Specific Induction of Ca$^{2+}$ Transport Activity in MATa Cells of Saccharomyces cerevisiae by a Mating Pheromone, $\alpha$ Factor*

Yoshinori Ohsumi and Yasuhiro Anraku

From the Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

Incubation with a high concentration of a mating pheromone, $\alpha$ factor, of Saccharomyces cerevisiae induced the accumulation of Ca$^{2+}$ ion in MATa cells, but not in MATa or MATa/\alpha cells, after a lag of 30–40 min. The $\alpha$ factor did not cause a nonspecific lesion of the membrane barrier, but induced Ca$^{2+}$ transport activity specifically. This induction of Ca$^{2+}$ transport activity correlated with formation of a projection on the cells, or with localized cell elongation, but not with G1 arrest or agglutinin induction. The increased Ca$^{2+}$ transport activity was maintained only in the continuous presence of a high concentration of $\alpha$ factor and de novo protein synthesis. Kinetic studies of induction of Ca$^{2+}$ transport by $\alpha$ factor and effects of ethylene glycol bis(\beta-aminoethyl ether)-N,N,N',N''-tetraacetic acid and Ca$^{2+}$ ionophore, A23187, on mating suggested an essential role of this physiological reaction in the initiation of sexual conjugation.

A useful system for studies on the molecular mechanisms of intercellular interaction and developmental control in eukaryotic cells is that of mating of the two different haploid cell types of Saccharomyces cerevisiae to form diploid cells. The two haploid cells, MATa and MATa cells, synthesize and secrete the sex-specific oligopeptide pheromones $\alpha$ factor and a factor, respectively (1, 2). The $\alpha$ factor plays essential roles in the initial stage of the mating process, acting on target MATa cells and causing the following biochemical and morphological changes: 1) cell division arrest at a specific point "start" in the G1 phase (3, 4); 2) production of an agglutination substance on the cell surface that promotes the aggregation of opposite cell types (5, 6); and 3) formation of projections that are called the "shmoo" and may act as copulation tubes (7, 8).

These physiological responses are believed to be mediated by the binding of $\alpha$ factor to specific receptor(s) on the target cells. Jenness et al. (9) reported the specific binding of $\alpha$ factor to MATa cells using $^{35}$S-labeled $\alpha$ factor and suggested the existence of a large number of binding sites per cell (9 $\times$ 10$^5$/cell), which are encoded by the STE2 gene. Recently, Baffi et al. (10) studied the effects of various synthetic analogues of $\alpha$ factor and suggested that the expressions of these $\alpha$ factor-induced phenomena involve more than one receptor. Moreover, Moore (11) examined the dose-response curves for these pheromone-induced reactions and showed that the amount of $\alpha$ factor required for morphological changes is 2 orders of magnitude higher (1.4 $\times$ 10$^4$ M) than that for G1 arrest or agglutinin induction. However, the actual reaction mechanism of $\alpha$ factor and the causal relations of these three phenomena are still unknown.

Since we are interested in the nature of the internal signals for onset of conjugation and progression of the cell division cycle, we are studying the genetics and physiology of calcium metabolism in yeast in the hope of finding that Ca$^{2+}$ ion triggers some of the cell function mentioned above (12). Here we report the specific induction by $\alpha$ factor of Ca$^{2+}$ transport activity in MATa cells. The relevance of this novel phenomenon to the mechanism that triggers conjugation is discussed.

MATERIALS AND METHODS

Strains and Culture Conditions—Haploid strains X2180-1A (MATa), X2180-1B (MATa), and temperature-sensitive mutants (MATa, prt-1, and prt-2) were obtained from the Yeast Genetic Stock Center, Berkeley, CA. The diploid strain X2180-1D (MATa/\alpha) was constructed by crossing X2180-1A and X2180-1B. Cells were grown in YEPD medium containing 1% yeast extract (Difco), 2% polypeptide from Difco Etryo Chemicals, Saitama, Japan), and 2% glucose (Wako Chemicals, Tokyo) at 30 °C with shaking.

Chemicals—$\alpha$ factor, synthesized chemically at the Protein Research Foundation, Osaka, was stocked as a 0.3 mM solution in 0.01 M HCl, 0.1 mM EDTA, and 1 mM $\beta$-mercaptoethanol. Radioactive compounds, $^{45}$CaCl$_2$ (57 MBq/86 $\mu$g) and $^{85}$RbCl (37 MBq/906 $\mu$g) were purchased from Amersham, England. A23187 was gift from Eli Lilly S.A. Other chemicals were standard commercial products of analytical grade.

Assay of Ca$^{2+}$ Accumulation—Exponentially growing cells were harvested, washed twice with distilled water, and suspended in fresh YEPD medium at a cell density of 1 $\times$ 10$^7$/ml. The cell suspension (1.2 ml) was placed in two test tubes; one tube received $\alpha$ factor to 3 $\mu$M final concentration and the other received the same solution without $\alpha$ factor as a control. At time zero, 1.8 $\mu$Ci of $^{45}$CaCl$_2$ was added to each tube and incubation at 30 °C was started. Every 10 min, 100-$\mu$l samples of the cultures were diluted with 5 ml of ice-cold 10 mM MES/Tris (pH 6.0)/5 mM CaCl$_2$, filtered quickly on a membrane filter (Toyo Roshi Co., Ltd. Type TM-2, 0.45 $\mu$m), and washed with 10 ml of the same solution. The radioactivity retained on the filter was measured with toluene base scintillator in a Beckman LS-9000 liquid scintillation counter. The amount of Ca$^{2+}$ in the YEPD medium used was reported to be 180 $\mu$M (12).

Assays of Ca$^{2+}$ and Rb$^{+}$ Transport Activities—Exponentially growing X2180-1A cells (1.0 $\times$ 10$^7$/ml) were incubated with or without $\alpha$ factor (3 $\mu$M) at 30 °C for 80 min, then harvested, washed twice with distilled water, and suspended in 10 mM MES/Tris (pH 6.0)/100 mM glucose to the original volume. The cell suspension was divided into 90-$\mu$l aliquots and preincubated at 30 °C for 30 s. Uptake activities were measured by adding 10 $\mu$l of a 1 $\mu$m solution of $^{45}$CaCl$_2$ or $^{85}$RbCl. Reactions were stopped by diluting the mixtures with ice-cold 10 mM MES/Tris (pH 6.0)/5 mM MgCl$_2$/25 mM KCI. Radioactivities were measured as described.

Other Analytical Procedures—Magnesium and calcium contents of cells were determined after cells were washed twice with distilled
Induction of S. cerevisiae Ca²⁺ Transport by α Factor

RESULTS

We found that addition of a high concentration of α factor to the culture medium induced about 30-fold more accumulation of ⁴⁰Ca²⁺ ion in MATa cells than in MATα cells after a lag period of 30-40 min (Fig. 1). The α factor did not induce Ca²⁺ accumulation in diploid cells (MATa/α). Therefore, this Ca²⁺ accumulation is under the control of mating type locus, MAT. A crude preparation of natural α factor prepared by the method of Duntze et al. (7) had effects similar to those of the synthetic α factor at concentrations of 10 units/ml or more. Since even after incubation with a high concentration of α factor for 3 h, almost all of the cells were still viable, this marked increase in the level of cellular Ca²⁺ was not simply due to unphysiological events, such as membrane disintegration or adsorption of Ca²⁺ to dead cells. Changes in the amounts of magnesium and calcium ions associated with cells during α factor treatment were examined. As shown in Fig. 2, changes in the calcium content in the cells increased markedly and reached a level about 5-fold that in normal cells after incubation for 160 min with 3 pM α factor, while the magnesium content did not change. The increase of Ca²⁺ ion induced by α factor was calculated to be 1.4 x 10⁻¹⁸ mol/cell, and this large value could not be explained by simple surface binding, but rather by pheromone-induced stimulation of Ca²⁺ transport.

Next we treated MATa cells with 3 μM α factor for 80 min, harvested them, washed them with distilled water, and then measured their Ca²⁺ and Rb⁺ transport activities. As shown in Fig. 3, treatment with α factor greatly increased Ca²⁺ uptake activity, but did not affect Rb⁺ uptake activity. These results suggested that α factor does not cause a nonspecific change in the membrane barrier for cations but induces Ca²⁺ transport activity specifically. Labeled Ca²⁺ ions taken up by cells remained in the cells and were not released even on prolonged incubation, suggesting that most of the Ca²⁺ ions taken up are sequestered in vacuoles by a Ca²⁺/H⁺ antiport system (14).

in Fig. 3, treatment with α factor greatly increased Ca²⁺ uptake activity, but did not affect Rb⁺ uptake activity. These results suggested that α factor does not cause a nonspecific change in the membrane barrier for cations but induces Ca²⁺ transport activity specifically. Labeled Ca²⁺ ions taken up by cells remained in the cells and were not released even on prolonged incubation, suggesting that most of the Ca²⁺ ions taken up are sequestered in vacuoles by a Ca²⁺/H⁺ antiport system (14).

Fig. 4 shows dose-response curves for a change in Ca²⁺
accumulation by $\alpha$ factor. The Ca$^{2+}$ uptake continued for a longer time with higher concentrations of $\alpha$ factor, resulting in much higher steady levels of accumulation. As shown in the inset of Fig. 4, the apparent initial rate of Ca$^{2+}$ accumulation increased cooperatively with an increase in the concentration of $\alpha$ factor and reached a plateau with more than 3 $\mu$M $\alpha$ factor, while the lag period for progression of Ca$^{2+}$ accumulation did not change with the amount of $\alpha$ factor added.

Under our experimental conditions, 1 $\mu$M $\alpha$ factor was sufficient to arrest almost all the cells in the G1 phase. In fact, we observed that on treatment with 1 $\mu$M pheromone for 90 min, the cells mostly showed round and unbudded morphology, but had bizarre shapes after 3 h. On the other hand, after treatment with more than 3 $\mu$M $\alpha$ factor, most of the cells had a pointed projection. The lag period for induction of Ca$^{2+}$ transport corresponded well with that for appearance of cells with this pointed projection. Moreover, the Ca$^{2+}$ ionophore A23187 at 10 $\mu$M had a negligible effect on growth of X2180-1A cells and did not affect the timing and efficiency of G1 arrest or agglutination induction by $\alpha$ factor, but it inhibited formation of a pointed projection and also reduced $\alpha$ factor-induced Ca$^{2+}$ accumulation (data not shown). These results suggest that $\alpha$ factor-induced Ca$^{2+}$ transport is correlated with formation of the projection, but not with the G1 arrest or agglutination induction.

When cells were treated with 3 $\mu$M $\alpha$ factor for 80 min, then washed quickly, and resuspended in fresh YEPD medium, their enhanced Ca$^{2+}$ transport activity decreased to the normal level within 15 min, whereas on readdition of 3 $\mu$M $\alpha$ factor immediately after washing the cells, their higher Ca$^{2+}$ transport activity remained unchanged during further incubation for 30 min (Fig. 5). Thus, this high induced Ca$^{2+}$ transport activity requires the continuous presence of a sufficient amount of $\alpha$ factor; in other words, the $\alpha$ factor functions as a modulator of Ca$^{2+}$ transport activity. The cell density ($1 \times 10^7$/ml) used in this experiment was fairly high, but was necessary for assay of transport activity. Under these conditions, $\alpha$ factor seemed to be degraded quickly by a MATa cell-associated proteinase (15), which may explain why Ca$^{2+}$ accumulation decreased with time in the case of lower dose of $\alpha$ factor.

Addition of cycloheximide (2 $\mu$g/ml) at the same time with $\alpha$ factor completely blocked the induction of Ca$^{2+}$ accumulation, although the antibiotic itself enhanced the Ca$^{2+}$ uptake activity of normal cells slightly for some unknown reason. We have also observed that the temperature-sensitive protein synthesis mutants (prt-1 and prt-2) could not induce this $\alpha$ factor-dependent Ca$^{2+}$ transport activity at the restrictive temperature.

To determine the duration of protein synthesis necessary for the induction of the Ca$^{2+}$ accumulation activity, cycloheximide was introduced to aliquots of the culture at various time after addition of $\alpha$ factor. Then, these cultures were incubated for a total period of 120 min and the amounts of $^{45}$Ca$^{2+}$ accumulated were measured (Fig. 6a, closed circles). When cycloheximide was added within 30 min after addition of $\alpha$ factor, no induction of Ca$^{2+}$ transport activity was observed. However, when cycloheximide was added at or after 30 min, Ca$^{2+}$ accumulation increased gradually with the time, giving plots parallel to that of the standard time course of Ca$^{2+}$ accumulation (Fig. 6a, open circles).

When cycloheximide was added to the cells at 80 min after addition of $\alpha$ factor, where the Ca$^{2+}$ accumulation activity had been fully induced, Ca$^{2+}$ accumulation continued about 15 min, then stopped completely (Fig. 6b). These results suggest that de novo protein synthesis during and after the lag period...
is indispensable for the induction of Ca\textsuperscript{2+} transport activity. In other words, a protein(s) necessary for the Ca\textsuperscript{2+} accumulation is synthesized inducibly with cycloheximide and incubation with cycloheximide), and the amount of \textsuperscript{45}Ca\textsuperscript{2+} accumulated was measured as described under "Materials and Methods".

![Effect of cycloheximide on induction of Ca\textsuperscript{2+} accumulation](image)

**FIG. 6.** Effect of cycloheximide on induction of Ca\textsuperscript{2+} accumulation. a, exponentially growing X2180-1A cells were harvested, washed, suspended in fresh YEPD medium at a cell density of 1 × 10\textsuperscript{7}/ml, and received 3 μM α factor. One tube (1.5 ml of the cell suspension) was incubated to measure the standard time course of Ca\textsuperscript{2+} accumulation at 30 °C after addition of 2.25 μCi of \textsuperscript{45}CaCl\textsubscript{2} (○). The other tube (2.0 ml of the cell suspension) was incubated at 30 °C with added 3.0 μCi of \textsuperscript{45}CaCl\textsubscript{2}. At the time indicated a 100-μl aliquot of the culture was transferred to a new tube to which cycloheximide was added at a final concentration of 2 μg/ml. Incubation was continued at 30 °C for a total period of 120 min (incubation without cycloheximide and incubation with cycloheximide), and the amount of \textsuperscript{45}Ca\textsuperscript{2+} accumulated was measured as described under "Materials and Methods" (●), b, at time zero X2180-1A cells (2.4 ml), suspended in YEPD medium as described under "Materials and Methods," received 3 μM α factor and 3.5 μCi of \textsuperscript{45}CaCl\textsubscript{2} and incubation was started at 30 °C. At 80 min, 1.0 ml of the culture was transferred to a new tube containing cycloheximide at a final concentration of 2 μg/ml and incubation was continued at 30 °C. The amounts of \textsuperscript{45}Ca\textsuperscript{2+} accumulated in control and cycloheximide-treated cells were examined periodically as indicated; ●, without cycloheximide; ○, with cycloheximide. The results suggest that α factor-induced Ca\textsuperscript{2+} transport actually takes place and is closely related with formation of a copulation tube in the mating process.

**DISCUSSION**

The effects of α factor on the morphology of a target cell have been reported by many investigators. They used their own terminologies (7, 11, 16), and there are some confusions. In the presence of α factor, the amount being dependent on the cell density and also strain, cell division is arrested in the G1 phase and the cells show large, round, and unbudded morphology and then acquire a bizarre shape after 2 or 3 h. With more α factor the cells become pear-shaped and with much more α factor they show oriented cell surface growth, forming the more pointed projections. The term "shmoo" is somewhat ambiguous so we use "projection formation" as the morphology induced by high dose of α factor.

In the present work we have analyzed carefully the cause-effect relationship between an α factor-dependent induction of Ca\textsuperscript{2+} transport and α factor-induced changes of the target cells. We have done similar experiments with α factor and observed essentially the same results obtained with MATα cells; that is, the induction of Ca\textsuperscript{2+} transport activity by α factor is related to projection formation of MATα cells (data not shown). Thus, based on all these results, we propose the following model for the mating process involving the function of the mating pheromones. When cells of opposite mating type are mixed, they first recognize low concentrations of their own terminologies (7, 11, 16), and there are some confusions. In the presence of α factor, the amount being dependent on the cell density and also strain, cell division is arrested in the G1 phase and the cells show large, round, and unbudded morphology and then acquire a bizarre shape after 2 or 3 h. With more α factor the cells become pear-shaped and with much more α factor they show oriented cell surface growth, forming the more pointed projections. The term "shmoo" is somewhat ambiguous so we use "projection formation" as the morphology induced by high dose of α factor.

In the present work we have analyzed carefully the cause-effect relationship between an α factor-dependent induction of Ca\textsuperscript{2+} transport and α factor-induced changes of the target cells. We have done similar experiments with α factor and observed essentially the same results obtained with MATα cells; that is, the induction of Ca\textsuperscript{2+} transport activity by α factor is related to projection formation of MATα cells (data not shown). Thus, based on all these results, we propose the following model for the mating process involving the function of the mating pheromones. When cells of opposite mating type are mixed, they first recognize low concentrations of...
pheromones of the opposite mating type. This induces the synthesis of agglutinin on the cell surface and then G1 arrest, as initial stages in the overall mating process. The induced agglutinability may increase the opportunity of mating, because it results in formation of tight aggregates of this nonmotile organism. The local concentration of α factor in the aggregate should be high, and once it becomes sufficient to bind to a low affinity receptor or to occupy most of a single receptor, induction of Ca\(^{2+}\) transport should take place.

Formation of a projection or copulation tube is thought to be due to oriented cell surface growth. When cells of opposite mating types are placed a short distance apart on an agar plate, their projections are always oriented towards each other, probably in response to the direction of the respective pheromone sources (17). This means that projection formation is a process that recognizes a concentration gradient of pheromone and then modulates the site of localized surface growth. Among mating pairs, the concentrations of pheromones must be particularly high at the contact sites of the cells and this high dose of α factor may modulate the Ca\(^{2+}\) transport activity at this specific site. In suspension culture, the concentration of α factor around the cell surface must be equal, but a projection may be formed at a birth scar or newly synthesized bud scar, since it has been shown that these are the sites most accessible to α factor (15).

Transient Ca\(^{2+}\) uptake from a specific site may be a signal for initiating localized cell surface growth, as proposed in the case of the algae Fucus and Pelvetia (18, 19), and then for formation of a projection or copulation tube as observed in this work. Local Ca\(^{2+}\) influx may trigger rearrangement of the cytoskeletal organization to ensure directional growth.

We have isolated yeasts with various mutations of regulation of Ca\(^{2+}\) metabolism, showing Ca\(^{2+}\)-dependent (12) and Ca\(^{2+}\)-sensitive phenotypes. Some of these mutants have defects in progression of the cell cycle, suggesting the fact that Ca\(^{2+}\) has important roles in the mitotic cycle. We are now using these mutants and various other mutants defective in mating processes, such as ast, ste, and cdc, in further analyses of the mechanism of induction of Ca\(^{2+}\) transport activity by the α factor.

Acknowledgment—We are grateful to Dr. I. Takano for a generous gift of α factor.

REFERENCES

\(^2\) Y. Ohyaa, Y. Ohsumi, and Y. Anraku, manuscript in preparation.