Transient increase in Ca\(^{2+}\) influx in *Saccharomyces cerevisiae* in response to glucose: effects of intracellular acidification and cAMP levels

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Influx of \(^{45}\text{Ca}^{2+}\) into *Saccharomyces cerevisiae* was measured under experimental conditions which enabled measurements of initial rate of transport across the plasma membrane, without interference by the vacuolar Ca\(^{2+}\) transport system. Addition of glucose or glycerol to the cells, after pre-incubation in glucose-free medium for 5 min, caused a rapid, transient increase in \(^{45}\text{Ca}^{2+}\) influx, reaching a peak at 3–5 min after addition of substrate. Ethanol, or glycerol added with antimycin A, had no effect on \(^{45}\text{Ca}^{2+}\) influx. We have shown previously that this increase is not mediated by an effect of the substrates on intracellular ATP levels. Changes in membrane potential accounted for only a part of the glucose-stimulated \(^{45}\text{Ca}^{2+}\) influx. The roles of intracellular acidification and changes in cellular cAMP in mediating the effects of glucose on \(^{45}\text{Ca}^{2+}\) influx were examined. After a short pre-incubation in glucose-free medium addition of glucose caused a decrease in the intracellular pH, \([\text{pH}]_{\text{i}}\), which reached a minimum value after 3 min. A transient increase in the cellular cAMP level was also observed. Addition of glycerol also caused intracellular acidification, but ethanol or glycerol added with antimycin A had no effect on \([\text{pH}]_{\text{i}}\). Artificial intracellular acidification induced by exposure to isobutyric acid or to CCCP caused a transient rise in Ca\(^{2+}\) influx but the extent of the increase was smaller than that caused by glucose, and the time-course was different. We conclude that intracellular acidification may be responsible for part of the glucose stimulation of Ca\(^{2+}\) influx. The role of the increase in cAMP level on Ca\(^{2+}\) influx was examined by measuring the effect of glucose and of artificial intracellular acidification on Ca\(^{2+}\) influx in a *cyr1* strain which lacks adenylate cyclase activity. In this strain, addition of glucose or isobutyric acid still led to a transient increase in Ca\(^{2+}\) transport. Therefore, we concluded that at least part of the increase in Ca\(^{2+}\) influx in response to glucose is cAMP-independent.

**Introduction**

Influx of Ca\(^{2+}\) into yeast cells is thought to be an energy-dependent process (Borst-Pauwels, 1981). This assumption is based on experiments showing marked stimulation of Ca\(^{2+}\) uptake by glucose (Roomans *et al.*, 1979). Recently, the process of Ca\(^{2+}\) uptake and accumulation in *Saccharomyces cerevisiae* was found to occur in two steps: (a) influx of Ca\(^{2+}\) across the plasma membrane and into the cytosol, and (b) accumulation of Ca\(^{2+}\) in the vacuoles (Eilam & Chernichovsky, 1987) via the \(n\text{H}^{+}/\text{Ca}^{2+}\) antiporter which depends on the activity of the vacuolar \(H^{+}\)-ATPase (Ohsumi & Anraku, 1983). Whereas the second step is clearly an energy-dependent process, the initial step of influx across the plasma-membrane proceeds down an electrochemical gradient for Ca\(^{2+}\) (Eilam & Chernichovsky, 1987; Eilam & Othman, 1990).

Using a new procedure to measure the initial Ca\(^{2+}\) influx across the plasma membranes without interference by the vacuolar Ca\(^{2+}\) transport system (Eilam & Chernichovsky, 1987), it was found that glucose and other metabolic substrates stimulated the rate of this transport. Removal of glucose led to a reduced rate of Ca\(^{2+}\) influx within 5–7 min; subsequent addition of glucose caused a transient increase in Ca\(^{2+}\) influx which reached a peak 5 min after glucose addition and thereafter decreased. A second increase in Ca\(^{2+}\) influx was observed 60–80 min later (Eilam & Othman, 1990).

Investigations on the mechanism of the initial increase in Ca\(^{2+}\) influx have established that in cells with the same value for membrane potential, \(^{45}\text{Ca}^{2+}\) influx in the presence of glucose was always higher than in its absence. These results led to the conclusion that the changes in membrane potential, resulting from exposure
to glucose, could not account for the full extent of glucose-stimulated \( \text{Ca}^{2+} \) transport; hence, another mechanism must also be involved (Eilam & Othman, 1990). Changes in cellular ATP levels, which followed the addition of several metabolic substrates, did not correlate with changes in \( \text{Ca}^{2+} \) influx. It was concluded, therefore, that ATP levels could not mediate the glucose-induced increase in \( \text{Ca}^{2+} \) influx (Eilam & Othman, 1990).

In the present work, we investigated the mechanism of transient increase in \( \text{Ca}^{2+} \) influx induced by glucose and other substrates. Addition of glucose to glucose-starved, Gl- arrested, yeast cells triggers the cells to grow and divide (Kaibuchi et al., 1986). The glucose-induced signal is mediated by a transient increase in intracellular cAMP, occurring 0.5–2 min after addition of glucose (Purwin et al., 1982; Eraso & Gancedo, 1985). The signal transduction pathway requires the function of the CDC25 gene-product and one of the GTP binding proteins of the RAS family, which mediates the increase in adenylate cyclase activity (Field et al., 1987; Daniel et al., 1986; Toda et al., 1985; Broek et al., 1987; Mbonyi et al., 1988). The rapid decrease in intracellular pH which followed the addition of glucose was thought previously to mediate the cAMP increase (Caspiani et al., 1985; Purwin et al., 1986; Valle et al., 1986, 1987), but at present, it is uncertain to what extent this acidification is the sole cause for the rise in cAMP (Eraso et al., 1987; Thevelein et al., 1987).

In the present work, we examine whether the glucose-induced transient rise in \( \text{Ca}^{2+} \) influx is mediated by intracellular acidification or/and by the increase in cAMP levels which follows the exposure of the cells to glucose.

### Methods

**Organism and culture conditions.** *Saccharomyces cerevisiae* strain N123 (genotype \( MATA/a \) hisl) was maintained at 4 °C on YPD agar slopes and grown at 30 °C in YPD broth (Bacto-yeast extract 10 g l⁻¹, Bacto-peptone 20 g l⁻¹, glucose 20 g l⁻¹). Cells were collected from an overnight culture by centrifugation, washed three times by resuspension in distilled water and finally resuspended in the indicated medium. Strain AM7-11D (genotype \( MATA/cyl1 \)) (Matsumoto et al., 1982) was maintained at 4 °C on YPD agar slopes to which cAMP (1 mM final concentration) was added. Cells were grown for 40 h in YPD broth containing cAMP (1 mM) at 30 °C with shaking, then washed and resuspended as above.

**\( \text{Ca}^{2+} \) influx measurements.** We demonstrated previously that the initial rate of \( ^{45}\text{Ca}^{2+} \) influx across the plasma membrane may be determined without interference by uptake into intracellular compartments by a 20 s \( ^{45}\text{Ca}^{2+} \) influx measurement which follows a period of pre-incubation in buffer–glucose medium (Eilam & Chernichovsky, 1987). Every experiment was therefore initiated by pre-incubating the cells for 90 min at 30 °C with shaking, in medium containing MES/Tris (10 mM, pH 6.0) and glucose (100 mM) (buffer–glucose medium) at a density of \( 10^8 \) cells ml⁻¹. After this pre-incubation cells were collected by centrifugation, washed once with MES/Tris buffer (10 mM, pH 6.0) (buffer medium) and resuspended in buffer medium. Strain N123 was resuspended at a density of \( 10^8 \) cells ml⁻¹ and strain AM7-11D at a density of \( 2 \times 10^8 \) cells ml⁻¹, since the cells of the haploid strain are smaller. The cell suspensions were incubated at 30 °C for 5 min then glucose or another metabolic substrate was added in small aliquots to 1 ml samples of the cell suspension. At different time intervals, before and after the addition of substrate, \( ^{45}\text{Ca}^{2+} \) transport was initiated by the addition of \( \text{CaCl}_2 \), labelled with \( ^{45}\text{Ca}^{2+} \) in a small aliquot to yield a final concentration of 1 µM-Ca²⁺ (1 µCi ml⁻¹ (37 kBq ml⁻¹)); the cell suspension was then mixed immediately. Transport was terminated after 20 s by the addition of 1 ml washing solution containing MgCl₂ (20 mM) and LaCl₃ (0.1 mM); the cells were then filtered immediately on membrane filters (0.45 µm pore size), and washed five times with 2 ml portions of the washing solution. The filters were dried and the radioactivity was determined, after the addition of toluene-based scintillation fluid, using a liquid scintillation counter. \( \text{Ca}^{2+} \) binding was determined from zero-time measurements, which were obtained by the addition of 1 ml of cell suspension to 1 ml of washing solution; \( \text{CaCl}_2 \), labelled with \( ^{45}\text{Ca}^{2+} \) was then added in a small aliquot (concentration as above) (see Eilam & Chernichovsky, 1987). The zero-time measurement was subtracted from the transport results. In preliminary experiments, it was determined that the time-courses of \( \text{Ca}^{2+} \) uptake were linear for 20 s under all conditions tested in the present work.

**Determination of [pH].** Intracellular pH values were determined from the distribution of propionic acid between the intracellular water and the medium using cells pre-incubated as for \( \text{Ca}^{2+} \) influx measurement. \( ^{14}\text{C} \)-Propionic acid (1 µM; 0.1 µCi ml⁻¹ (3.7 kBq ml⁻¹)) was added to the pre-incubation medium 30 min before termination of the pre-incubation, and to the buffer medium in which the cells were incubated for 5 min before the addition of the substrate. Samples (1 ml) were filtered on membrane filters (0.45 µm pore size) at different time intervals before and after the addition of the substrate. The cells on the filters were washed four times with 2 ml of distilled water. The filters were dried and the radioactivity determined as described above. The water volume of the cells was determined as described previously (Eilam et al., 1985b). The pH was calculated as described by De La Peña et al. (1982).

**\( ^{3}\text{H} \)TPP⁺ measurements:** These were based on the steady-state distribution of \( ^{3}\text{H} \)TPP⁺ between cells and medium. Samples of cell suspension underwent the same procedure of pre-incubation and washing as described above but \( ^{3}\text{H} \)TPP⁺ (1 µM; 0.05 µCi ml⁻¹ (1.85 µCi ml⁻¹)) was included in the pre-incubation medium for 90 min, and in the buffer medium for the 5 min pre-incubation, followed by the addition of glucose or CCCP. The samples were filtered as described for \( \text{Ca}^{2+} \) influx measurements but glass fibre filters were used instead of membrane filters. All filters were prewashed in washing solution. The results were corrected for \( ^{3}\text{H} \)TPP⁺ binding.

**Measurements of cellular cAMP content.** Cells were grown and pre-incubated as for \( ^{45}\text{Ca}^{2+} \) influx measurements. After pre-incubation in buffer–glucose medium cells were collected by centrifugation, washed once with buffer medium and resuspended in buffer medium as above. After incubation for 5 min, glucose was added. Samples for measurements of cAMP content were taken at different time intervals before and after the addition of glucose. The cells were extracted with 7% (v/v) HClO₄, and the extracts were prepared for cAMP assay with the Amersham cAMP determination kit.

**Reproducibility of results.** Each experiment was repeated at least four times with similar results.
Glucose-induced Ca\(^{2+}\) influx in yeast: \([\text{pH}]\) and cAMP effects

Results

In the first group of experiments, the effects of several substrates on Ca\(^{2+}\) influx and on the intracellular pH \(([\text{pH}])\) were examined. After pre-incubation in buffer–glucose medium for 90 min to obtain the necessary conditions for Ca\(^{2+}\) transport measurements, and incubation in buffer–medium for 5 min, substrate was added, and \(^{45}\text{Ca}^{2+}\) influx was measured for 20 s. In parallel experiments, accumulation of \(^{14}\text{C}\)propionic acid was measured.

As reported previously (Eilam & Othman, 1990) Ca\(^{2+}\) influx increased after the addition of glucose, reaching a peak at 5 min and decreased thereafter. Under similar experimental conditions glucose caused a transient intracellular acidification of 0.45 pH units. The lowest \([\text{pH}]\) value was observed 3 min after addition of glucose. At 6 min, \([\text{pH}]\) returned to a level of 0.1 pH units below the initial value (Fig. 1).

Glycerol caused similar changes in \(^{45}\text{Ca}^{2+}\) influx and in \([\text{pH}]\), as did glucose but the magnitude of the changes was smaller (Fig. 2). On the other hand, glycerol, in the presence of antimycin A, did not cause any changes in \(^{45}\text{Ca}^{2+}\) influx, nor in \([\text{pH}]\) (Fig. 2). Similarly, addition of ethanol (20 mM) did not affect either \(^{45}\text{Ca}^{2+}\) influx or \([\text{pH}]\) (not shown).

To assess the role of intracellular acidification in mediating the transient increase in \(^{45}\text{Ca}^{2+}\) influx, we induced acidification by two different methods: (1) by exposing the cells to isobutyric acid, and (2) by exposing the cells to the protonophore CCCP. (1) Cells were pre-incubated as in the previous experiments, but isobutyric acid (100 mM) was added instead of a substrate. \(^{45}\text{Ca}^{2+}\) influx and \([\text{pH}]\) were measured before, and at different time intervals after the addition of isobutyric acid (Fig. 3). A transient acidification (0.5 pH units) was observed 3 min after the addition of isobutyric acid. By 5 min the value of \([\text{pH}]\) increased to a level of 0.25 pH units below the control, and remained at that level up to the end of measurements at 10 min. \(^{45}\text{Ca}^{2+}\) influx increased transiently by 3-2-fold. The kinetics of the changes in \([\text{pH}]\) and in Ca\(^{2+}\) influx resembled those observed with glucose and glycerol. The steady-state level of \(^{45}\text{Ca}^{2+}\) influx was below the control level.

CCCP is a protonophore which causes cellular acidification in cells incubated in a medium of acidic pH. However, since CCCP also causes membrane depolarization, we examined whether addition of a low concentration of TFP restored the \(\Delta\psi\) of the cells exposed to CCCP back to the control level. We found previously that TFP causes a concentration-dependent hyper-
polarization of yeast cells by inducing K+ efflux (Eilam, 1984). Table 1 shows that addition of 5 \mu M-TFP restored the $\Delta \psi$ of cells exposed to CCCP to that of cells incubated in buffer–glucose medium. Therefore, experiments with CCCP were done in the presence of 5 \mu M-TFP. The results in Fig. 4 indicate that CCCP caused a transient acidification similar to that caused by isobutyric acid. The [pH] reached a minimum level approximately 3 min after CCCP addition and thereafter increased to a level below the control. $^{45}\text{Ca}^{2+}$ influx seems to mirror-image the changes in [pH].

It is well established that glucose induces a transient rise in cAMP in yeast cells which are derepressed by incubation in medium containing a very low concentration of glucose, or by growth in sugar-free medium (Caspiani et al., 1985; Purwin et al., 1986; Valle et al., 1986, 1987). Under our experimental procedure, the cells were 'glucose-derepressed' for only 5 min. We examined whether, under these conditions, glucose induces an increase in cAMP. The results in Fig. 5 show that following this short incubation in glucose-free medium, an increase in cAMP level was observed reaching a peak 2 min after addition of glucose. This increase was not observed in the cyr1 strain (Fig. 5) which lacks the catalytic subunit of adenylate cyclase (Matsumoto et al., 1982). Cultures of the cyr1 strain were grown in the presence of cAMP (1 mM); consequently, the intracellular cAMP level measured at the time of the experiments presumably originated from the exogenous cAMP added during growth. This level was similar to the level in strain N123 before addition of glucose, and was not changed by glucose addition (Fig. 5).

To investigate the role of cAMP in mediating the increase in $^{45}\text{Ca}^{2+}$ influx, the experiments shown in Figs
glucose causes, within 0.5 min, a 3-5-fold increase in the cell cycle, and initiation of cell growth and division. The mode of action of glucose in inducing cell division is not (Beullens et al., 1988), followed by a transition to the S phase of the cell cycle (Kaibuchi et al., 1988). Thus, glucose acts in yeast in a similar way to certain hormones and growth factors in mammals (for a review see Engelberg et al., 1989).

In recent years it has become evident that in Saccharomyces cerevisiae the key regulatory proteins are similar to those in mammalian cells: examples are the GTP regulatory proteins, adeylate cyclase, phosphodies- terases and different protein kinases, which function similarly in yeast and mammalian cells (Watson et al., 1987; Matsumoto et al., 1985). Calmodulin, the Ca$^{2+}$- binding regulatory protein, is an essential protein in S. cerevisiae (Davis et al., 1986). Several Ca$^{2+}$-dependent mutants, which require a high concentration of Ca$^{2+}$ for growth, have been isolated (Ohya et al., 1984, 1986; Baum et al., 1986; Schmit et al., 1988). The mutant call arrests at nuclear division upon shifting to low Ca$^{2+}$ medium at a non-permissive temperature (Ohya et al., 1984), whereas els$^4$ arrests before bud formation (Ohya et al., 1986). Low concentrations of the calmodulin antagonist TFP cause cell cycle arrest at two stages of the cell cycle – at 'start' and at nuclear division (Eilam & Chernichovsky, 1988). These results may indicate that Ca$^{2+}$/calmodulin regulates the cell cycle in yeast at the above two stages, similar to its function in mammalian cells (Mean & Rasmussen, 1988). Based on these studies, a regulatory role of Ca$^{2+}$ in yeast has been suggested (Davis & Thorner, 1986).

Many external messages in mammalian cells are transduced by transient changes in the intracellular Ca$^{2+}$ concentration. Recently, the role of elevated rates of Ca$^{2+}$ cycling across the plasma membrane in maintaining sustained responses to transient changes in the intracellular Ca$^{2+}$ concentration have been inferred (Alkon & Rasmussen, 1988). Whether changes in the intracellular Ca$^{2+}$ concentration or in Ca$^{2+}$ cycling across membranes serve as signals in yeast is still unknown. Also, little is known about factors which regulate Ca$^{2+}$ transport across membranes.

Addition of glucose to yeast cells has long been known to stimulate Ca$^{2+}$ uptake. These results have been interpreted as being due to the energy requirement of the Ca$^{2+}$ uptake process (Borst-Pauwels, 1981). However, in these experiments the process of transport across plasma membranes was not separated from subsequent uptake into the vacuole, which is indeed an energy-requiring process (Ohsumi & Anraku, 1983). Our kinetic Ca$^{2+}$ efflux experiments first established the presence of two cellular compartments for Ca$^{2+}$ in yeast (Eilam, 1982a, b). These experiments were done by measuring the concentration of Ca$^{2+}$ in the cytosolic and the vacuolar compartments by differential extraction. Cytosolic Ca$^{2+}$ homeostasis, at a low concentration of Ca$^{2+}$ was observed (Eilam et al., 1985a). These results were confirmed recently by direct measurements (Halachmi & Eilam, 1989). Further experiments done by a procedure

**Discussion**

Starvation of yeast cells of glucose causes arrest at the G1 stage of the cell cycle (Kaibuchi et al., 1986). Addition of glucose causes, within 0.5 min, a 3-5-fold increase in the cAMP level, followed by a transition to the S phase of the cell cycle, and initiation of cell growth and division. The mode of action of glucose in inducing cell division is not by increasing energy supply, since metabolism of this sugar beyond the sugar phosphate step is not necessary (Beullens et al., 1988), but is by activation of adenylate cyclase. The signal is transduced by the CDC25 gene product and by two GTP-binding proteins from the RAS family. Thus, glucose acts in yeast in a similar way to

![Fig. 7. Effects of isobutyric acid on $^{45}$Ca$^{2+}$ influx and on [pH] in the cyrl strain. Experiments were done as in Fig. 6, but isobutyric acid (100 mM) was added instead of glucose.](image-url)
which enables measurement of Ca\(^{2+}\) transport across plasma membranes without interference by the vacuolar Ca\(^{2+}\) transport system (Eilam & Chernichovsky, 1987) led to the conclusion that this transport is stimulated strongly by an increase in \(\Delta \psi\) (Eilam & Chernichovsky, 1987). Removal of glucose caused rapid inactivation of Ca\(^{2+}\) influx, whereas re-addition of glucose caused a transient increase, followed by a decrease and then a second increase in transport rate. The initial increase was found not to be mediated by the intracellular ATP levels. Changes in \(\Delta \psi\) in response to glucose account for part of this effect (Eilam & Othman, 1990). In the present work, we examined two other possible mechanisms for glucose effects on Ca\(^{2+}\) influx: cellular acidification and cAMP levels.

Addition of glucose to \textit{S. cerevisiae} incubated in a sugar-free medium induces a transient intracellular acidification (Purwin et al., 1982; Valle et al., 1986, 1987; Caspani et al., 1985). The mechanism of this glucose-induced acidification is not clear. In the present work, it was found that incubation of yeast cells for 5 min in glucose-free medium was sufficient for a subsequent intracellular acidification upon addition of glucose. Acidification was also induced by glycerol, but not by ethanol or by glycerol in the presence of antimycin A, which prevents its metabolism. The transient nature of the acidification may be explained by the increase in cAMP, induced by glucose or by intracellular acidification (Caspani et al., 1985; Purwin et al., 1986; Eraso et al., 1987; Thevelein et al., 1987). Activation of the H\(^+\)-ATPase by cAMP, as recently reported by Ulaszewski et al. (1989), may lead to the restoration of [pH], following intracellular acidification.

A transient increase in the rate of Ca\(^{2+}\) transport was observed under all experimental conditions in which intracellular acidification was observed, namely, following the addition of glucose, glycerol, isobutyric acid and CCCP. Agents which did not cause acidification such as ethanol, or glycerol in the presence of antimycin A, did not stimulate Ca\(^{2+}\) influx. These results lead to the conclusion that the increase in Ca\(^{2+}\) influx may be mediated in part by intracellular acidification. Since the magnitude of the increase in influx was higher when glucose was added, as compared with isobutyric acid or CCCP, and the time-course was different, acidification may account for part of the glucose effect on Ca\(^{2+}\) transport, whereas part may be mediated by the glucose-induced membrane hyperpolarization (Van de Mortel et al., 1988).

The mechanism by which acidification may lead to stimulation of Ca\(^{2+}\) influx was investigated. Since acidification causes an increase in cAMP, Ca\(^{2+}\) influx may be stimulated by this cAMP increase. In several types of mammalian cells, Ca\(^{2+}\) influx is enhanced by an increase in cAMP, which leads to phosphorylation of Ca\(^{2+}\) channels by a cAMP-dependent protein kinase (Reuter, 1983; Hosey et al., 1986). To examine this possibility, the effect of glucose was measured in the cyrl strain. In this strain, which has a defective catalytic subunit of adenylate cyclase, glucose did not cause an increase in cAMP. Growth and cell division in this strain is dependent on uptake of cAMP from the medium (Matsumoto et al., 1982). Both acidification and the increase in \(^{45}\)Ca\(^{2+}\) influx following addition of glucose were observed in the cyrl strain, but the extent of the acidification and the initial increase in \(^{45}\)Ca\(^{2+}\) influx were smaller than in strain N123. The rate of \(^{45}\)Ca\(^{2+}\) influx in glucose-free medium in the cyrl strain was faster than in N123. We cannot explain these differences between the strains. It is not clear also why the decrease in [pH], induced by glucose or by isobutyric acid was smaller in the cyrl strain. The results lead to the conclusion that at least part of the increase in Ca\(^{2+}\) influx which follows the addition of glucose is cAMP-independent.

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References


Glucose-induced Ca\(^{2+}\) influx in yeast: [pH], and cAMP effects


