

Renata Tisi · Fiorella Belotti · Stefaan Wera
Joris Winderickx · Johan M. Thevelein · Enzo Martegani

Evidence for inositol triphosphate as a second messenger for glucose-induced calcium signalling in budding yeast

Received: 12 August 2003 / Revised: 21 October 2003 / Accepted: 23 October 2003 / Published online: 15 November 2003
© Springer-Verlag 2003

Abstract The *Saccharomyces cerevisiae* phospholipase C Plc1 is involved in cytosolic transient glucose-induced calcium increase, which also requires the Gpr1/Gpa2 receptor/G protein complex and glucose hexokinases. Differing from mammalian cells, this increase in cytosolic calcium concentration is mainly due to an influx from the external medium. No inositol triphosphate receptor homologue has been identified in the *S. cerevisiae* genome; and, therefore, the transduction mechanism from Plc1 activation to calcium flux generation still has to be identified. Inositol triphosphate (IP₃) in yeast is rapidly transformed into IP₄ and IP₅ by a dual kinase, Arg82. Then another kinase, Ipk1, phosphorylates the IP₅ into IP₆. In mutant cells that do not express either of these kinases, the glucose-induced calcium signal was not only detectable but was even wider than in the wild-type strain. IP₃ accumulation upon glucose addition was completely absent in the *plc1Δ* strain and was amplified

both by deletion of either *ARG82* or *IPK1* genes and by overexpression of *PLC1*. These results taken together suggest that Plc1p activation by glucose, leading to cleavage of PIP₂ and generation of IP₃, seems to be sufficient for raising the calcium level in the cytosol. This is the first indication for a physiological role of IP₃ signalling in *S. cerevisiae*. Many aspects about the signal transduction mechanism and the final effectors require further study.

Keywords Calcium · Inositol triphosphate · Glucose signalling

Communicated by S. Hohmann

R. Tisi · F. Belotti · E. Martegani (✉)
Dipartimento di Biotecnologie e Bioscienze,
Università degli Studi di Milano-Bicocca,
Piazza della Scienza 2,
20126, Milan, Italy
E-mail: enzo.martegani@unimib.it
Tel.: +39-02-64483533
Fax: +39-02-64483565

S. Wera · J. M. Thevelein
Laboratorium voor Moleculaire Celbiologie,
Katholieke Universiteit Leuven and Departement voor Moleculaire
Microbiologie,
Vlaans Interuniversitair Instituut voor Biotechnologie,
Kasteelpark Arenberg 31,
3001 Leuven-Heverlee, Belgium

J. Winderickx
Functional Biology,
Plantkunde en Microbiologie,
Katholieke Universiteit Leuven,
Kasteelpark Arenberg 31,
3001 Heverlee, Belgium

Introduction

Phosphatidylinositol (PI)-specific phospholipases C (PLCs) have a key role in calcium signalling in animal cells, where they catalyse the hydrolysis of PI-(4,5)-bisphosphate to diacylglycerol and *D*-myo-inositol-(1,4,5)-triphosphate (IP₃). Diacylglycerol directly activates protein kinase C, while IP₃ mediates the release of calcium from internal stores (Noh et al. 1995).

In budding yeast, a glucose-responding signal transduction pathway was reported, requiring the unique PI-specific PLC in *Saccharomyces cerevisiae*, Plc1 (Cocchetti et al. 1998). In fact, inhibition of Plc1 activity or deletion of the *PLC1* gene blocks the glucose-induced PI turnover and activation of the yeast plasma membrane H⁺-ATPase (Cocchetti et al. 1998; Tisi et al. 2001). Phospholipase C is also involved in the generation of a transient elevation in cytosolic calcium concentration (Tisi et al. 2002), but very little is known so far about the mechanism that triggers this calcium signalling. Glucose phosphorylation and Gpr1/Gpa2 GPCR complex activation are involved in this pathway (Tisi et al. 2002); and the same has been observed for another well known glucose-responsive signal transduction pathway in yeast, the cAMP/protein kinase A pathway (Thevelein and de Winde 1999). Differing from mammalian cells, the calcium accumulation in yeast is most likely due to calcium

influx from the external medium, mainly through the Cch1/Mid1 plasma membrane calcium channel (Tokes-Fuzesi et al. 2002).

Glucose-induced plasma membrane H^+ -ATPase activation also requires the yeast protein kinase C, Pkc1 (Brandão et al. 1994; Souza et al. 2001), and activation of the related MAP kinases cascade (de la Fuente and Portillo 2000). It is tempting to speculate that a pathway could exist in yeast also, connecting Plc1 activation to calcium and the Pkc1/Mpk1 signalling cascade. Nevertheless, it is not clear yet whether Pkc1 in yeast is actually responsive to a rise in calcium concentration. To date, the identified Pkc activators are cell wall stress sensors, such as Mid2 (Ketela et al. 1999), Wsc1/Hsc77/Slg1, Wsc2, Wsc3 (Delley and Hall 1999) and the Tor signalling proteins (Schmidt et al. 1997) via activation of the GTPase, Rho1 (Nonaka et al. 1995; Kamada et al. 1996), in addition to the yeast homologue of mammalian 3'-phosphoinositide-dependent kinase 1, Pkh2 (Inagaki et al. 1999). Consequently, it is possible that the phospholipase C-dependent calcium signalling pathway in yeast could lead to different effectors than in mammalian cells. At present, no physiological role has ever been proposed for glucose-induced calcium signalling; and the only indication about a possible effector of this signal is that both calcium signalling and H^+ -ATPase activation show a requirement for Plc1 activity.

No homologues of the mammalian IP_3 receptors have been found in the yeast genome (Wera et al. 2001), but two IP_3 -responsive calcium channels, not homologous to the mammalian channels, are reported in *Neurospora crassa* (Silverman-Gavrila and Lew 2002). This suggests that novel types of IP_3 receptor could exist in fungi. These observations raise the question of which mechanism could lead to calcium uptake after Plc1 activation and whether a second messenger (IP_3 or diacylglycerol) is involved.

In yeast, a complete degradation pathway seems to exist that sequentially dephosphorylates IP_3 to $I(1,4)P_2$, IP and *myo*-inositol (York et al. 1999). Nevertheless, none of the phosphatases involved have been identified up to now, except for two inositol monophosphatases (Imp1, Imp2) highly homologous to animal and plant ones (Lopez et al. 1999). Surprisingly, inhibition or deletion of these inositol monophosphatases leads to a decrease in the IP_3 level, in contrast to what is observed in mammalian cells (Navarro-Aviñó et al. 2003), indicating that IP_3 metabolism in *S. cerevisiae* is quite unusual when compared with mammalian cells.

The inositol phosphates phosphomonoesterase activity is outweighed by the 100-fold higher IP_3 kinase activity (Robinson et al. 1996). IP_3 kinase activity in yeast is represented by the *ARG82* (also called *ARGRIII* or *IPK2*) gene product, Arg82, a dual kinase which is active on both IP_3 and IP_4 , leading to IP_5 production. Arg82 was reported to be involved in arginine-responsive transcription regulation (Saiardi et al. 2000). In fact, the regulation of arginine

metabolism requires the integrity of four regulatory proteins, ArgRI, ArgRII, Arg82/ArgRIII and Mcm1. Arg82 seems to be involved in maintaining the stability of ArgRI and Mcm1, but this protein surely has some additive functions that are not Mcm1-dependent (El Bakkoury et al. 2000).

The $I(1,3,4,5,6)P_5$ is then phosphorylated to IP_6 by the kinase encoded by *IPK1* (also called *GSL1*), which is required together with Arg82 for efficient mRNA export from the nucleus (York et al. 1999).

In this paper, we analyse the IP_3 level upon glucose stimulation and the effect of the deletion of *IPK1* and *ARG82* genes on Plc1-dependent calcium signalling. Our findings suggest that IP_3 production could be sufficient as a signal for calcium increase and no further phosphorylation is required. This is the first report suggesting a role for IP_3 in *S. cerevisiae*, and further work is required to clarify many aspects about the signal transduction mechanism and the physiological effectors of this signalling pathway.

Materials and methods

Yeast strains and plasmids

The wild-type strain W303-1a (*MATa ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 GAL suc2*) and the isogenic mutant *plc1Δ* (*plc1::URA3*) were kindly provided by Laura Popolo (Università di Milano, Milan, Italy). The W303-1a strain carrying the multicopy plasmid pWP101, containing the *PLC1* coding sequence, was described by Coccetti et al. (1998). The SWY1659 strain (*Matz ipk1::KanMX2*) and the isogenic wild type, W303-1b, were a gift from Susan Wente and James York (Washington University School of Medicine, St. Louis, Mo., USA; York et al. 1999). Strain PJ69-4A (Clontech) and its derivative *arg82::KanMX2* (Saiardi et al. 2000) were kindly given by James Caffrey and Stephen Shears (National Institute of Environmental Health Sciences, Research Triangle Park, N.C., USA).

Plasmid YEplac195-*PDE1* was described by Ma et al. (1999); and the wild-type strain FDL33.6B (*leu2 ura3 trp1 arg4 ade*) and its derivatives, *cde25::HIS3 sdc25::HIS3* YE*pRAS2*^{tle152} and *cde25::HIS3 sdc25::HIS3* YE*pTPK1*, were obtained from M. Jacquet, Orsay (Colombo et al. 1998).

In vivo monitoring of cytosolic free calcium concentration after nutrient starvation

The cytosolic free calcium concentration was measured as described by Tisi et al. (2002). Briefly, exponentially growing cells in liquid rich medium (YPD; Tisi et al. 2002) at 24 °C were harvested by filtration, washed three times with water and resuspended in 0.1 M Mes/Tris buffer, pH 6.5. Cells were incubated for 1.5 h at 24 °C, collected by centrifugation and resuspended in the same nutrient-free buffer (2.5×10^9 cells ml^{-1}). To reconstitute functional aequorin, the cell suspension was incubated in the dark for 20 min at 24 °C, with 50 μM coelenterazine (stock solution at 1 mg ml^{-1} , dissolved in methanol; Molecular Probes). After washing the cells three times with Mes/Tris buffer, a suspension containing 2.5×10^7 cells in the same buffer was transferred to a luminometer tube and 10 mM $CaCl_2$ (final concentration) was added. Light emission was monitored with 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 glucose to a final concentration of 100 mM (or diacylglycerol, 2 mM final concentration; Sigma-Aldrich); and it was reported in relative luminescence units (RLU) s^{-1} . The figures represent typical RLU lines that were reproduced in at least three different experiments. At the end of each experiment, the aequorin expression and activity were tested by lysing cells with 0.5% Triton X-100. Typically, less than 10% of the total light emission (TLE) was expended during the calcium response assay.

Calibration of aequorin-dependent light signal

The raw data obtained from the luminometer were recorded and transferred to a personal computer for analysis. In order to make different experiments comparable, variations in the cytosolic free calcium concentration $[Ca^{2+}]_i$ were evaluated from the ratio of the aequorin light intensity of the yeast cells vs TLE, calculated from the total light yield obtained by lysing an aliquot of the same cells with 0.5% Triton X-100, referring to a standard curve reported in the literature (Matsumoto et al. 2002). The Ca^{2+} -independent luminescence of cells carrying the corresponding empty vector was subtracted as a blank value from all other data.

Determination of IP_3

Nutrient-starved cells were resuspended at room temperature at a density of 93.75 mg (wet weight) ml^{-1} in Mes/Tris buffer. At time-point zero, glucose was added to a final concentration of 100 mM. Treatment with 3-nitrocoumarin (synthesised as described by Tisi et al. 2001) was performed in Mes/Tris buffer at a concentration of 50 $mg\ l^{-1}$ for 15 min before the stimulus. Then, 1-ml samples, containing 75 mg of cells, were quenched in 4 ml of 60% methanol pre-cooled in a dry-ice/acetone bath and subsequently harvested by centrifugation. Ice-cold perchloric acid (1 ml) and 1.5 g of glass beads (0.5 mm diameter) were added in order to break the cells by vigorous shaking at 4 °C. The cell homogenates were centrifuged, after which the soluble fraction (extract) was used to determine the IP_3 content. IP_3 was measured in neutralised cell extracts, as described by Bergsma et al. (2001), using an IP_3 bovine adrenal-binding protein-based radioligand assay (Amersham Pharmacia Biotech). As demonstrated by Bergsma et al. (2001), this test was highly specific for IP_3 ; and the glycerophosphoinositol (phosphate) produced upon glucose stimulation (Hawkins et al. 1993) did not interact with this radioligand assay. Briefly, this assay was based on competitive binding to an insoluble protein between a radioactive IP_3 and the cold IP_3 contained in the samples.

Calculation of IP_3 content

The raw data obtained from the radioligand assay were the levels of radioactivity bound to the IP_3 bovine adrenal-binding protein when it was incubated with nearly 100 Bq of $[^3H]IP_3$ together with the appropriate volume of cell extract. These raw data were treated as follows.

The IP_3 content (in relative units) was calculated with the formula: $IP_3 = [(B_0 - X) / (X - NSB)]$, where B_0 was the raw value obtained when water substituted the cell extract, X was the raw value obtained with the sample and NSB (for *not specifically bound*) was the raw value obtained when 120 pmol of cold IP_3 substituted the cell extract. The IP_3 contents (in relative units) of at least four different standards were calculated, using known amounts of cold IP_3 . These standards were utilised to establish a linear correlation between the IP_3 (in relative units) and the real IP_3 content in the assays. The error in the IP_3 content calculation was estimated from the standard deviations in X , NSB, B_0 and standard raw data, all of which were always performed in duplicate. Each curve here reported is typical of at least three different experiments.

Results

Mutants impaired in IP_3 metabolism show an increase in calcium response

In order to clarify whether IP_3 production could be sufficient as a signal for calcium peak stimulation or whether further phosphorylation is required, we utilised yeast strains deleted either in *IPK1* or *ARG82*. In both strains, the calcium response was not only detectable but was higher than in the corresponding wild-type strain (Figs. 1, 2). In the *ipk1 Δ strain, the calcium accumulation was slightly increased, as was observed previously in the strain carrying *PLC1* on a multicopy plasmid*

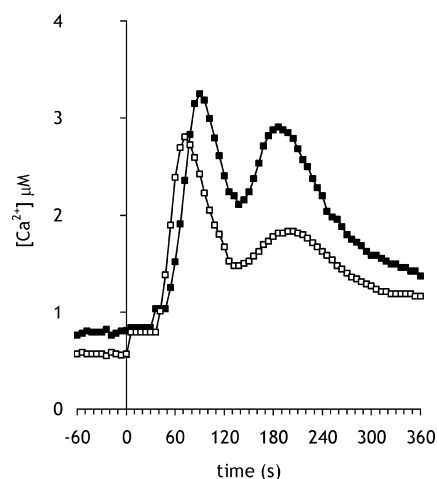


Fig. 1 Glucose-induced calcium signalling in an *ipk1*-deleted strain. Apoaequorin-expressing cells were starved for all nutrients for 2 h and then treated with coelenterazine as described in the Materials and methods. At time zero, 100 mM glucose was added. *White squares* Wild-type strain (W303-1b), *black squares* *ipk1 Δ strain*

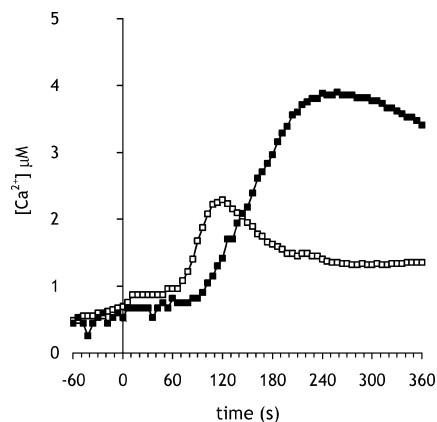


Fig. 2 Glucose-induced calcium signalling in an *arg82*-deleted strain. Apoaequorin-expressing cells were starved for all nutrients for 2 h and then treated with coelenterazine as described in the Materials and methods. At time zero, 100 mM glucose was added. *White squares* Wild-type strain (PJ69-4a), *black squares* *arg82 Δ strain*

(Tisi et al. 2002). In the *arg82Δ* strain, the calcium concentration increase was much higher, even at saturating concentration of glucose (100 mM), and the calcium peak appeared broader than in the wild-type strain, suggesting that the lack of IP₃/IP₄ kinase could enhance the accumulation of calcium in the cytosol.

Since the addition of diacylglycerol, the other product of Plc1 activation, was reported to activate the plasma membrane H⁺-ATPase (Brandão et al. 1994), we also tested whether this compound had any effect on calcium levels, but the exposition of cells to 2 mM diacylglycerol did not cause any change in the cytosolic calcium concentration (data not shown).

IP₃ levels increase after glucose stimulation of nutrient-starved cells

In the wild-type strain, a small but reproducible accumulation of IP₃ was observed after glucose stimulation of starved cells (Fig. 3). The low IP₃ content of yeast cells (about 1 nmol g⁻¹ wet weight) and the small amount of IP₃ accumulated upon glucose stimulation could explain why it was reported in the literature that only nitrogen, but not glucose, stimulation induces IP₃ production (Schomerus and Kuntzel 1992). The IP₃ increase was small but significant and showed a characteristic oscillatory behaviour, which we observed in all experiments done and also with a different wild-type strain (see Fig. 5).

Overexpression of *PLC1* caused a small increase in the calcium response (Tisi et al. 2002). The accumulation of IP₃ observed in this strain was also slightly higher than in the wild-type strain (Fig. 3) with an increase in the amplitude of oscillations, suggesting that the two events could be correlated. In contrast, no IP₃ production was

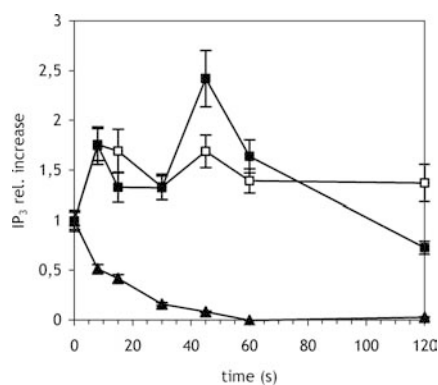


Fig. 3 Inositol (1,4,5) triphosphate (IP₃) content after glucose addition to wild-type, *PLC1*-overexpressing and *plc1*-deleted strains. Cells were starved for all nutrients for 2 h. At least three samples were taken for determining the basal level of IP₃ content, then 100 mM glucose was added and samples were taken at the indicated times. The IP₃ relative increase was calculated by taking the basal level as 1. *White squares* Wild-type strain (W303-1a), *black squares* W303-1a strain carrying pWP101 (multicopy *PLC1*), *black triangles* *plc1Δ* strain

observed in the *plc1Δ* strain, which is consistent with the complete inhibition of calcium signalling. Moreover, the IP₃ level decreased immediately after glucose addition (Fig. 3); and a similar lack of response was observed in a wild-type strain after the addition of 3-nitrocoumarin (data not shown).

The amplitude of IP₃ oscillations increased also in the *ipk1Δ* strain (Fig. 4), but the greatest accumulation was observed in the *arg82Δ* strain (Fig. 5). Even if the relative increase in IP₃ after glucose addition was not very high, this corresponded to a really high amount of IP₃ accumulation, which correlated with the higher accumulation of calcium in the cytosol (Fig. 2). In this mutant strain, the oscillatory behaviour was less evident and a more persistent increase of IP₃ was observed.

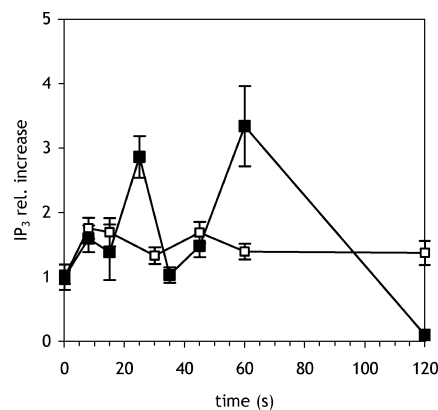


Fig. 4 IP₃ content after glucose addition in an *ipk1*-deleted strain. Cells were starved for all nutrients for 2 h. At least three samples were taken for determining the basal level of IP₃ content, then 100 mM glucose was added and samples were taken at the indicated times. The IP₃ relative increase was calculated by taking the basal level as 1. *White squares* Wild-type strain (W303-1a), *black squares* *ipk1Δ* strain

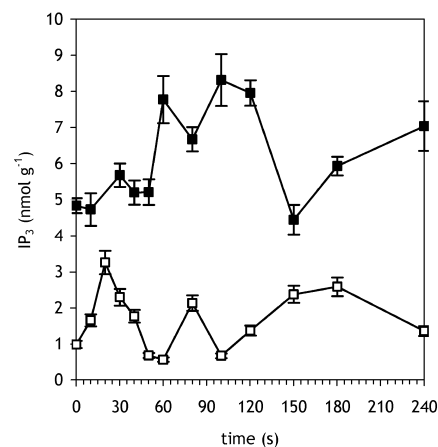


Fig. 5 Inositol (1,4,5) triphosphate content after glucose addition in an *arg82*-deleted strain. Cells were starved for all nutrients for 2 h. At least three samples were taken for determining the basal level of IP₃ content, then 100 mM glucose was added and samples were taken at the indicated times. *White squares* Wild-type strain (PJ69-4a) *black squares* *arg82Δ* strain

The *arg82Δ* strain showed a nearly 5-fold increase in the basal level of IP₃, likely depending on its slow conversion in the absence of the Arg82 kinase. Saiardi et al. (2000) indicated a much higher IP₃ basal level in this strain. However, those authors tested the IP₃ content in cells grown in YPD after a 2-h shift to a non-permissive temperature (37 °C) whereas, in our experimental conditions, cells were starved of nutrients for 2 h. These completely different conditions could explain the contrast in our results.

cAMP signalling is not required for calcium signalling

Since we previously showed that both the Gpr1/Gpa2 system (Tisi et al. 2002) and hexokinase/glucokinase activity (Tisi et al. 2002; Tokes-Fuzesi et al. 2002) were required for glucose-induced calcium signalling, the calcium response appears to have the same activation requirements as the cAMP signalling pathway (Rolland et al. 2000). Therefore, we tested whether cAMP signalling could be a prerequisite for calcium signalling. A strain was used carrying the phosphodiesterase-encoding gene *PDE1* on a multicopy plasmid, which was reported to completely abolish cAMP accumulation upon glucose stimulation (Ma et al. 1999). This strain was not defective in the glucose-induced calcium peak (Fig. 6). Moreover, we also tested a strain lacking both Ras-guanine nucleotide exchange factors (GEFs), Cdc25 and Sdc25, which was therefore completely impaired in glucose-induced cAMP signalling (Van Aelst et al. 1990). The *CDC25* deletion is lethal in a wild-type background, so this strain needs a suppressor of lethality. We used two different suppressors: overexpression of the catalytic subunit of protein kinase A, Tpk1, or expression of a mutated version of Ras, Ras2^{Ile152}, which was reported not to require a GEF (Camonis and Jacquet 1988). Both

strains were able to generate a normal glucose-induced calcium peak (data not shown).

Discussion

Since Plc1 is required for both glucose-induced calcium signalling (Tisi et al. 2002), glucose-induced activation of PI turnover and the stimulation of H⁺-ATPase (Cocchetti et al. 1998), it is reasonable to suppose that a phospholipase C-dependent signalling pathway could also exist in yeast, involving a classic second messenger for PLC signalling, which is IP₃. Unfortunately, the accumulation of IP₃ is quite difficult to detect in yeast, because IP₃ is quickly converted to inositol polyphosphates (York et al. 1999; Saiardi et al. 2000), rather than dephosphorylated to inositol monophosphate and inositol, following a peculiar scheme of inositol phosphate metabolism (Robinson et al. 1996). Moreover, IP₃ has a very low basal level (about 1 nmol g⁻¹ wet weight).

It was reported that the addition of a nitrogen source to nitrogen-starved cells induces the generation of a peak of IP₃ (Schomerus and Kuntzel 1992; Bergsma et al. 2001), whereas glucose addition to nutrient-starved cells was reported either to induce (Kaibuchi et al. 1986) or not to induce (Schomerus and Kuntzel 1992) IP₃ accumulation. Surprisingly, nitrogen-induced IP₃ accumulation is independent of Plc1 activity (Bergsma et al. 2001), indicating that IP₃ can be produced through at least two different biosynthetic pathways. This could account for the basal level of IP₃ in the *plc1Δ* strain being not dramatically lower than in the wild type, in our experimental conditions. The same effect on IP₃ basal level in a Plc1-deficient background was observed in another organism, *Dictyostelium*, where IP₃ can be produced in a Plc1-independent pathway (Van Dijken et al. 1995).

In our experimental system, it was possible to reveal a glucose-induced accumulation of IP₃ (Fig. 3). The increase in IP₃ was small and showed a characteristic oscillatory behaviour that was observed in all experiments and in two different wild-type yeast strains (W303-1a, PJ69-4a). Oscillations could be related to either a positive or a negative feedback mechanism, coupled to a non-linear behaviour caused either by a threshold or by a delay in response to a stimulus (Goldbeter 2002). At present, the basis for this oscillatory behaviour is not clear, but the indication that the oscillations are much less evident in the *arg82* deletion mutant would suggest that IP₃ phosphorylation could be relevant for this phenomenon.

Due to its rapid conversion to inositol polyphosphate in a wild-type strain, this accumulation was more clearly detected either in mutants impaired in IP₃ phosphorylation or (with an increase in the amplitude and/or duration of the oscillations) in the Plc1-overexpressing strain (Figs. 4, 5). All these strains also showed a higher accumulation of calcium in the cytosol (Figs. 1, 2; Tisi et al. 2002), suggesting a possible involvement of this second messenger in the generation of a calcium

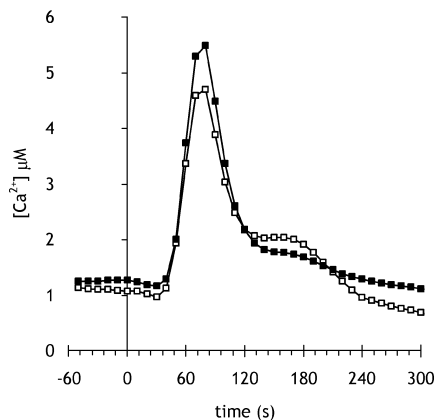


Fig. 6 Glucose-induced calcium signalling in a cAMP signalling-impaired strain. Apoaquorin-expressing cells were starved for all nutrients for 2 h, then treated with coelenterazine as described in the Materials and methods. At time zero, 100 mM glucose was added. *White squares* Wild-type strain (W303-1a), *black squares* wild-type strain carrying YEplac195-*PDE1*

pulse. In addition, it can be inferred that further phosphorylation of IP₃ is evidently not required to elicit the calcium signal.

Interestingly, the higher basal level of IP₃ in the *arg82Δ* strain does not involve a serious defect in the basal level of calcium. This could suggest that either IP₃ is not the signalling molecule that triggers calcium entry from the external medium or the IP₃ accumulated in the *arg82Δ* strain due to its genetic defect has no effect on calcium levels because of a different compartmentalisation. The former hypothesis leaves an open question: since diacylglycerol addition does not evoke any effect on the calcium level (data not shown) and IP₃ is not responsible for stimulating calcium uptake, should a different mechanism be considered which does not involve any second messenger, e.g. a direct interaction between Plc1 and the calcium channel. However, the hypothesis of a proper IP₃ localisation requirement for calcium signalling could also justify the different cellular behaviour following nitrogen stimulation. In fact, the IP₃ generated independently of Plc1 does not trigger any calcium mobilisation (Bergsma et al. 2001), which could indicate that its accumulation resides in a different location from the IP₃ generated via Plc1 activity after glucose stimulation.

PLC1 deletion causes the complete inhibition of not only the glucose-induced calcium entry (Tisi et al. 2002) but also the IP₃ accumulation (Fig. 3), in agreement with the report that PI turnover was also completely inhibited (Cocchetti et al. 1998). Further, the level of IP₃ following glucose addition falls to undetectable levels, suggesting that not only could Plc1-mediated PIP₂ cleavage be induced, but also inositol phosphate kinase activity could be stimulated. That could explain why the IP₃ accumulation in a wild-type strain is so faint, due to the immediate transformation of IP₃ into inositol polyphosphates.

The importance of inositol polyphosphates was recently stressed as regulators of the activity of the Swi/Snf ATP-dependent chromatin remodelling complex (Rando et al. 2003; Shen et al. 2003; Steger et al. 2003). This could suggest a possible role for the Plc1 pathway in transcriptional regulation induced by glucose. It is interesting to note that the Swi/Snf complex is involved in the induction of phosphate-responsive genes such as *PHO5* and *PHO84*. Moreover, the *plc1* null mutant can be partially rescued by activation of the PHO pathway either by genetic modification or by growth on a low-phosphate medium (Flick and Thorner 1998). This is consistent with a putative requirement of Plc1 to generate the inositol phosphates implicated in transcriptional activation of PHO pathway-controlled genes.

We previously found that glucose-induced calcium signalling requires the activation of the Gpr1/Gpa2 GPCR system (Tisi et al. 2002). As demonstrated for cAMP signalling (Rolland et al. 2000), glucose transport and phosphorylation are also required for the glucose induction of calcium uptake (Tisi et al. 2002; Tokes-Fuzesi et al. 2002). These observations prompted us to investigate whether cAMP signalling could be involved in

the generation of calcium signalling. However, neither Ras activation by its exchange factors, Cdc25 or Sdc25, nor cAMP accumulation were required for cytosolic calcium elevation. This suggests that the two pathways immediately diverge after initial activation by the same glucose-sensing system. This finding is also in agreement with the previous observation that glucose-induced PI turnover (requiring Plc1 activity) was not dependent upon the Cdc25/Ras pathway in yeast (Frascotti et al. 1990).

The physiological significance of glucose-induced IP₃/calcium signalling is still unknown. Since Pkc1p is required for glucose-induced H⁺-ATPase activation (Brandão et al. 1994; Souza et al. 2001), a connection could exist between Plc1 and Pkc1 activation upon glucose stimulation. These could provide different contributions to the general rearrangement occurring in yeast metabolism and physiology during the shift from a condition of nutrient starvation or slow growth in respiratory conditions to a condition of nutrient availability or rapid growth in fermentable conditions.

Acknowledgements We thank Laura Popolo, Enrico Ragni, Susan Wente, James York, James Caffrey and Stephen Shears for kindly providing the materials indicated in the Materials and methods. Our particular thanks go to Jan C.T. Bergsma for helpful discussion and advice. This work was supported by grants from the Fund for Scientific Research—Flanders and the Research Fund of the Katholieke Universiteit Leuven (Concerted Research Actions) to S.W., J.W. and J.M.T. and by a grant from FAR (formerly MURST 60%) to E.M.

References

- Bergsma JC, Kasri NN, Donaton MC, De Wever V, Tisi R, Winde JH de, Martegani E, Thevelein JM, Wera S (2001) PtdIns(4,5)P₂ and phospholipase C-independent Ins(1,4,5)P₃ signals induced by a nitrogen source in nitrogen-starved yeast cells. *Biochem J* 359:517–523
- Brandão RL, Magalhaes-Rocha NM de, Alijo R, Ramos J, Thevelein JM (1994) Possible involvement of a phosphatidylinositol-type signaling pathway in glucose-induced activation of plasma membrane H⁺-ATPase and cellular proton extrusion in the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1223:117–124
- Camonis JH, Jacquet M (1988) A new *RAS* mutation that suppresses the *CDC25* gene requirement for growth of *Saccharomyces cerevisiae*. *Mol Cell Biol* 8:2980–2983
- Cocchetti P, Tisi R, Martegani E, Teixeira LS, Brandão RL, Miranda Castro I de, Thevelein JM (1998) The *PLC1* encoded phospholipase C in the yeast *Saccharomyces cerevisiae* is essential for glucose-induced phosphatidylinositol turnover and activation of plasma membrane H⁺-ATPase. *Biochim Biophys Acta* 1405:147–154
- Colombo S, Ma P, Cauwenberg L, Winderickx J, Crauwels M, Teunissen A, Nauwelaers D, Winde JH de, Gorwa MF, Colavizza D, Thevelein JM (1998) Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. *EMBO J* 17:3326–3341
- Delley PA, Hall MN (1999) Cell wall stress depolarizes cell growth via hyperactivation of *RHO1*. *J Cell Biol* 147:163–174
- El Bakkoury M, Dubois E, Messenguy F (2000) Recruitment of the yeast MADS-box proteins, ArgR1 and Mcm1 by the pleiotropic factor ArgR3 is required for their stability. *Mol Microbiol* 35:15–31

- Flick JS, Thorner J (1998) An essential function of a phosphoinositide-specific phospholipase C is relieved by inhibition of a cyclin-dependent protein kinase in the yeast *Saccharomyces cerevisiae*. *Genetics* 148:33–47
- Fraschetti G, Baroni D, Martegani E (1990) The glucose-induced polyphosphoinositides turnover in *Saccharomyces cerevisiae* is not dependent on the *CDC25-RAS* mediated signal transduction pathway. *FEBS Lett* 274:19–22
- Fuente N de la, Portillo F (2000) The cell wall integrity/remodeling MAPK cascade is involved in glucose activation of the yeast plasma membrane H^+ -ATPase. *Biochim Biophys Acta* 1509:189–194
- Goldbeter A (2002) Computational approach to cellular rhythms. *Nature* 420:238–245
- Hawkins PT, Stephens LR, Piggott JR (1993) Analysis of inositol metabolites produced by *Saccharomyces cerevisiae* in response to glucose stimulation. *J Biol Chem* 268:3374–3383
- Inagaki M, Schmelzle T, Yamaguchi K, Irie K, Hall MN, Matsumoto K (1999) *PDK1* homologs activate the Pkc1-mitogen-activated protein kinase pathway in yeast. *Mol Cell Biol* 19:8344–8352
- Kaibuchi K, Miyajima A, Arai K, Matsumoto K (1986) Possible involvement of *RAS*-encoded proteins in glucose-induced inositolphospholipid turnover in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 83:8172–8176
- Kamada Y, Qadota H, Python C P, Anraku Y, Ohya Y, Levin DE (1996) Activation of yeast protein kinase C by Rho1 GTPase. *J Biol Chem* 271:9193–9196
- Ketela T, Green R, Bussey H (1999) *Saccharomyces cerevisiae* mid2p is a potential cell wall stress sensor and upstream activator of the *PKC1-MPK1* cell integrity pathway. *J Bacteriol* 181:3330–3340
- Lopez F, Leube M, Gil-Mascarell R, Navarro-Aviñó JP, Serrano R (1999) The yeast inositol monophosphatase is a lithium- and sodium-sensitive enzyme encoded by a non-essential gene pair. *Mol Microbiol* 31:1255–1264
- Ma P, Wera S, Van Dijck P, Thevelein JM (1999) The *PDE1*-encoded low-affinity phosphodiesterase in the yeast *Saccharomyces cerevisiae* has a specific function in controlling agonist-induced cAMP signaling. *Mol Biol Cell* 10:91–104
- Matsumoto TK, Ellsmore AJ, Cessna SG, Low PS, Pardo JM, Bressan RA, Hasegawa PM (2002) An osmotically induced cytosolic Ca^{2+} transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of *Saccharomyces cerevisiae*. *J Biol Chem* 277:33075–33080
- Navarro-Aviñó JP, Bellés JM, Serrano R (2003) Yeast inositol mono- and trisphosphate levels are modulated by inositol monophosphatase activity and nutrients. *Biochem Biophys Res Commun* 302:41–45
- Noh D-Y, Shin SH, Rhee SG (1995) Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim Biophys Acta* 1242:99–114
- Nonaka H, Tanaka K, Hirano H, Fujiwara T, Kohno H, Umikawa M, Mino A, Takai Y (1995) A downstream target of *RHO1* small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J* 14:5931–5938
- Rando OJ, Chi TH, Crabtree GR (2003) Second messenger control of chromatin remodeling. *Nat Struct Biol* 10:81–83
- Robinson KS, Wheals AE, Rose AH, Dickinson JR (1996) Unusual inositol triphosphate metabolism in yeast. *Microbiology* 142:1333–1334
- Rolland F, Winde JH de, Lemaire K, Boles E, Thevelein JM, Winderickx J (2000) Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. *Mol Microbiol* 38:348–358
- Saiardi A, Caffrey JJ, Snyder SH, Shears SB (2000) Inositol polyphosphate multikinase (ArgR11) determines nuclear mRNA export in *Saccharomyces cerevisiae*. *FEBS Lett* 468:28–32
- Schmidt A, Bickle M, Beck T, Hall MN (1997) The yeast phosphatidylinositol kinase homolog *TOR2* activates *RHO1* and *RHO2* via the exchange factor *ROM2*. *Cell* 88:531–542
- Schomerus C, Kuntzel H (1992) *CDC25*-dependent induction of inositol 1,4,5-trisphosphate and diacylglycerol in *Saccharomyces cerevisiae* by nitrogen. *FEBS Lett* 307:249–252
- Shen X, Xiao H, Ranallo R, Wu W-H, Wu C (2003) Modulation of ATP-dependent chromatin-remodeling complexes by inositol polyphosphates. *Science* 299:112–114
- Silverman-Gavrila LB, Lew RR (2002) An IP_3 -activated Ca^{2+} channel regulates fungal tip growth. *J Cell Sci* 115:5013–5025
- Souza MA, Tropia MJ, Brandão RL (2001) New aspects of the glucose activation of the H^+ -ATPase in the yeast *Saccharomyces cerevisiae*. *Microbiology* 147:2849–2855
- Steger DJ, Haswell ES, Miller AL, Wente SR, O'Shea EK (2003) Regulation of chromatin remodeling by inositol polyphosphates. *Science* 299:114–116
- Thevelein JM, Winde JH de (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* 33:904–918
- Tisi R, Cocchetti P, Banfi S, Martegani E (2001) 3-Nitrocoumarin is an efficient inhibitor of budding yeast phospholipase-C. *Cell Biochem Funct* 19:229–235
- Tisi R, Baldassa S, Belotti F, Martegani E (2002) Phospholipase C is required for glucose-induced calcium influx in budding yeast. *FEBS Lett* 520:133–138
- Tokes-Fuzesi M, Bedwell DM, Repa I, Sipos K, Sumegi B, Rab A, Miseta A (2002) Hexose phosphorylation and the putative calcium channel component Mid1p are required for the hexose-induced transient elevation of cytosolic calcium response in *Saccharomyces cerevisiae*. *Mol Microbiol* 44:1299–1308
- Van Aelst L, Boy-Marcotte E, Camonis JH, Thevelein JM, Jacquet M (1990) The C-terminal part of the *CDC25* gene product plays a key role in signal transduction in the glucose-induced modulation of cAMP level in *Saccharomyces cerevisiae*. *Eur J Biochem* 193:675–680
- Van Dijck P, Haas JR de, Craxton A, Erneux C, Shears SB, Van Haastert PJ (1995) A novel, phospholipase C-independent pathway of inositol 1,4,5-trisphosphate formation in *Dictyostelium* and rat liver. *J Biol Chem* 270:29724–29731
- Wera S, Bergsma JCT, Thevelein JM (2001) Phosphoinositides in yeast: genetically tractable signalling. *FEMS Yeast Res* 1406: 1–5
- York JD, Odom AR, Murphy R, Ives EB, Wente SR (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. *Science* 285:96–100