Regulation of the cAMP Level in the Yeast

_Saccharomyces cerevisiae_: Intracellular pH and the Effect of Membrane Depolarizing Compounds

By JOHAN M. THEVELEIN,*, MONIQUE BEULLENS, FANNY HONSHOVEN, GREET HOEBEECK, KATRIEN DETREMERIE, JAN A. DEN HOLLANDER AND ARNOLD W. H. JANS

1 Laboratorium voor Cellulaire Biochemie, Katholieke Universiteit te Leuven, Kardinaal Mercierlaan 92, B-3030 Leuven – Heverlee, Belgium
2 Philips Medical Systems, PO Box 218, 5600 MD Eindhoven, The Netherlands
3 Max-Planck-Institut für Systemphysiologie, Rheinlanddamm 201, D-4600 Dortmund 1, FRG

(Received 1 December 1986; revised 3 March 1987)

Addition of plasma membrane depolarizing agents, such as dinitrophenol (DNP) and azide, to cells of _Saccharomyces cerevisiae_ under aerobic conditions, is known to cause an increase in the cAMP level within 15 s. We found that both compounds lowered the intracellular pH (measured by in _vivo_ $^3$P-NMR) drastically within the same time period. Plasma membrane depolarization, however, was much slower: DNP and azide had no effect on the membrane potential during, respectively, the first 2 min and the first 10 min after addition. Apparently, the intracellular pH of yeast is much more sensitive to perturbation than the membrane potential. The effect of both compounds on the cAMP level was highly dependent on the extracellular pH: when the latter was raised, the effect disappeared completely between pH 6 and 7. A similar dependence on the extracellular pH was observed for the lowering of intracellular pH. Addition of organic acids, such as acetate and butyrate, at low pH and under aerobic conditions, also caused an immediate increase in the cAMP level and an immediate drop in the intracellular pH. These results suggest that agents such as DNP and azide do not raise the cAMP level in yeast cells because of their membrane depolarizing properties but because they lower the intracellular pH. Under anaerobic conditions, DNP, azide and organic acids were much less effective in increasing the cAMP level. Addition of a small amount of glucose, however, restored their capacity to enhance the cAMP level. This suggests that under anaerobic conditions and in the absence of glucose the ATP level is a limiting factor for cAMP synthesis.

INTRODUCTION

A range of membrane depolarizing conditions causes a rapid increase in the cAMP level in cells of different fungi, including _Saccharomyces cerevisiae_ (Pall, 1977; Trevillyan & Pall, 1977). This has been confirmed by several authors (Uno & Ishikawa, 1981; Mazon et al., 1982; Thevelein & Beullens, 1985). Pall (1981) suggested that the fungal adenyl cyclase, which is localized in the plasma membrane, is in some way sensitive to the membrane potential. However, the evidence that the effect was specifically due to membrane depolarization has always been weak.

In the present paper the effect of membrane depolarizing compounds on the cAMP level in yeast cells was critically reinvestigated. Evidence is presented that intracellular acidification rather than membrane depolarization is responsible for the effect of membrane depolarizing

Abbreviations: DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide m-chlorophenylhydrazone; TPP+, tetraphenylphosphonium ion.
compounds which cause increases in the cAMP level. Recently, evidence supporting the same conclusion was obtained by Caspani et al. (1985), Purwin et al. (1986) and indirectly also by Valle et al. (1986), who studied the activation by membrane depolarizing compounds of trehalase, an enzyme regulated by cAMP-dependent protein phosphorylation.

METHODS

Yeast strain and culture conditions. Cells of the diploid (and homothallic) strain Y55 of the yeast Saccharomyces cerevisiae (formerly called Pichia pastoris strain Y55: see Thevelein & Beullens, 1985) were grown to a density of about 8 mg wet wt ml⁻¹ in a medium containing 2% (w/v) potassium acetate, pH 6, 1% (w/v) peptone (Merck 7224) and 0.5% yeast extract. The cultures were quickly cooled in ice, and the cells were harvested by centrifugation and washed with ice-cold 25 mM-MES/NaOH buffer, pH 6.

Incubation conditions. For time-course measurements of the cAMP level, cells were incubated at a density of 30 mg wet wt ml⁻¹ in a reciprocating water-bath shaker at a temperature of 25 °C ('shake culture'). Incubation was done in 25 mM-MES/NaOH buffer, pH 6, for 10 min before addition of the compounds mentioned. In experiments at other external pH values, the following buffers were used (each at 25 mM): pH 4-5, and 5-5, glycylglycine/HC1; pH 6, 6-25 and 6-5, MES/NaOH; pH 7, Tris/HC1. In the experiments labelled 'aerobic' or 'anaerobic' in Figs 4 to 9 the cells suspensions (300 mg cells ml⁻¹) were supplied with pure O₂ or pure N₂.

Determination of the membrane potential. The membrane potential was determined from the uptake of [3H]tetraphenylphosphonium ion (TPP⁺). Cells were suspended for 2-5 h in 25 mM-MES/NaOH buffer, pH 6 (shake culture) in the presence of 200 nM-[3H]TPP⁺ [4 μCi nmol⁻¹ (118 Bq nmol⁻¹)]. Samples (1 ml) were removed before and after addition of effectors. The samples were added to 10 ml ice-cold 25 mM-MES/NaOH buffer pH 6, containing 20 mM-MgCl₂, and then quickly filtered on Whatman GF/C filters. The filters were washed twice with 20 ml of the same buffer, dried and counted in a liquid scintillation counter with 3-5 ml scintillation fluid. Controls were taken for unspecific binding of TPP⁺ to the filters.

cAMP determination. Samples containing 75 mg cells were quickly filtered on Whatman glass fibre filters (GF/C, 25 mm diameter) in a Gelman grid filter system and immediately immersed in liquid nitrogen. The filters were rapidly (without thawing) broken into pieces and transferred into 7 ml Teflon containers (Braun) pre-frozen in liquid nitrogen and containing 3.3 g glass beads of 3 mm diameter, 1 ml of 1 M-HClO₄ and a little bit of liquid nitrogen. The containers were allowed to warm up to -20 °C in a freezer and then vibrated in a Braun microdisembrator for about 7 min at an amplitude of 10 mm. Under these conditions the frozen mixture is first pulverized; when it subsequently thaws the cells are killed and broken at a temperature substantially lower than 0 °C. The perchloric acid extracts were centrifuged for 2 min at 10000 g to remove potassium perchlorate. Samples (250 μl) of the supernatant were transferred into tubes placed in ice and mixed with 10 μl of a saturated thymol blue solution. Thereafter, a volume of 5 M-K₂CO₃ (about 40-50 μl) was added until the colour changed to blue. The tubes were left (open) in ice for about 15 min and subsequently centrifuged for 2 min at 10000 g; 200 μl of the supernatant was removed and 1 M-HCl was added until the colour changed to yellow. Thereafter, 10 μl 2 M-Tris/HCl, pH 7-5, was added, the tubes were centrifuged again for 2 min at 10000 g and 50 μl of the supernatant was taken for cAMP determination. cAMP assay kits (Amersham), based on competition of cAMP with [3H]cAMP for the regulatory subunit of CAMP-dependent protein kinase, were used for all cAMP determinations. Substitution of other commercially available binding proteins for the CAMP binding protein in the kit gave inconsistent and irreproducible results. Control experiments were done as described by Thevelein (1984). cAMP values for time zero are the means of two duplicates. Under the conditions of our experiments the cAMP level is stable before addition of effectors.

In vivo 31P-NMR spectroscopy. High-resolution 31P-NMR spectra of living cells were obtained on a Bruker AM-400 WB NMR spectrometer operating in the Fourier transform mode at a frequency of 161.9 MHz. The spectra were accumulated at 25 °C for periods of 15 s (26 scans each). The spectra shown represent the mean value for a block of 15 s. The polyphosphate peak was used for calibration (−22-45 p.p.m.). For each experiment 4 g wet wt cells were used in 13 ml 25 mM-MES/NaOH buffer, pH 6 (or Tris/HC1 buffer pH 8, as indicated in the Figure legends). The samples were suspended in 20 mm NMR tubes while N₂ or O₂ were continuously bubbled through the suspensions by means of two glass capillaries. Addition of effectors was done by injection of a small volume from a concentrated solution through capillary tubing.

The intracellular pH was determined from the chemical shift of the phosphate peak of the cytoplasm with a previously obtained titration curve (den Hollander et al., 1981). pH values below 5-5 are only approximate.

Reproducibility of results. All experiments were repeated at least twice with consistent results. Representative results are shown.

RESULTS

Figs 1 and 2 show the rapid increase in the cAMP level in yeast cells after addition of DNP and azide. Under the conditions used, DNP and azide had no effect on the membrane potential
Regulation of the cAMP level in yeast

Fig. 1. pH dependence of the stimulating effect of 2 mM-DNP on the cAMP level in acetate-grown yeast cells. ●, pH 4.5; ▲, pH 5; △, pH 6; ■, pH 6.25; ○, pH 6.5; ▽, pH 7 (for the buffers used, see Methods).

Fig. 2. pH dependence of the stimulating effect of 5 mM-azide on the cAMP level in acetate-grown yeast cells. ●, pH 4.5; ▲, pH 5; △, pH 6; ■, pH 6.25; ○, pH 6.5; ▽, pH 7 (for the buffers used, see Methods).

Fig. 3. Effect of DNP and azide on the uptake of TPP·. ●, Control (no addition); ○, 2 mM-DNP; ▲, 5 mM-azide.

during, respectively, the first 2 min and the first 10 min after addition (Fig. 3). In vitro 31P-NMR experiments showed that both compounds rapidly lowered the intracellular pH with a response time similar to the response time of the increase in the cAMP level (Figs 4 and 5). (For technical reasons the conditions in these NMR-experiments could not be made identical to those used in Figs 1, 2 and 3.)

Examination of the pH dependence of the effect of DNP (Fig. 1) and azide (Fig. 2) showed that their effect was most pronounced at low pH. Above pH 6 the effect gradually diminished and at pH 7 it practically disappeared. When azide or DNP were added at pH 6.3 and a few minutes later the pH was suddenly lowered to pH 5.5, a sharp increase in the cAMP level was observed.

Under anaerobic conditions, DNP and azide had a much smaller effect or no effect at all. Under these conditions however, the intracellular pH was very low (Fig. 4). Their effect could be restored by addition of a small amount of glucose, which in itself had only a small effect on the cAMP level (Fig. 6). Under these conditions, glucose markedly enhanced the ATP level, as measured by in vivo 31P-NMR spectroscopy (not shown).

Addition of acetate (Fig. 7) and butyrate at pH 4 and pH 5 under aerobic conditions also caused pronounced increases in the cAMP level. At pH 6 there was no effect. The difference in the cAMP response at pH 4 and pH 5 (Fig. 7) was reproducible. Under anaerobic conditions the
Fig. 4. Effect of DNP on the intracellular pH under aerobic (■, pH 5; ●, pH 6; △, pH 7) and anaerobic (○, pH 6; △, pH 7) conditions.

Fig. 5. Effect of azide on the intracellular pH under aerobic conditions. ●, pH 6; △, pH 6-25; ○, pH 7.

Fig. 6. Synergistic effect of 2 mM-DNP and low glucose concentration on the cAMP level under anaerobic conditions. ●, 10 mM-glucose added at time zero (first arrow) followed by addition of 2 mM-DNP after 1 min in the same medium (second arrow). Controls: 10 mM-glucose (○) and 2 mM-DNP (△), each added in separate experiments at time zero.

Although the stimulating effect of membrane depolarizing compounds on the cAMP level in fungal cells is well documented, evidence that the effect is specifically due to membrane depolarization was either much smaller or virtually absent. Under both aerobic and anaerobic conditions acetate addition at low pH caused a dramatic drop in the intracellular pH in just a few seconds (Fig. 8). Under aerobic conditions the pH gradually recovered afterwards.

DISCUSSION

Although the stimulating effect of membrane depolarizing compounds on the cAMP level in fungal cells is well documented, evidence that the effect is specifically due to membrane depolarization was either much smaller or virtually absent. Under both aerobic and anaerobic conditions acetate addition at low pH caused a dramatic drop in the intracellular pH in just a few seconds (Fig. 8). Under aerobic conditions the pH gradually recovered afterwards.
Regulation of the cAMP level in yeast

Trevillyan & Pall (1979) showed that the addition of a high concentration of KCl to Neurospora crassa hyphae also produced a cAMP increase and concluded that the cAMP response was not specific for proton influx. The effect of KCl, however, was very small compared to the effect of DNP, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) or nystatin. Furthermore, one depolarizing treatment, osmotic shock, failed to produce an increase in the cAMP level (Pall, 1981).

The membrane depolarizing agents DNP, CCCP, azide, nystatin etc. act as proton ionophores and they are capable of rapidly lowering the intracellular pH in yeast (Ballarin-Denti et al., 1984; Valle et al., 1986). We have looked for effectors which would depolarize the membrane without causing (rapid) intracellular acidification, but apparently the intracellular pH of yeast is much more sensitive to perturbation than the membrane potential. With our strain no rapid depolarization was observed with 200 mM-KCl + valinomycin (results not shown) or with azide (Fig. 3). Even DNP did not depolarize the membrane within the period of the cAMP response. The intracellular pH, on the other hand, decreased very rapidly after addition of DNP or azide (Figs 4 and 5). These results indicate that the effect of DNP and azide is not due to their membrane depolarizing effect, but to some other property such as, for example, their lowering effect on the intracellular pH.

Investigation of the pH dependence of the DNP and azide effect showed that it was highly dependent on the extracellular pH (Figs 1 and 2). Between pH 6 and 7 the effect completely disappeared. Lowering the extracellular pH in the presence of DNP or azide caused sharp increases in the cAMP level. These results support the suggested pH dependence of cAMP synthesis in vivo. They are in agreement with the difference in pH optima published for adenyl cyclase (pH 6: Londesborough & Nurminen, 1972) and phosphodiesterase (pH 8: Londesborough, 1977). This difference suggests that lowering the intracellular pH, which is normally under aerobic conditions somewhere between 6.5 and 7.4 in yeast (den Hollander et al., 1981; Thevelein et al., 1987; this paper Figs 4, 5 and 8), should cause increased cAMP synthesis and decreased cAMP breakdown (Londesborough, 1977; Busa & Nuccitelli, 1984). To check this hypothesis further we did experiments in which the intracellular pH was deliberately lowered by addition of organic acids at low external pH (Fig. 8). Addition of acetate and butyrate at pH 4 and pH 5 caused immediate increases in the cAMP level. These results confirmed that lowering the intracellular pH is associated with an increase in the cAMP level. These experiments alone, however, do not allow us to make with certainty an unequivocal distinction between membrane depolarization and pH effects.
depolarization and intracellular acidification because of results obtained by Sanders et al. (1981) with *N. crassa*. They showed that addition of organic acids also caused depolarization of the plasma membrane, probably because of unspecific changes in the passive permeability of the membrane caused by the lowered intracellular pH.

We have also done experiments with membrane depolarizing compounds and organic acids under anaerobic conditions (Figs 4, 6, 7 and 8). In this case the stimulating effect on the cAMP level was virtually absent or at least much slower compared to that under aerobic conditions. The drop in the intracellular pH, on the other hand, was at least as pronounced. The stimulating effect on the cAMP level could be restored by adding a small amount of glucose which in itself had only a small effect on the cAMP level (Fig. 6). We suggest that under anaerobic conditions and in the absence of glucose the low ATP level is a limiting factor for cAMP synthesis.

J. M. T. gratefully acknowledges receipt of a fellowship from the Belgian National Fund for Scientific Research ('Research Associate'). We thank Dr M. C. Loureiro-Dias, Professor N. van Uden and Dr D. Kuschmitz for stimulating discussions. We are also indebted to Professor R. Kinne for critical reading of the manuscript. This work was supported in part by grants from the Belgian National Fund for Scientific Research, the Research Fund of the KU Leuven and the North Atlantic Treaty Organization (grant no. 5-2-05/RG 86/174).

REFERENCES


