Activation of yeast plasma membrane ATPase by phorbol ester

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Addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to yeast cells produces a 2-fold activation of the plasma membrane ATPase. The activation is reversible and time- and dose-dependent. The activated enzyme shows an increased affinity for its substrate, ATP, and its optimum pH is shifted to a more alkaline range. These changes are similar to those observed in the reported activation by glucose. Upon incubation of yeast cells with $^{32}P_i$ incorporation of radioactivity in a membrane polypeptide of 105 kDa is observed after addition of either glucose or TPA.

ATPase *Plasma membrane* *(Yeast)* *Phorbol ester* *Phosphorylation*

1. INTRODUCTION

Yeast cells create and maintain a proton electrochemical gradient by actively extruding protons through their plasma membrane. This gradient is necessary to support active transport of nutrients required by the cell. The enzyme responsible for this proton gradient is the ATPase located on the plasma membrane [1]. Recently [2] it was found that glucose activates 'in vivo' yeast ATPase in a reversible manner. The activation appears to be a covalent modification but nothing is known about its mechanism.

It has been observed in several systems [3–5] that those phorbol esters that are tumor promoters and activators of protein kinase C stimulate the Na^+/H^+-ATPase. This paper shows that 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C in other systems [6], stimulates the yeast plasma membrane ATPase and produces in it similar changes to those produced by glucose.

2. METHODS

2.1. *Yeast strain and growth conditions*

Saccharomyces cerevisiae A364A (Yeast Genetic Stock Center) was used. The yeasts were grown to stationary phase (25–35 mg wet wt/ml) on a medium containing 1% yeast extract (Difco), 1% bacto-peptone (Difco) and 2% glucose.

2.2. *Conditions for ATPase activation*

Yeast cells were harvested by centrifugation, washed twice with deionized water and resuspended (150 mg wet wt/ml) in a medium with 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes) adjusted to pH 6.5 with Tris. The yeast suspension was incubated at 30°C with agitation. After 30 min TPA (Sigma) or 0.1 M glucose was added. After the indicated times samples of 4 ml were taken, frozen in liquid nitrogen and stored at −70°C until use. Prior to freezing, a concentrated solution of Tris, EDTA and dithioerythritol to give final concentrations of 50, 5 and 5 mM, respectively, was quickly added.

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2.3. *Cell homogenization and membrane preparation*

Frozen samples prepared as in section 2.2 were thawed and homogenized by vortex-mixing for 5 min with 6 g glass beads (0.5 mm diameter). Total membrane fraction and purified plasma membrane were prepared as in [2].

2.4. *$^{32}P_i$ labeling of yeast and SDS-polyacrylamide gel electrophoresis of membrane proteins*

Carrier-free $^{32}P_i$ (Amersham) was added to a yeast suspension prepared as in section 2.2 at 50 μCi/ml and labeling was allowed to proceed for 5 h at 30°C with aeration. In these conditions up to 20% of the radioactivity added was incorporated into the yeast. After labeling, yeast cells were incubated for 5 min with glucose or TPA and samples were processed as described above. Particulate fractions were subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli [7] except that 0.1 mM phenylmethylsulfonyl fluoride was added during solubilization of the samples. After electrophoresis the gels were treated with 10% trichloroacetic acid to hydrolyze ribonucleic acid, stained, destained, dried and exposed to X-Omat S-5 X-ray film for 12 h at −70°C with an amplifier screen.

2.5. *ATPase assay*

Plasma membrane ATPase of total membranes (20–40 μg) or purified plasma membranes (10–20 μg) was assayed as in [2] except that lysolecithin was omitted.

2.6. *Protein determination*

Protein was measured by a modified Bradford procedure [8] with bovine serum albumin as standard.

2.7. *Determination of cAMP*

Sampling of yeast for determination of cAMP and cAMP measurements were as in [9]. To calculate the intracellular concentration of cAMP it was considered that 1 g wet yeast contains 0.6 ml cell sap [10].

3. RESULTS

3.1. *Activation of ATPase by TPA in vivo*

It has recently been found that the addition of glucose to a yeast suspension activates several-fold the proton pumping ATPase activity assayed in vitro [2]. The observed activation is reversible; upon washing, the cells become free of glucose and enzyme activity returns to basal levels.

In an attempt to characterize this activation we explored the in vivo effect of the tumor promoter TPA on the ATPase activity. After 5 min incubation with TPA a 2-fold increase in ATPase activity was observed, while, in the same conditions, glucose caused a 4-fold activation. When both agents were added simultaneously, or when one was allowed to act for 5 min prior to the addition of the other, a slightly higher activation than that obtained with glucose was observed (not shown). The activation produced by TPA was totally reversed after washing the cells.

3.2. *TPA effect is time- and dose-dependent*

To study further the observed TPA effect, the time and dose dependence were investigated. The effect of TPA on ATPase activity was time- and dose-dependent, 25 ng/ml being the optimal dose (fig.1), and maximal effect was achieved in 5 min (not shown). Accordingly, subsequent experiments were performed at 25 ng/ml of TPA for 5 min.

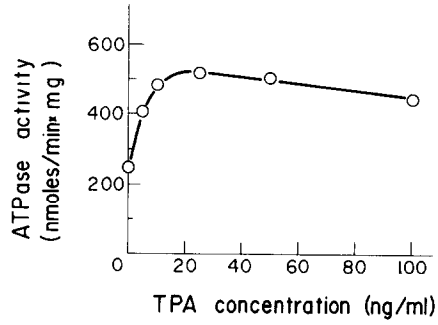

Fig.1. Dose response for TPA-induced ATPase activation. TPA in dimethyl sulfoxide was added to a yeast suspension prepared as described in section 2. Incubation was performed for 5 min at 30°C and samples taken as described. The amount of DMSO in the samples (0.0005%) was considered negligible.

3.3. *TPA mimics the effect of glucose on ATPase*

Glucose affected the enzyme in 2 ways [2], the affinity of the enzyme for the substrate was increased 4-fold and optimal pH for the enzymatic activity was shifted to a more alkaline one.

To compare the effect of TPA with the effect caused by glucose on ATPase, the apparent K_m of the enzyme for ATP and the optimum pH were measured in purified plasma membranes after 5 min treatment with 25 ng/ml TPA. The apparent affinity constant for ATP decreased from 4 to 2 mM (fig.2) and the optimum pH range moved from 5.5–5.7 to 6.0–6.4 (not shown). These results indicate that TPA and glucose modify ATPase in a similar manner.

3.4. *The concentration of intracellular cAMP is not changed by TPA*

It is known that addition of glucose to a yeast suspension causes a sharp and transitory increase in the cAMP concentration [9]. Since TPA seemed to mimic the glucose effect on ATPase it was of interest to test whether it would also produce an increase in cAMP. However, TPA treatment did not change the concentration of cAMP over 20 min or modify the increase caused by glucose (fig.3).

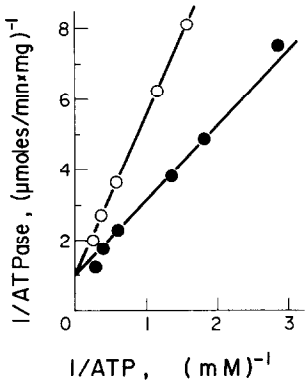

Fig.2. Effect of TPA on the apparent affinity constant of ATPase for ATP. Purified plasma membranes of yeast cells treated (●—●) or not (○—○) with 25 ng/ml of TPA were used to determine the effect of ATP concentration on ATPase activity by the double-reciprocal plot. The ATP concentration plotted corresponds to the average during the 10 min reaction. The ATP consumption was <20%.

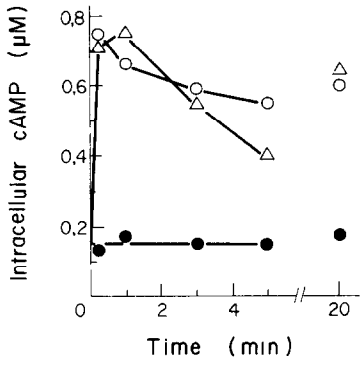

Fig.3. Effect of TPA, glucose and glucose plus TPA on the intracellular cAMP concentration. cAMP was determined as described in section 2 after addition of 25 ng/ml of TPA (●—●), 0.1 M glucose (○—○) or 25 ng/ml TPA plus 0.1 M glucose (△—△).

3.5. *Glucose and TPA increase the phosphorylation of a 105 kDa membrane protein*

Recent studies have provided evidence that protein kinase C is a target for phorbol ester [6]. When a yeast suspension was labeled in vivo with $^{32}P_i$ and then incubated for 5 min with glucose, TPA or glucose plus TPA, an incorporation of label was observed in a membrane polypeptide of M_r ~105000 (fig.4).

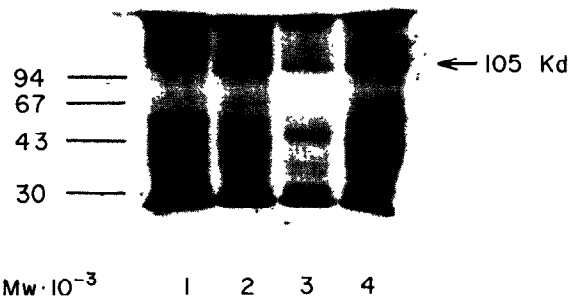

Fig.4. Effect of glucose, TPA and glucose plus TPA on the $^{32}P_i$ labeling of yeast plasma membrane proteins. Yeast suspensions labeled for 5 h with 50 μCi $^{32}P_i$ were incubated with glucose, TPA or glucose plus TPA for 5 min. Particulate fractions from these cells, prepared as in section 2 were subjected to SDS-gel electrophoresis. The figure shows the autoradiography of the gel: Lanes: 1, no addition; 2, 0.1 M glucose added; 3, 25 ng/ml TPA added; 4, 0.1 M glucose plus 25 ng/ml TPA added. The first lane shows the M_r markers.

The amount of radioactivity in the 105 kDa band was lower in membranes of TPA-treated cells, a result consistent with the lower activation observed with TPA as compared with glucose. The 105 kDa labeled band was not observed in control cells. It is known that the catalytic subunit of yeast ATPase is a polypeptide of about 105 kDa and that no other proteins are observed in this molecular mass range when yeast plasma membrane proteins are subjected to SDS gel electrophoresis [11].

4. DISCUSSION

The results reported here show that the phorbol ester, TPA, is able to activate yeast ATPase and that its action is very similar to the effect produced by glucose: both glucose and TPA effects are reversed by washing the cells, the optimum pH shifts to a more basic one closer to the yeast intracellular pH and the affinity for ATP is increased.

Glucose is known to produce an increase in cAMP when added to a yeast culture [9]. Therefore, the possibility that a cAMP-dependent phosphorylation of ATPase was taking place was explored. In fact, as shown in section 3, upon incubation of $^{32}P_i$-labeled cells with glucose, or TPA, or both, phosphorylation of a 105 kDa band, probably the catalytic subunit of ATPase was shown. The fact that phosphorylation of ATPase caused by glucose has not been observed previously [2] can probably be attributed to different electrophoretic conditions. Our finding points to phosphorylation of ATPase as the mechanism responsible for the reported glucose activation.

Since no change in the cAMP concentration was found after TPA treatment we can conclude that TPA does not act through a cAMP-dependent process, but we cannot exclude the possibility that glucose and TPA act through different pathways that converge at the end giving an activation of ATPase. Glucose could act through a cAMP-dependent process while TPA would act through a TPA-activated kinase, presumably protein kinase C. An alternative explanation is that glucose and TPA activate ATPase through one and the same pathway but that TPA bypasses the first steps of the pathway. These first steps, elicited by glucose and not by TPA, could account for the different degree of activation observed with glucose and TPA. With the available data it is difficult to distinguish between the 2 alternatives and more work is needed to clarify this point.

The fact that yeast ATPase is stimulated by incubation with TPA can be taken as an indication of the presence of protein kinase C in this organism. Protein kinase C has been shown to be present in several mammalian tissues [6] and its physiological substrates are now beginning to be identified. Its crucial role in signal transduction for a variety of substances which activate cell proliferation makes more interesting the possibility that protein kinase C may be involved in the activation of yeast ATPase since it could imply that this enzyme is involved in the control of proliferation.

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