Inhibition of membrane Ca^{2+} -ATPase of *Saccharomyces cerevisiae* by mating pheromone α -factor *in vitro*

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Plasma membrane Ca^{2+} -ATPase of *Saccharomyces cerevisiae* was solubilized and partially purified by calmodulin-affinity chromatography. The activity of Ca^{2+} -ATPase isolated from *MATa* cells was inhibited by physiological concentrations of the mating pheromone α -factor in a dose-dependent manner. The enzyme prepared from a receptor-deficient sterile mutant cells (Δste2) was similarly inhibited by α -factor, but the enzyme from *MATa* cells was resistant to the mating pheromone. We suggest that the inhibition may be involved in the α -factor-induced increase of Ca^{2+} uptake reaction of *MATa* cells.

Introduction

Haploid cells of a given mating type (*MATa* or *MAT α*) of *Saccharomyces cerevisiae* differentiate to gamete cells, responding to the mating pheromone secreted by the cell of the opposite mating type (reviewed by Cross *et al.*, 1988). This process is required for mating. The pheromone induces a morphological change ('shmoo' formation) in the target cells, and arrests the growth of these cells in the G1 phase of the cell division cycle. Recent studies on the signalling mechanism of mating pheromones have demonstrated the existence of a signal-transduction pathway involving the pheromone receptors and a G protein (Cross *et al.*, 1988).

We have previously demonstrated that one of the earliest physiological effects of the mating pheromones, in both *Rhodospiridium toruloides* and *S. cerevisiae*, is a very rapid and transient uptake of Ca^{2+} from the medium. The change appears to be involved in mating pheromone signal transduction (Miyakawa *et al.*, 1985, 1986; Tachikawa *et al.*, 1987).

It has been suggested that in mammalian cells, membrane-bound Ca^{2+} -ATPase is linked with the plasma membrane Ca^{2+} -extrusion pump that maintains cytoplasmic free Ca^{2+} concentrations at a 10^4 -fold lower level than that of the extracellular fluid (Schatzmann & Vincenzi, 1969). Using solubilized and partially purified membrane Ca^{2+} -ATPase of *R. toruloides*, a basidiomycetous yeast, we have recently shown that the enzyme

activity is inhibited by rhodotorucine A, a lipopeptide mating pheromone. This inhibition was observed only with enzyme prepared from the cells of mating type *a* (pheromone target cell) and not with that from mating type *A* (pheromone producer cell). Also, from the studies with pheromone analogues, it was found that the inhibition correlated highly with the biological activity of the mating pheromone (Miyakawa *et al.*, 1987). The inhibition of Ca^{2+} -ATPase could be responsible for the pheromone-induced intracellular Ca^{2+} increase (Miyakawa *et al.*, 1985, 1986). In the present study, we report the effect of mating pheromone α -factor on the membrane Ca^{2+} -ATPase of *S. cerevisiae*.

Methods

Strains and materials. The following strains of *Saccharomyces cerevisiae* were used: X2180-1A (*MATa SUC2 mal mel gal2 CUP1*); X2180-1B (*MAT α SUC2 mal mel gal2 CUP1*); NNY11 (*MATa STE2*); NNY14 (*MATa ste2::URA3*). Chemically synthesized α -factor was purchased from the Peptide Institute, Osaka, Japan. [γ - ^{32}P]ATP was purchased from ICN Biochemicals. Syringomycin was a gift from Dr J. Takemoto of Utah State University, USA. Calmodulin (CaM) isolated from bovine brain by the procedure of Gopalakrishna & Anderson (1982) was used for the preparation of CaM-affinity columns. For the Ca^{2+} -ATPase assay, yeast CaM purified from a protease-deficient (*pep4*), CaM-overproducing strain of *S. cerevisiae* harbouring plasmid pGCAM104 (Ohya & Anraku, 1989) was used.

Purification and assay of membrane Ca^{2+} -ATPase. Plasma membranes of *S. cerevisiae* were isolated by the concanavalin A method developed by Duran *et al.* (1975). Membrane proteins were solubilized in buffer containing 4% (w/v) n-octylglucoside, and the resulting suspension was centrifuged at 100000 g for 60 min. CaM-binding

Abbreviations: CaM, calmodulin; MM, minimal medium.

proteins were isolated by CaM-affinity chromatography as previously described (Miyakawa *et al.*, 1987). Ca^{2+} -ATPase activity was measured using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as substrate by the procedure described previously (Miyakawa *et al.*, 1987).

Ca^{2+} efflux studies. Exponentially growing cells were cultivated for 60 min at 28 °C in 7 ml of minimum medium (MM) containing $^{45}\text{Ca}^{2+}$ ($2\ \mu\text{Ci ml}^{-1}$, $74\ \text{kBq ml}^{-1}$, $50\ \mu\text{M-Ca}^{2+}$), and the cells were collected by centrifugation in Ca^{2+} -free MM. The efflux experiment was initiated by suspending the ^{45}Ca -loaded cells at a concentration of $10^8\ \text{cells ml}^{-1}$ in 7 ml of MM containing $0.68\ \text{mM-Ca}^{2+}$ and the indicated concentrations of syringomycin. The cell suspension was incubated at 28 °C with gentle shaking. Samples (700 μl) were taken at the indicated times and rapidly centrifuged in a microfuge at 4 °C. Supernatant (500 μl) was taken and spotted on a filter paper to determine radioactivity in a liquid scintillation counter as previously described (Miyakawa *et al.*, 1985). The results are expressed as percentages of the initial radioactivity present in the cells.

Results and Discussion

Ca^{2+} -pump ATPases have been isolated from solubilized plasma membranes of mammalian cells by purification procedures including CaM-affinity chromatography (Niggli *et al.*, 1981; Caroni & Carafoli, 1981; Wuytack *et al.*, 1981). We used a similar procedure to isolate the plasma membrane Ca^{2+} -ATPase of *S. cerevisiae*. Proteins were solubilized from the isolated plasma membranes of *S. cerevisiae* by n-octyl glucoside, and the CaM-binding proteins, purified by CaM-Sepharose affinity chromatography, were used for ATPase assay. CaM-binding proteins purified by the above procedure usually constituted about 0.006% of total cellular protein. The enzyme activity, which was stimulated about 4-fold by the addition of CaM, was strongly inhibited by EGTA, showing that the enzyme is a Ca^{2+} -dependent, CaM-stimulated ATPase, and by vanadate, indicating that the Ca^{2+} -ATPase is a plasma membrane enzyme (Duran *et al.*, 1975). Syringomycin, a phytotoxin, which is also toxic to *S. cerevisiae* at concentrations above $0.5\ \mu\text{g ml}^{-1}$ (Zhang & Takemoto, 1986) and affects cellular Ca^{2+} levels (J. Takemoto, unpublished; also see below), strongly inhibited Ca^{2+} -ATPase activity (Fig. 1).

Ca^{2+} -ATPase activity from *MATa* cells was measured in the presence of the mating pheromone α -factor. α -Factor inhibited the Ca^{2+} -ATPase in a dose-dependent manner at concentrations of 20 to 500 ng ml^{-1} (Fig. 2). The inhibition was about 50% at 100 ng ml^{-1} . The Ca^{2+} ATPase activity measured in the presence of 500 ng ml^{-1} α -factor was similar to that measured in the presence of 1 mM-EGTA. When trypsin-digested, biologically inactive α -factor (500 ng ml^{-1}) was added instead of α -factor, no inhibition was observed (Fig. 2). The amounts of mating pheromone required to inhibit the Ca^{2+} -ATPase were roughly comparable to those required for inducing

Fig. 1. Properties of the plasma membrane Ca^{2+} -ATPase of *S. cerevisiae* (*MATa*) purified by CaM affinity chromatography. The Ca^{2+} -ATPase activity was measured in the absence of inhibitors (●), or the presence of 1 mM-EGTA (X), 50 ng syringomycin ml^{-1} (▲), 50 μM -trifluoperazine (○) or 0.5 mM-sodium vanadate (△). The activity was also measured in the absence of CaM (□).

Fig. 2. Effect of α -factor on CaM-affinity-purified membrane Ca^{2+} -ATPase. The activity of Ca^{2+} -ATPase was measured in the presence of 0 (●), 20 (■), 100 (□), and 500 (▲) ng α -factor ml^{-1} , 500 μg trypsin-digested α -factor ml^{-1} (△), or 1 mM-EGTA (X).

Fig. 3. Effect of α -factor on Ca^{2+} -ATPase prepared from *MATa* (a), *MATa ste2* (b) and *MATα* (c) cells. Symbols are as in Fig. 2.

'shmoo' formation in the same number of *MATa* cells as were used to prepare the enzyme for the ATPase assay (data not shown).

α -Factor exerts its effect by binding to the (*STE2*-encoded) mating type-specific receptor located at the cell surface of *MATa* cells. To see whether the α -factor receptor is involved in the inhibition of the Ca^{2+} -ATPase, the effects of α -factor on the activity of the enzyme prepared from a receptor-deficient mutant and from *MATα* cells were tested. The Ca^{2+} -ATPase activity of the receptor-deficient mutant ($\Delta\text{ste2}::\text{URA3}$, Nakayama *et al.*, 1987) was inhibited by mating pheromone in a dose-dependent manner similar to that of the parental strain (Fig. 3). The results suggest that the α -factor receptor is not necessary for inhibition. In contrast, the Ca^{2+} -ATPase prepared from *MATα* cells, the α -factor producer strain, was resistant to α -factor (Fig. 3), showing that the inhibition is mating-type-specific. It is not yet clear whether the specificity of the enzyme inhibition is determined by the catalytic subunit of the Ca^{2+} -ATPase or by unidentified subunits associated with the ATPase of *MATa* cells.

To estimate the physiological roles of the Ca^{2+} -ATPase, we determined the effect of syringomycin, a potent inhibitor of the Ca^{2+} -ATPase (see Fig. 1) on the maintenance of cellular Ca^{2+} . The apparent Ca^{2+} uptake rate of the cell was stimulated markedly by the presence of $1 \mu\text{g ml}^{-1}$ syringomycin (T. Tachikawa, unpublished). Also, the rate of Ca^{2+} efflux, as measured by incubating the $^{45}\text{Ca}^{2+}$ -loaded cells in the media that contain nonradioactive Ca^{2+} and various concentrations of syringomycin, was inhibited significantly by the toxin (Fig. 4). The inhibition was about 50% in the presence of $3 \mu\text{g ml}^{-1}$ of the toxin. At higher concentrations of syringomycin ($5 \mu\text{g ml}^{-1}$), Ca^{2+} efflux was strongly inhibited during the first 10 min of treatment, and was followed by a very rapid Ca^{2+} efflux. The latter effect may be due to extensive damage to cell membranes such

Fig. 4. Effect of syringomycin on Ca^{2+} efflux. Cells loaded with $^{45}\text{Ca}^{2+}$ were suspended in media containing 0 (●), 1 (○), 3 (△) or 5 (□) $\mu\text{g syringomycin ml}^{-1}$ and the radioactivity of the cells after various periods of incubation was determined. The results are expressed as percentages of the initial radioactivity present in the cells.

as loss of the permeability barrier caused by the high concentration of the toxin. These *in vivo* effects of syringomycin on cellular Ca^{2+} appear to be mediated by the inhibition of membrane Ca^{2+} -ATPase that serves as the Ca^{2+} -extrusion pump of the plasma membrane. Similarly, the inhibition of membrane Ca^{2+} -ATPase by α -factor could be responsible for the increase in cellular Ca^{2+} uptake induced by the mating pheromone (Miyakawa *et al.*, 1985, 1986; Tachikawa *et al.*, 1987). The transient nature of the α -factor-induced increase in the apparent Ca^{2+} uptake may be due partly to the Ca^{2+} /CaM-stimulated regulatory mechanism of the ATPase. From these results, we suggest that there may be some unknown mechanism involved in α -factor

signalling. This mechanism appears to be involved in the control of cellular Ca^{2+} concentration, and is different from the well-characterized signalling pathway mediated by G protein and the α -factor receptor encoded by *STE2*.

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