phosphorylation is required for signal transduction through

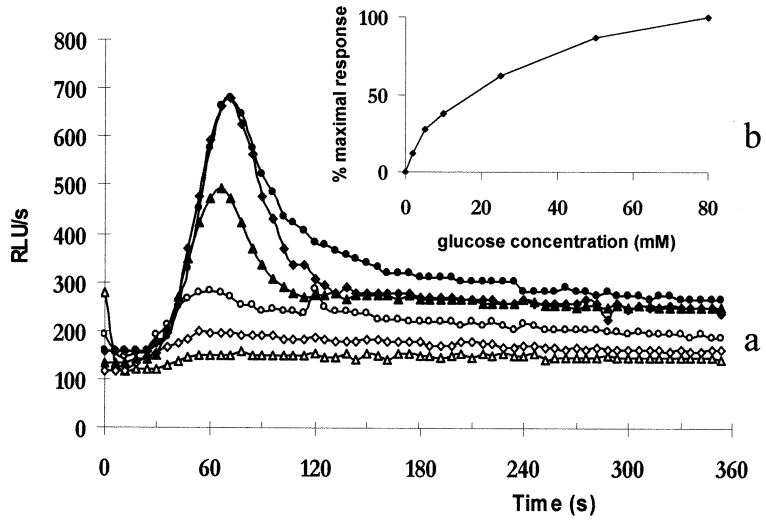

Fig. 2. Coelenterazine-treated apoaequorin-expressing cells were starved for nutrients for 2 h, then, at time zero, different concentrations of glucose were added as indicated below. Bioluminescence was monitored for 6 min. ●, 80 mM; ♦, 50 mM; ▲, 25 mM; ○, 10 mM; ◇, 5 mM; △, 2 mM. b: Total emission was expressed as percentage of the higher emission, obtained with 80 mM glucose.

the Gpr1p/Gpa2p system not only for cAMP generation but also for calcium influx.

3.3. Hypotonic shock

In mammalian cells, PLC activity leads to activation of protein kinase C. Since in yeast there are only one PI-specific PLC and one protein kinase C, it was tempting to look for a link between the Plc1p and Pkc1p pathways. In yeast Pkc1p activates a MAP kinase cascade pathway that is induced by different kinds of stress, one of which is hypotonic shock [32]. Consequently, we wondered if the hypotonic shock-triggered calcium influx could also be Plc1p-dependent. In order to investigate this, we monitored the calcium levels after hypotonic shock in the wild-type strain WPY260 and in the *plc1*Δ strain WPY300. A fast response was observed both in the wild-type strain and in the *plc1*Δ strain. Surprisingly, the calcium increase was even higher in this latter than in the wild-type strain (Fig. 7). This does not exclude an involvement of Plc1p in the regulation of this pathway, but Plc1p is certainly not required for the transduction of the hypotonic stress signal.

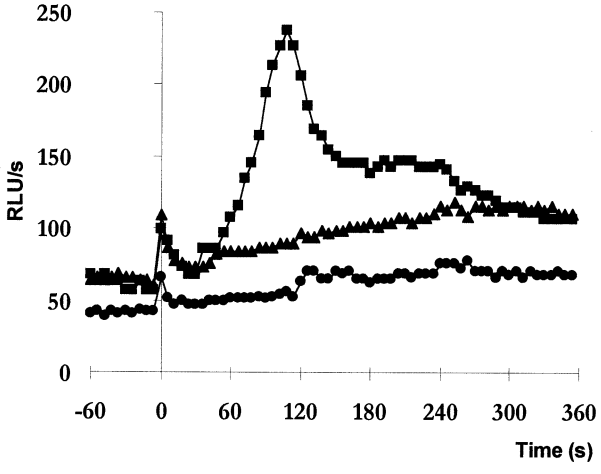

Fig. 3. Coelenterazine-treated apoaequorin-expressing cells were starved for nutrients for 2 h and 25 mM glucose was added at time zero. Glucose-induced calcium influx was observed in WPY260 cells resuspended in MES/Tris buffer (●), in MES/Tris buffer supplemented with 10 mM $CaCl_2$ (■) or with 0.5 mM BAPTA (▲).

4. Discussion

The *PLC1*-encoded PLC was suggested to act in a signal-

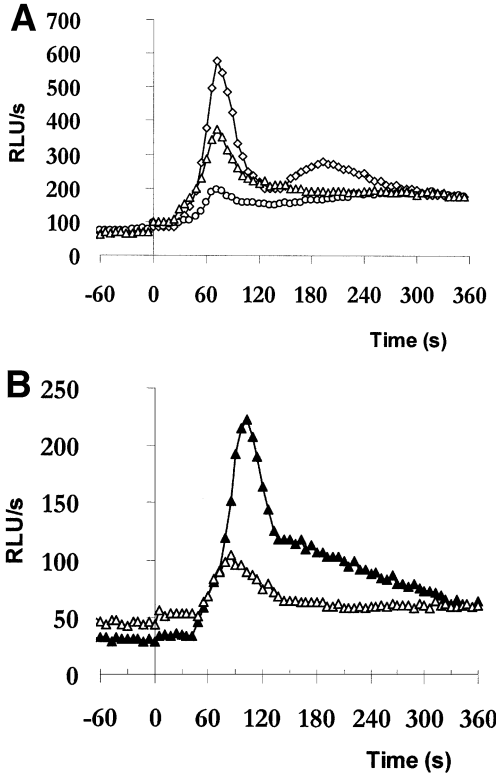

Fig. 4. Coelenterazine-treated apoaequorin-expressing cells were starved for nutrients for 2 h, then 50 mM of different fermentable sugars were added and bioluminescence was monitored for 6 min. A: W303 wild-type challenged with 50 mM: ◇, glucose; △, fructose; ○, mannose. B: 50 mM fructose was added at time zero. ▲, WPY260 wild-type cells, △, WPY300 *plc1*Δ cells.

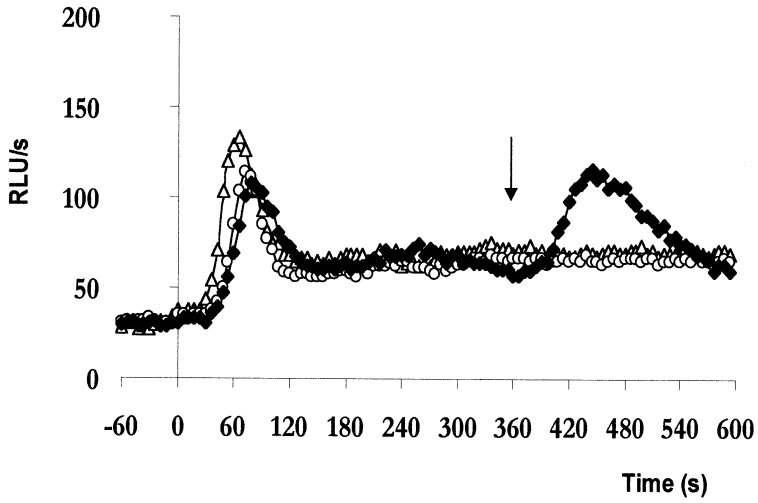

Fig. 5. Coelenterazine-treated apoaequorin-expressing cells were starved for all nutrients for 2 h, then first 5 mM glucose at time zero and after 6 min 50 mM (as indicated by the arrow) were added. Bioluminescence was monitored as described. ♦, W303 wild-type strain; ○, *gpr1*Δ strain; △, *gpa2*Δ strain.

ling pathway triggered by glucose leading to activation of the plasma membrane H^+-ATPase [20].

Our data on glucose-induced calcium signalling confirm a role for Plc1p activity in transducing signal after glucose stimulation, and this role is specific for glucose, since other sugars (such as fructose and mannose) can still raise a calcium influx even in the absence of an active Plc1p. Even if the absence of Plc1 causes a strong reduction in the Ca^{2+} signal induced by fructose, the presence of this small signal indicates that there is at least another mechanism that can trigger calcium concentration variations upon nutrient signalling independently of Plc1p activity. One possibility is that the calcium concentration increase is due to a transient cytosolic acidification. However, this is excluded by our results obtained with other carbon sources that when added to nutrient-deprived cells cause intracellular acidification (e.g. glycerol) [8], without any calcium level variation (data not shown). Otherwise, the calcium increase could be influenced by oscillations in ATP levels. Anyway, no simple correlation was observed between the variations of these two parameters [7].

The results we obtained with BAPTA, a non-permeable calcium chelator, indicate that the major component of the calcium flux comes from the external medium, while a minor contribution comes from the internal stores. It has been reported that glucose induces both an influx from the external medium and an efflux from internal stores [33]. However, this was observed after longer times of stimulation with glucose (20–30 min instead of 3–5 min), and so probably involved a completely different phenomenon.

The Gpr1/Gpa2 complex activates cAMP signalling after glucose (but not fructose) addition to derepressed cells [26]. Low concentrations of glucose added prior to high glucose stimulation could inhibit the response to high glucose in the *gpr1*Δ or *gpa2*Δ mutants, but not in the wild-type strain [26,34]. The same pattern was observed for intracellular calcium increase, suggesting that glucose signal can be trans-

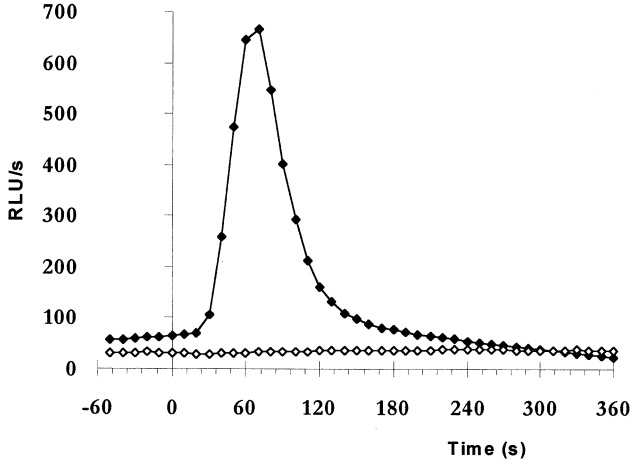

Fig. 6. Coelenterazine-treated apoaequorin-expressing cells were grown in YPGal, starved for all nutrients for 2 h, then 100 mM glucose was added at time zero. Bioluminescence was monitored as described. ♦, W303 wild-type strain; ◇, *hxk1*Δ *hxk2*Δ *glk1*Δ strain.

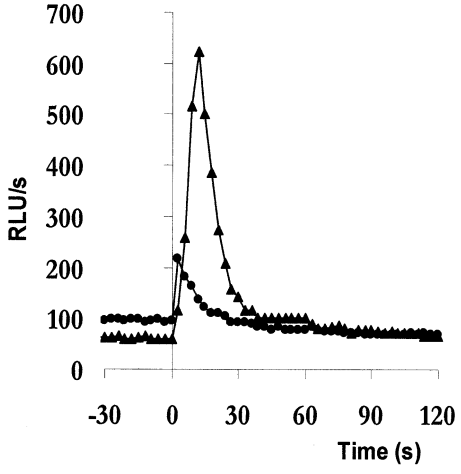

Fig. 7. Coelenterazine-treated apoaequorin-expressing cells were resuspended in YPD medium and then diluted with four volumes of distilled water at time zero. Bioluminescence was monitored for 2 min. ●, WPY260 wild-type strain; ▲, WPY300 (*plc1*Δ strain).

duced in at least two different ways, one of which is dependent on the Gpr1p/Gpa2p complex. It was suggested that the initial answer to low glucose concentration would not be dependent on Gpr1p/Gpa2p complex: in fact, this complex seems not to be significantly activated at glucose concentrations lower than 20 mM [26], which are of the same order as the apparent K_m we found for the calcium response. Even though different possibilities have been suggested (intracellular acidification, hexokinase activity or glucose transporters acting as sensors), the signalling mechanism that is supposed to act at lower concentrations of glucose is still unknown [22,26,34]. Anyway, unlike *GPR1* or *GPA2* deletions, deletion of the hexokinase- and the glucokinase-encoding genes can arrest the glucose signal transduction through both of these systems.

It has been proposed that Plc1p would be required to allow an interaction between Gpr1p and Gpa2p [22]. If that was the only role of this protein, one would expect the *gpr1*Δ and *gpa2*Δ strains to have a phenotype as severe as the *plc1*Δ strain, and that is not the case: deletion of *GPR1* or *GPA2* genes does not abolish the low glucose-induced calcium signal, which is totally absent in the *plc1*Δ strain. Moreover, a constitutive allele of *GPA2*, $GPA2^{R273A}$, would be expected to bypass the requirement of an active Plc1p to generate the calcium signal. In contrast, even a multicopy plasmid carrying that allele is not able to rescue 3-nitrocoumarin inhibition of the calcium response (data not shown). This evidence suggests instead a model that proposes a role for the GPCR system acting immediately upstream of Plc1p, while the hexokinase system would act upstream of both GPCR system and Plc1p. Another possibility would be that Plc1p could be involved in the generation of the hexokinases system, which might also explain why deletion of PLC reduces the fructose-induced Ca^{2+} signal. A consequence of Plc1p requirement for hexokinase system activation would be that also cAMP signalling would require Plc1p activity, and that is not the case, since cAMP signalling is normal in the *plc1*Δ strain (K. Lemaire, unpublished data).

Finally, our data on hypotonic shock-induced cytosolic calcium influx indicate that this is not dependent on Plc1p. This calcium uptake was suggested to be dependent on a stretch-activated channel [5]. Since calcium concentration increase is higher in the *plc1*Δ strain than in the wild-type strain, it is possible that Plc1p could be involved in the down-regulation of the response. Anyway, these data exclude a general requirement of Plc1p for calcium channel activity, suggesting that Plc1p involvement is very specific for glucose-induced calcium flux.

Acknowledgements: We thank M. Fitzgerald-Hayes for the WPY260 and WPY300 strains, J. Thevelein for the *gpr1*Δ, *gpa2*Δ and YSH757 strains and J. Winderickx for the strain expressing $GPA2^{R273A}$. In addition we also thank J. Thevelein and J. Winderickx for helpful advice and discussion. This work was supported by MURST-Cofin 40% to E.M. and by ex-MURST-60% to E.M.

References

[1] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[2] Berridge, M.J. (1993) Nature 365, 388–389.
[3] Iida, H., Yagawa, Y. and Anraku, Y. (1990) J. Biol. Chem. 265, 13391–13399.
[4] Nakajima-Shimada, J., Sakaguchi, S., Tsuji, F.I., Anraku, Y. and Iida, H. (2000) Cell Struct. Funct. 25, 125–131.
[5] Batiza, A.F., Schulz, T. and Masson, P.H. (1996) J. Biol. Chem. 271, 23457–23462.
[6] Kanzaki, M., Nagasawa, M., Kojima, I., Sato, C., Naruse, K., Sokabe, M. and Iida, H. (1999) Science 285, 882–886.
[7] Eilam, Y. and Othman, M. (1990) J. Gen. Microbiol. 136, 861–866.
[8] Eilam, Y., Othman, M. and Halachmi, D. (1990) J. Gen. Microbiol. 136, 2537–2543.
[9] Nakajima-Shimada, J., Iida, H., Tsuji, F.I. and Anraku, Y. (1991) Proc. Natl. Acad. Sci. USA 88, 6878–6882.
[10] Fu, L., Miseta, A., Hunton, D., Marchase, R.B. and Bedwell, D.M. (2000) J. Biol. Chem. 275, 5431–5440.
[11] Saavedra-Molina, A., Villalobos, R. and Borbolla, M. (1983) FEBS Lett. 160, 195–197.
[12] Iida, H., Sakaguchi, S., Yagawa, Y. and Anraku, Y. (1990) J. Biol. Chem. 265, 21211–21222.
[13] Anraku, Y., Ohya, Y. and Iida, H. (1991) Biochim. Biophys. Acta 1093, 169–177.
[14] Ohya, Y., Ohsumi, Y. and Anraku, Y. (1984) Mol. Gen. Genet. 193, 389–394.
[15] Halachmi, D. and Eilam, Y. (1989) FEBS Lett. 256, 55–61.
[16] Halachmi, D. and Eilam, Y. (1993) FEBS Lett. 316, 73–78.
[17] Flick, J.S. and Thorner, J. (1993) Mol. Cell. Biol. 13, 5861–5876.
[18] Yoko-o, T., Matsui, Y., Yagisawa, H., Nojima, H., Uno, I. and Toh-e, A. (1993) Proc. Natl. Acad. Sci. USA 90, 1804–1808.
[19] Payne, W.E. and Fitzgerald-Hayes, M. (1993) Mol. Cell. Biol. 13, 4351–4364.
[20] Coccetti, P., Tisi, R., Martegani, E., Teixeira, L.S., Brandão, R.L., de Miranda Castro, I. and Thevelein, J.M. (1998) Biochim. Biophys. Acta 1405, 147–154.
[21] Heinisch, J.J., Lorberg, A., Schmitz, H.P. and Jacoby, J.J. (1999) Mol. Microbiol. 32, 671–680.
[22] Ansari, K., Martin, S., Farkasovsky, M., Ehbrecht, I.M. and Kuntzel, H. (1999) J. Biol. Chem. 274, 30052–30058.
[23] Xue, Y., Batle, M. and Hirsch, J.P. (1998) EMBO J. 17, 1996–2007.
[24] Kraakman, L., Lemaire, K., Ma, P., Teunissen, A.W.R.H., Donaton, M.C.V., Van Dijck, P., Winderickx, J., de Winde, J.H. and Thevelein, J.M. (1999) Mol. Microbiol. 32, 1002–1012.
[25] 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.
[26] Rolland, F., de Winde, J.H., Lemaire, K., Boles, E., Thevelein, J.M. and Winderickx, J. (2000) Mol. Microbiol. 38, 348–358.
[27] Tisi, R., Coccetti, P., Banfi, S. and Martegani, E. (2001) Cell Biochem. Funct. 19, 229–235.
[28] Wach, A., Brachat, A., Pohlmann, R. and Philippsen, P. (1994) Yeast 10, 1793–1808.
[29] Schiestl, R. and Gietz, R.D. (1989) Curr. Genet. 16, 346–349.
[30] Blinks, J.R., Mattingly, P.H., Jewell, B.R., van Leewen, M., Harrer, G.C. and Allen, D.G. (1978) Methods Enzymol. 57, 292–328.
[31] Cunningham, K.W. and Fink, G.R. (1996) Mol. Cell. Biol. 16, 2226–2237.
[32] Davenport, K.R., Sohaskey, M., Kamada, Y., Levin, D.E. and Gustin, M.C. (1995) J. Biol. Chem. 270, 30157–30161.
[33] Kaibuchi, K., Miyajima, A., Arai, K. and Matsumoto, K. (1986) Proc. Natl. Acad. Sci. USA 83, 8172–8176.
[34] Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J.H., Gorwa, M.F., Colavizza, D. and Thevelein, J.M. (1998) EMBO J. 17, 3426–3441.