Glucose-stimulated cAMP increase may be mediated by intracellular acidification in *Saccharomyces cerevisiae*

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It has been reported that addition of glucose to cells of *Saccharomyces cerevisiae* grown on a sugar-free medium causes a peak of intracellular cAMP levels. Also, it has been proposed that this effect might be mediated by plasma membrane depolarization. However, here, we observed a hyperpolarizing effect of glucose in *S. cerevisiae* and, in addition, no change in cAMP levels when depolarization was induced by valinomycin in the presence of K^+. In contrast, treatments that induced a rapid intracellular acidification such as addition of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at pH 5.5 but not at pH 8.0, extracellular pH shift from 8.5 to 3.5, and glucose itself, also increased the cyclic nucleotide. Thus, our data strongly support the hypothesis that intracellular acidification mediates the effect of glucose on cAMP levels.

Yeast *cAMP* *Glucose* *Membrane potential* *Intracellular acidification*

1. INTRODUCTION

Addition of glucose to yeast cells adapted to a sugar-free medium causes a time-dependent inactivation of several enzymes [1]. The gluconeogenetic enzyme fructose-1,6-bisphosphatase has been shown to underly this 'catabolite inactivation' [2], via a rapid phosphorylation with a concomitant decrease of 60% in the initial activity [3–6], followed by a much slower proteolytic degradation [4,7]. The abrupt increase in intracellular cAMP levels that also occurred immediately after sugar addition suggests that phosphorylation might occur via the activation of the cAMP-dependent protein kinase by the cyclic nucleotide [8–11]. Indeed, we could demonstrate the cAMP dependence of the process by employing a yeast mutant lacking adenylate cyclase, isolated by Matsumoto et al. [12], since the mutation prevented fructose-1,6-bisphosphatase inactivation, which in addition was strongly stimulated by extracellularly added nucleotide [13,14].

In the described sequence of events triggered by glucose, little is known about the mechanism by which the sugar induces the cAMP overshoot. It has been proposed that such an effect might be mediated by plasma membrane depolarization [15]. In fact, different depolarizing treatments, including uncouplers of oxidative phosphorylation, give rise to a cAMP peak in *Neurospora crassa* [16], *Rhodotorula gracilis* [17] and *Saccharomyces cerevisiae* [11,18]. However, a recent paper reported a hyperpolarizing effect induced by glucose in *S. cerevisiae* [19], which is confirmed here. We have also shown that the well-known depolarizing effect of the potassium ionophore, valinomycin, in the presence of K^+ does not cause any change in cAMP levels. Treatments here employed which were able to induce a rapid intracellular acidification also effectively increased the cyclic nucleotide. Finally, the same effect was obtained by means of a pH shift from 8.5 to 3.5 in the incubation medium. On the basis of the present data, intracellular acidification thus seems to be

Abbreviations: TPP^+, tetraphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mes, 4-morpholineethanesulfonic acid

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the most probable candidate in mediating the effect of glucose on cAMP levels.

2. MATERIALS AND METHODS

Reagents for enzyme assay, and cAMP determination, valinomycin, Mes and Hepes were purchased from Boehringer (Mannheim). [^{3}H]TPP$^+$ was from Amersham International (Amersham, England). FCCP was obtained from Fluka (Buchs, Switzerland). Fluorescein diacetate was from Koch-Light Laboratories (Colnbrook, England). Cellulose nitrate filters were from Sartorius (Göttingen). Yeast growth media and all other chemicals were purchased from Merck (Darmstadt). The haploid strain of *S. cerevisiae* X2180-1B was kindly supplied by Professor H. Holzer, University of Freiburg i.Br. Cells were grown to the stationary phase for 24 h at 30°C under vigorous shaking. Growth was started by inoculating, with 1 ml of preculture, 100 ml of the growth medium containing 1% yeast extract, 2% universal peptone M66 and 2% glucose. For TPP$^+$ uptake determinations a modification of the method reported by De la Peña et al. [20] was employed. At the end of growth yeasts were collected on cellulose nitrate filters (25 mm diameter; pore size, 0.65 μm) and washed with one of the following buffers: 20 mM sodium Hepes, pH 7.0; 100 mM sodium Mes, pH 5.5; 100 mM Tris-HCl, pH 8.5. Cells were subsequently resuspended in the washing buffer to a density of 5 mg/ml; [^{3}H]TPP$^+$ (24 Ci/mmol) to a final 13 nM concentration was added and allowed to equilibrate between the interior and the exterior of the cells for 150 min at 30°C under shaking. Equilibration of the lipophilic cation was then attained, and effectors were added. At the indicated times after effector additions, 400-μl samples were withdrawn, diluted in 2 ml incubation buffer plus 20 mM $MgCl_2$ at 0°C, and collected on Whatman GF/A glass fiber filters (25 mm diameter). Filters were washed twice with 20 ml of the same buffer at 0°C, dried and counted for radioactivity in a liquid scintillation counter. Zero time samples were withdrawn immediately before effector addition. Uptake values were expressed as nmol incorporated cation per g yeast fresh wt. Intracellular cAMP levels and fructose-1,6-bisphosphatase specific activities were determined as reported [9]. For such tests, cells were suspended to a final density of 20 mg/ml in the same buffers adopted in the parallel measurements of TPP$^+$ uptake. For all described tests, each reported value is the mean of at least 3 determinations. Intracellular pH changes were determined according to the procedure of Slavik [21], adapted as follows: at the end of growth, cells were resuspended to a density of 20 mg/ml in 20 mM sodium Hepes, pH 7.0, 0.1 mM fluorescein diacetate and preincubated at 30°C for 30 min. Subsequently, cells were collected on cellulose nitrate filters (25 mm diameter; pore size 0.65 μm), washed twice at 0°C with 5 ml of one of the incubation buffers, and resuspended in the same buffer to a cell density of 5 mg/ml. Samples of 3 ml were placed in a cuvette of a Jasco FP-550 recording spectrofluorometer, and fluorescence intensity at 520 nm was recorded after excitation at 435 and 490 nm. Effectors were added in volumes not exceeding 0.2 ml; the resulting effect of dilution on fluorescence intensity was allowed for by means of parallel determinations carried out after addition of an equal volume of water.

3. RESULTS AND DISCUSSION

The effect of the protonophore FCCP on the uptake of TPP by yeast cells was tested at pH 5.5 and 8.0 (fig.1A). At both pH values TPP efflux occurred, which indicates plasma membrane depolarization. Such an effect cannot be explained only in terms of stimulation of proton fluxes across the plasma membrane by the protonophore, since intracellular pH in *S. cerevisiae* is in the range 6–7 under different physiological conditions [21,22]. Thus, in the presence of FCCP, one might expect proton influx with concomitant depolarization at pH 5.5 and proton efflux with hyperpolarization at pH 8.0. The detected effect is probably mediated at the mitochondrial membrane level by oxidative phosphorylation uncoupling, with subsequent cell deenergization; this in turn should inhibit proton pumping of plasma membrane ATPase with a concomitant drop in membrane potential [23]. In any case, cyclic nucleotide formation was stimulated only at pH 5.5 (fig.1B), which does not support the hypothesis of the depolarization-induced cAMP overshoot.

We therefore investigated the possible role of

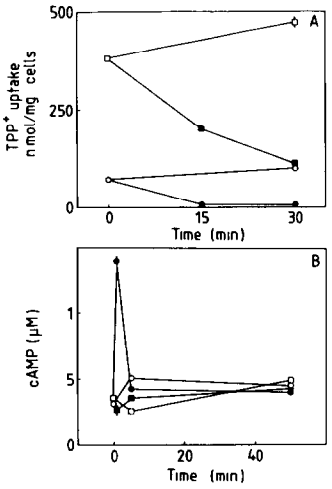

Fig.1. Effect of FCCP on TPP^+ uptake (A) and on intracellular cAMP levels (B) in yeast cells. Cells were preincubated for 150 min with 13 nM [^{3}H]TPP^+ (A) or 15 min without (B), in 0.1 M sodium Mes, pH 5.5 (○, ●) or in 0.1 M Tris-HCl, pH 8.0 (□, ■). At zero time 40 μM FCCP was added (closed symbols), and determinations were carried out at the indicated times. Open symbols represent control tests without FCCP addition. Each point represents the mean ± SE; when not shown, the SE bar was smaller than the symbol used.

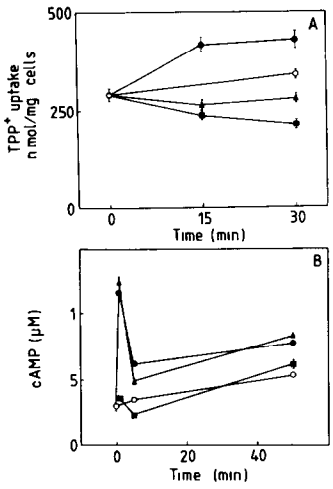

Fig.2. Effect of different treatments on TPP^+ uptake (A) and on intracellular cAMP levels (B) in yeast cells. Cells were preincubated for 150 min with 13 nM [^{3}H]TPP^+ (A) or without (B) in 20 mM sodium Hepes, pH 7.0. At zero time the following effectors were added and determinations were carried out at the indicated times: (○) no effector added; (●) 0.1 M glucose; (■) 50 mM KCl, 54 μM valinomycin; (▲) 0.1 M glucose, 50 mM KCl, 54 μM valinomycin. Each point represents the mean ± SE; when not shown, the SE bar was smaller than the symbol used.

membrane depolarization on cAMP peak stimulation by employing as effectors: glucose; valinomycin in the presence of K^+; and glucose, K^+ and valinomycin at the same time. In accordance with [19], glucose addition to yeast cells caused TPP^+ uptake, which indicates membrane hyperpolarization (fig.2A); this effect might be mediated by plasma membrane ATPase stimulation with subsequent proton extrusion [23]. K^+ plus valinomycin caused the expected depolarization due to K^+ influx, whereas simultaneous addition of glucose, K^+ and valinomycin did not induce significant changes in membrane potential (fig.2A). Moreover, parallel experiments showed that depolarization induced by K^+ plus valinomycin did not evoke the cAMP peak, which instead occurred under non-depolarizing conditions, i.e. in the presence of glucose alone, or glucose and K^+ plus valinomycin (fig.2B). Thus, there was no correlation between plasma membrane depolarization and the rapid increase in the cyclic nucleotide.

We therefore considered the alternative hypothesis, that intracellular acidification is responsible for the rapid rise in cAMP levels, which was suggested by FCCP effectiveness at external low pH only. This was checked by measuring intracellular pH changes resulting from the treatments adopted in figs 1 and 2. The data presented in fig.3 strongly support the hypothesis, since any treatment inducing a transient intracellular acidification induced the cAMP peak as well, and since, in contrast, treatments ineffective on intracellular pH values did not affect cyclic nucleotide levels. Further evidence was provided by experiments where a pH shift from 8.5 to 3.5 in the incubation medium caused a concomitant drop in intracellular pH (fig.4A) and an increase in cAMP levels (fig.4B). The fact that HCl triggered a somewhat stronger initial acidification and a much faster cAMP increase with respect to HNO_3 probably depends on different proton permeabilities which in turn may be due to different permeation pathways and/or intracellular fates of Cl^- and

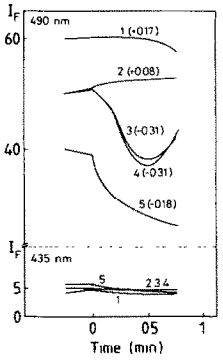

Fig.3. Intracellular pH changes after addition of different effectors. After a 30 min preincubation in 20 mM sodium Hepes, pH 7.0, and 0.1 mM fluorescein diacetate, cells were washed and resuspended in the same buffer, pH 7.0, in 0.1 M sodium Mes, pH 5.5, or in 0.1 M Tris-HCl, pH 8.0. At zero time the effectors listed below were added, and fluorescence intensity at 435 and at 490 nm expressed as relative units (ordinate) was recorded as a function of time. Effectors: (1) 40 μM FCCP, pH 8.0; (2) 50 mM KCl, 54 μM valinomycin, pH 7.0; (3) 40 mM glucose, 50 mM KCl, 54 μM valinomycin, pH 7.0; (4) 40 mM glucose, pH 7.0; (5) 40 μM FCCP, pH 5.5. Intracellular pH values before effector addition: (1) 7.14; (2–4) 6.83; (5) 6.17. For each treatment pH changes at 0.5 min are also reported (brackets). For other details see section 2.

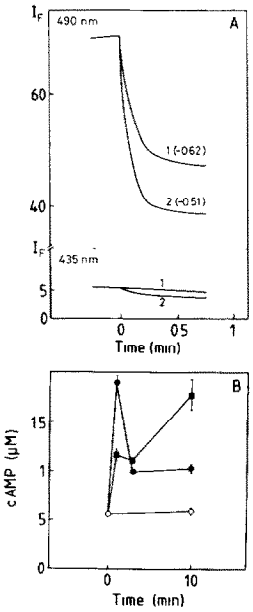

Fig.4. Effect of medium pH shift from 8.5 to 3.5 on intracellular pH (A) and on intracellular cAMP (B). Intracellular pH measurements were carried out as reported in fig.3, except that after equilibration in fluorescein diacetate cells were resuspended in 20 mM Tris, 20 mM lactate, pH 8.5, and HCl (1) or HNO_3 (2) was added. Intracellular pH before effector addition was 7.34. For each treatment pH changes at 0.5 min are also reported (brackets). For cAMP determinations, cells were preincubated for 15 min in the above-mentioned buffer, pH 8.5; at zero time appropriate amounts of concentrated HCl (●) or HNO_3 (■) for the desired shift were added. (○) No effector added. Each point represents the mean ± SE; when not shown, the SE bar was smaller than the symbol used.

NO_3^- counterions. Thus, our data strongly support the stimulation of cyclic nucleotide formation by intracellular acidification.

As further support of the hypothesis, short-term inactivation of fructose-1,6-bisphosphatase was promoted by acidifying treatments, whereas treatments displaying no such effect did not significantly influence enzyme activity (not shown).

The effect of intracellular acidification exerted by glucose addition to cells of *S. cerevisiae* has been described by Den Hollander et al. [24]. However, a still unanswered question arising from these results is: how does lowering of pH stimulate cAMP formation? A plausible explanation has been recently proposed by Busa and Nuccitelli [25] on the basis of pH-activity profiles of adenylate cyclase and 'low-K_m' phosphodiesterase; they accounted for the cAMP peak in terms of an activity increase in the former enzyme and an activity decrease in the latter in response to a lowering of pH in the physiological range. Nevertheless, one cannot rule out that other concurring effects promoted by glucose play a role in the investigated phenomenon. Work is in progress with glucose analogues to elucidate whether other mechanisms triggered by the sugar concur to stimulate cAMP overshoot.

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