Essential Role for Induced Ca\(^{2+}\) Influx Followed by [Ca\(^{2+}\)]\(_{i}\) Rise in Maintaining Viability of Yeast Cells Late in the Mating Pheromone Response Pathway

A STUDY OF [Ca\(^{2+}\)], IN SINGLE SACCHAROMYCES CEREVISIAE CELLS WITH IMAGING OF FURA-2*

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We established an experimental system for measuring the cytosolic-free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) in individual Saccharomyces cerevisiae cells using fura-2 as a Ca\(^{2+}\)-specific probe in conjunction with digital image processing and examined changes in [Ca\(^{2+}\)]\(_{i}\) in response to α-factor in single cells of a mating type. The addition of α-factor to a cells raised [Ca\(^{2+}\)]\(_{i}\) to several hundred nanomolar in the cells from a basal level of approximately 100 nM, simultaneous with the induction of Ca\(^{2+}\) influx. When the cells were incubated with α-factor in a Ca\(^{2+}\)-deficient medium, Ca\(^{2+}\) influx was greatly reduced, and the rise in [Ca\(^{2+}\)]\(_{i}\) was not detected. This indicates that the α-factor-induced rise in [Ca\(^{2+}\)]\(_{i}\) is generated by Ca\(^{2+}\) influx through the plasma membrane and not by release from internal stores. In the Ca\(^{2+}\)-deficient medium, a cells died specifically after they had changed into cells with one projection on the cell surface. This indicates that the rise in [Ca\(^{2+}\)]\(_{i}\) is essential for the late response to α-factor. The duration of Ca\(^{2+}\) requirement for maintaining viability was limited to this stage, and the earlier and later stages were not affected by Ca\(^{2+}\) deprivation. Mating between a and α mating type cells was impaired in this medium due to cell death at and before the stage of conjugation. These findings are the first evidence for an essential role for mobilized Ca\(^{2+}\) in the yeast life cycle.

In higher eukaryotic cells, Ca\(^{2+}\) plays essential roles in the regulation of cellular functions, including hormone secretion, neurotransmitter release, muscle contraction, fertilization, and lymphocyte activation by acting as a second messenger in response to extracellular stimuli (for reviews, see Refs. 1, 2). These stimuli induce a rapid and transient rise in [Ca\(^{2+}\)]\(_{i}\) in target cells either by opening Ca\(^{2+}\) channels in the plasma membrane to allow extracellular Ca\(^{2+}\) to enter the cytoplasm or by activating cell surface receptors that trigger hydrolysis of membrane phosphoinositide lipids and thereby produce cytosolic inositol-1,4,5-triphosphate that releases free Ca\(^{2+}\) from internal stores.

In Saccharomyces cerevisiae, the mating process of haploid cells is controlled by the mating pheromones, α- and α-factors, that are synthesized and secreted by a and α mating type cells, respectively (for reviews, see Refs. 3, 4). The mating pheromones induce several responses in cells of the opposite mating type: in the early stage, they rapidly alter the pattern of gene expression (5, 6), induce cell surface agglutinins that facilitate (but are not essential for) mating (7, 8), and arrest mitotic cell division in the G1 phase (9, 10). In the late stage, they induce morphological changes into cells (so-called shmoo) with one or more projections on the cell surface that act as the points of contact between mating cells (11, 12), and lead to cell and nuclear fusions (13-15).

The mating pheromone response pathway has been shown to involve mechanisms similar to those found in mammalian cells, such as the pheromone-receptor interaction (16-18) and the function of the pheromone receptor-coupled guanine nucleotide-binding regulatory (G) protein (19-21), which are essential for mating pheromone signal transduction. However, nothing is known about the role of extracellular and intracellular Ca\(^{2+}\) in this pathway except that Ca\(^{2+}\) influx in α cells is stimulated by α-factor after a lag of 30-40 min in a dose-dependent manner (22).

The study presented here was designed to investigate whether Ca\(^{2+}\) influx induced by α-factor correlates with changes in [Ca\(^{2+}\)]\(_{i}\) that may have a regulatory role for signal transduction of the mating pheromone and to elucidate the role of induced Ca\(^{2+}\) influx and changes in [Ca\(^{2+}\)]\(_{i}\) in the mating pheromone response pathway. To measure [Ca\(^{2+}\)]\(_{i}\) in individual yeast cells, we employed fura-2 as a Ca\(^{2+}\)-specific probe, which has been widely used to measure [Ca\(^{2+}\)]\(_{i}\) in mammalian cells, in conjunction with digital image processing (23, 24). To elucidate the role of induced Ca\(^{2+}\) influx and changes in [Ca\(^{2+}\)]\(_{i}\), we investigated the effect of Ca\(^{2+}\) deprivation on Ca\(^{2+}\) influx, [Ca\(^{2+}\)]\(_{i}\), changes and progression of the mating process.

The results show that [Ca\(^{2+}\)]\(_{i}\) rises following an influx of Ca\(^{2+}\) which is induced by α-factor. When this influx and consequent rise are prevented by incubating α cells with α-factor in a Ca\(^{2+}\)-deficient medium, the cells die specifically after they have changed into shmoos. The duration of the Ca\(^{2+}\) requirement for maintaining viability is relatively short and the requirement is specific to this limited stage. Mating between a and α cells is thereby impaired due to cell death at or before the stage of conjugation. These results indicate that induced Ca\(^{2+}\) influx followed by a rise in [Ca\(^{2+}\)]\(_{i}\) is essential for the late stage of the mating pheromone response.
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**Materials and Methods**

**Strains**—Yeast strains used in this study were X2180-1A (MATa SUC2 mal gal2 CUP1; Yeast Genetic Stock Center (YGSC), University of California, Berkeley, CA), X2180-1B (MATu SUC2 mal gal2 CUP1; AS54A (MATu ade2 ura3-1 his3-12 ura3-52 trpl-1 289; this work), RC618 (MATu ade2-1 ura3 his3-1 cyh2 GAL; Ref. 25), RC629 (MATu a-stul-2 in RC618; Ref. 25), RC631 (MATu a-stul-2 in RC618; Ref. 25), OS1-1D (MATu sat2-1, a segregant of RC88 x X2180-1B cross); KMG4-8C (MATa ura3-52 leu2-3,112 trpl-1 289; Ref. 19, and KMG22 (MATa Delta ste2-2; LEU2 in KMG4-8C; Ref. 26). Strain KM64-8C carried plasmid YL100 which is a Ycp-type plasmid carrying the coding region of the GPA1 gene fused with the promoter region of the GAL1 gene so that the GPA1 gene is expressed only when galactose is used as a carbon source (19). Strain KM62 carried plasmid YCpGPA102 which is a Ycp-type plasmid carrying the 5-kilobase XhoI fragment containing the GPA1 gene (26). Strains RC618, RC629, RC631, and OS1-1D were provided by Y. Ohsumi. Cells were incubated at 30 °C throughout the experiment.

**Media**—A complete medium, SD, contained 6.7 g/liter yeast nitrogen base without amino acids and 10 g/liter dextrose. Yeast nitrogen base without amino acids was prepared according to the formula described in DIFCO Manual (10th ed., DIFCO Laboratories, Detroit, Michigan, 1984). Because SD medium contains 680.2 μM CaCl\(_2\), and 0.8 μM calcium pantothenate, in a Ca\(^{2+}\)-deficient medium, SD-Ca, CaCl\(_2\) was omitted and calcium pantothenate was replaced by sodium pantothenate. SSG medium contained 6.7 g/liter yeast nitrogen base without amino acids, 2 g/liter sucrose, and 40 g/liter galactose. SSG-Ca medium contained the same components as SSG except that CaCl\(_2\) was omitted and calcium pantothenate was replaced by sodium pantothenate. All the media described above were supplemented with autotrophic requirements. VPD contained 10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter dextrose.

**Measurement of Ca\(^{2+}\) Accumulation**—Cells of a mating type were grown for several generations to a density of 2 × 10\(^6\) cells/ml in SD or SD-Ca at 30 °C, and each culture was divided into two equal aliquots. One of the two aliquots received 6 μl of fura-2-containing stock solution, and the other received the same solution without a-factor as a control. At the same time, 4 CaCl\(_2\) (Du Pont-New England Nuclear) was added to final concentrations of 1.7 μCi (63 KBq)/ml and 0.14 μCi (5.2 KBq)/ml in the aliquots from SD and SD-Ca, respectively, and incubation was started. At the times indicated, samples (180 μl) were taken and filtrated on GF/C filters (Whatman) presoaked in 5 ml of water, and resuspended in the same medium, as described under "Materials and Methods." The other portion (0) was kept in the same growth conditions. Cell density was measured in a hemocytometer, and viability was examined by the methylene blue method after resuspending cells in the medium. Note that a decrease in the cell density for treated cells is due to the loss of cells during recovering them from the filtration apparatus.

**Measurement of [Cu\(^{+}\)]\(^{+}\) in Individual Yeast Cells**—Cells incubated under various conditions (2 × 10\(^6\) cells/ml) were washed three times with distilled water by filtration and resuspended in distilled water containing 80 μM fura-2 (Molecular Probes, Eugene, OR) at the density of 2 × 10\(^6\) cells/ml, and the suspensions (0.5 ml) were subjected to electroporation. One pulse of 10 ms at 800 V/0.2 cm was applied to the suspension using an electroporation apparatus (Gene Transfer, model GT-11; M&S Instrument Inc., Osaka, Japan). The cells were washed three times and resuspended in the same media used for incubation before electroporation at the density of 2 × 10\(^6\) cells/ml. The temperature of all the solutions used was kept constant, 30 °C. Fura-2-loaded cells were adhered to polylysine-coated slides immediately after being suspended, covered by coverslips, and sealed by nail varnish to prevent water evaporation. The suspensions were observed under the objective (Fluor 100/1.30 Oi Ph4DL of a Nikon Microphot-FX microscope with an integral fluorimeter system equipped with two synchronized light beams of selected wavelengths (340 and 380 nm). The time of excitation for one frame at each wavelength was 1/30 s, and eight frames were averaged for each wavelength. The time between excitation at each wavelength was approximately 1.2 s. Bleaching of fura-2 during this period was negligible. A 500-nm long-cut filter was used in the emission path. Images of fura-2 fluorescence at excitation wavelengths of 340 and 380 nm were acquired by a SIT camera (C2400-08H; Hamamatsu Photonics Ltd., Hamamatsu, Japan) and relayed both into a Hamamatsu Photonics PVM1371Q TV monitor and a Hamamatsu Photonics ARGS-100 image processor. The ratio (340/380 nm) was converted to [Ca\(^{2+}\)], using the equation described by Grynkiewicz et al. (23) and displayed essentially as described by Poenie et al. (24). The calibration curve was made by taking the 340/380 fluorescence ratio of a series of fura-2 containing calcium buffers (23) between slides and coverslips on the microscope stage. The cell-associated fluorescence at the end of the experiment was at least 50 times greater than the average autofluorescence. No leakage of fura-2 from cells was detected during the experiment. Pseudocolor images were printed by using a color video printer (G2-F11; Sharp Ltd., Osaka, Japan). The room temperature was kept constant, at 29–30 °C, during the measurement.

The conditions for loading fura-2 into yeast cells established in this study allow us to measure [Ca\(^{2+}\)], in about 10% or more of the total number of cells. The remainder has insufficient fura-2 in the cytosol for measurement. Viability of cells after electroporation is over 90%, and dead cells are distinguishable under a phase-contrast microscope during [Ca\(^{2+}\)] measurement because contrast of the cell wall of dead cells looks strong. We have confirmed that those cells are all methylene blue-positive (i.e. inviable) and the remainders are methylene blue-negative (i.e. viable). We have also confirmed that cells that have sufficient fura-2 for [Ca\(^{2+}\)] measurement are methylene blue-negative under the microscope. Furthermore, growth curves of cells in a culture tube containing SD medium are not affected by electroporation with fura-2 (Fig. 1), suggesting that budding and cell division are normal in cells with sufficient fura-2. However, it is still possible to speculate that the low percentage (approximately 10%) of cells with sufficient fura-2 may no longer be representative of the remainder of the population, even if they are viable. We therefore performed following two experiments, addressing this serious question. In the first experiment, cells of a mating type (strain RC629) were subjected to electroporation with fura-2 as described above and incubated with 0 μM a-factor in SD medium. One and 2 h after the start of the incubation, percentages of shmoo formed were determined for cells with sufficient fura-2 and those with insufficient fura-2 in the same culture under an epifluorescence microscope. Results showed that there was no essential difference in the efficiency of shmoo formation between cells with sufficient fura-2 and those with insufficient fura-2 (Table I and Fig. 2). In the second experiment, cells of a mating type (strain RC629) were again subjected to electroporation with fura-2 as described above and mixed with the same number of cells of a mating type (X2180-1B) in SD medium. The mixture was collected on the bottom of tubes as described below (see section for Determination of Mating Efficiency) and incubated for 4 h at 30 °C. The number of budded zygotyes, unbudded zygotyes, and vegetative cells were then counted under an epifluorescence microscope. Results showed that the efficiency of mating was essentially the same between cells with sufficient fura-2 and those with insufficient fura-2 and that zygotes with sufficient fura-2 budded at normal frequency (Table II and Fig. 2). The above results clearly indicate
Efficiency of morphological changes into shmoos in fura-2-loaded cells of a mating type

Cells of strain RC629 subjected to electroporation with fura-2 were incubated in SD medium containing 6 μM α-factor at 30 °C. One and 2 h after the start of the incubation, the percentage of shmoos formed was determined for cells with sufficient fura-2 and those with insufficient fura-2 in the same culture. Examples of such cells were shown in Fig. 2. At least 150 cells were examined for each cell population.

| Time (h) | % Shmoos formed |
|---------|----------------|
|         | Cells with sufficient fura-2 | Cells with insufficient fura-2 |
| 1       | 65                     | 62                     |
| 2       | 82                     | 81                     |

Efficiency of mating in fura-2-loaded cells

Cells of strain RC629 (a mating type, 2 × 10^6 cells) subjected to electroporation with fura-2 as described under "Materials and Methods" were mixed with 2 × 10^6 cells of strain X2180-1B (a mating type) that had not been subjected to electroporation, collected on the bottom of a tube as described under "Materials and Methods" and incubated in 2 ml of SD medium at 30 °C without shaking. Four h after mixing, cells were resuspended by vortexing and sonicated briefly. The number of budded zygotes, unbudded zygotes, and vegetative cells was counted for cells with sufficient fura-2 and those with insufficient fura-2. Examples of such cells are shown in Fig. 2.

| Cell type | Cells with sufficient fura-2 | Cells with insufficient fura-2 |
|-----------|------------------------------|------------------------------|
| Budded zygotes | 38                     | 35                     |
| Unbudded zygotes | 3                     | 2                     |
| Vegetative cells | 118                    | 124                    |
| Total       | 159                     | 161                    |

2, rates of Ca" efflux were measured for cells subjected to electroporation and those not subjected to it. Cells growing exponentially or those incubated with 6 μM α-factor for 40 min in SD or SD-Ca were pulse-labeled with 64CaCl2 (Du Pont-New England Nuclear; 20 μCi (740 KBq)/ml for SD and 2 μCi (74 KBq)/ml for SD-Ca) for 10 min. The cells were washed as described above and resuspended in distilled water. Portions of the suspensions were taken and radioactivity of the cells was measured after filtration on GF/C filters as described above. The remainders were divided into two equal parts. One received 80 μM fura-2 and was subjected to electroporation, washed, and resuspended in the same media used for incubation before electroporation as described above. The others were treated as above except that electroporation was omitted. The samples were then incubated for 5 and 10 min in the media and filtrated on GF/C filters. The radioactivity retained on the filter was measured. The results showed that the rates of Ca" efflux were essentially the same between cells of a mating type subjected to electroporation and those not subjected to it. We have also confirmed that Ca" content of cells that had been labeled with 64CaCl2 as described above was not perturbed by washing with distilled water during filtration which was used before and after electroporation. Fura-2 acetoxymethyl ester, a compound more permeable to the mammalian plasma membrane than fura-2, appeared not to be permeable to the yeast envelope. Loaded fura-2 has the disadvantage of being sequestered into an organelar compartment, the vacuole. A similar problem in mammalian cells has been reported (24). Thus, we measured [Ca"], as soon as possible after loading. Usually, 3 min after loading, cells with no fura-2 accumulation in the vacuole were examined for 10-10 min period. The concentration of the free Ca" in the vacuole was calculated to be 400-1000 nM, when cells with sufficient fura-2 in the vacuole were measured (see Fig. 2 as an example). However, it should be noted that vacuole pH is more acidic than cytosolic pH. Therefore, this value may be semiquantitative because pH of the calibration solutions used is 7.2 (23).

Determination of Viability by the Methylene Blue Method—Aliquots (0.1 ml) of cultures (2 × 10^6 cells/ml) were taken at the times indicated and were added to an equal volume of 0.01% methylene blue-2% sodium citrate solution (26). The mixtures were then sonicated briefly to dissociate cell clumps. The number of methylene blue-negative and positive cells were determined microscopically within 10 min after mixing. Methylene blue-negative cells are viable and methylene blue-positive cells are inviable. Viability was expressed as the number of methylene blue-negative cells as a percentage of the total number of cells at each time point.

Mating Pheromones—Synthetic α-factor was obtained from Sigma (No. T-6901). For α-factor preparation, a cell-free supernatant of strain X2180-1A grown in SD-Ca medium was concentrated 200-fold by ultrafiltration with a YM-30 membrane (Amicon Corp., Danvers, MA) which retains α-factor. The specific activity of the preparation was 80 units/ml, as determined by morphogenetic assay described by Strazdis and MacKay (28).

Determination of Mating Efficiency and Viability of Various Types of Cells after Mating—To determine mating efficiency in SD-Ca

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**Fig. 2.** Morphological changes into shmoos and the formation of zygotes in fura-2-loaded cells. Experimental conditions were described in the legends for Tables I and II. a-d, cells of a mating type subjected to electroporation with fura-2 were exposed to 6 μM α-factor for 2 h. Two shmoos are shown. One shmoos contains sufficient fura-2 in the vacuole and the other does not. e-h, cells of a mating type subjected to electroporation with fura-2 were mixed with cells of a mating type and incubated for 4 h as described in the legend for Table II. Two zygotes and an unbudded cell are shown. One zygote contains sufficient fura-2 in vacuoles and the other zygote and a cell do not. a and e, phase-contrast micrographs; b and f, fluorescence micrographs at excitation wavelength of 340 nm; c and g, fluorescence micrographs at excitation wavelength of 380 nm; d and h, pseudocolor hues coordinated with the fluorescence ratio (340:380 nm) indicating the free Ca" concentration (for relationships between hues and Ca" concentrations, see Fig. 3). Note that fluorescence of fura-2 is seen only in the vacuole because cells were incubated for long periods after fura-2 loading.

that cells with sufficient fura-2 can be representative of the remainder of the population.

To rule out the possibility that unusual Ca" efflux may occur after electroporation and falsify subsequent Ca" measurement with fura-
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A Rise in \([\text{Ca}^{2+}]_i\) Associated with Induced Ca\(^{2+}\) Influx—To study changes in the cytosolic-free Ca\(^{2+}\) concentration, \([\text{Ca}^{2+}]_i\), cells incubated under various conditions were loaded with fura-2 by electroporation and the ratio of 340 to 380 nm excitation of fura-2 fluorescence from a single cell was quantified and converted to \(\text{Ca}^{2+}\) concentration by a video camera and digital image processing as described under “Materials and Methods.”

Fig. 3 shows examples of \([\text{Ca}^{2+}]_i\), imaging and a summary of the average \([\text{Ca}^{2+}]_i\); in individual cells. \([\text{Ca}^{2+}]_i\); in a cells (strain RC629) growing exponentially in the complete synthetic medium SD was low (Fig. 3, a and e). The average \([\text{Ca}^{2+}]_i\); in the cells was found to be 116 nm (SD = 90 nm), although \([\text{Ca}^{2+}]_i\); appeared to vary from cell to cell (Fig. 3e). We found no significant difference in \([\text{Ca}^{2+}]_i\); between budded cells and un budded cells. When \(\alpha\)-factor was added to a concentration of 6 \(\mu M\), \(\alpha\) cells underwent a rise in \([\text{Ca}^{2+}]_i\); with the onset of \(\text{Ca}^{2+}\) influx. When \(\text{Ca}^{2+}\) influx was induced 40 min after the addition of \(\alpha\)-factor and 30% of the cells had changed into shmoos (Fig. 4a and b), \([\text{Ca}^{2+}]_i\); in some shmoos (5 out of 11) rose above 200 nm (281–720 nm) (Fig. 3e). \([\text{Ca}^{2+}]_i\); in cells that had not changed into shmoos remained low (data not shown). In addition, no significant rise in \([\text{Ca}^{2+}]_i\); was observed 5 min (data not shown) and 20 min (Fig. 3e) after the addition of \(\alpha\)-factor. One hour after the addition, when the level of \(\text{Ca}^{2+}\) accumulation was about half its plateau level and 56% of cells had changed into shmoos with one projection (Fig. 4, a and b), \([\text{Ca}^{2+}]_i\); rose above 200 nm (225–1091 nm) in 9 out of 11 shmoos (Fig. 3e). A similar result was obtained with cells incubated with \(\alpha\)-factor for 2 h in which \(\text{Ca}^{2+}\) accumulation had reached plateau level and 91% of the cells had changed into shmoos (Fig. 3e, Fig. 4a, c and d). It should be noted that \([\text{Ca}^{2+}]_i\); in all shmoos was not necessarily high. The reason for this apparent deviation in \([\text{Ca}^{2+}]_i\); will be given under “Discussion.” The \([\text{Ca}^{2+}]_i\); rise was not detected when \(\alpha\) cells were treated with \(\alpha\)-factor (data not shown) and when \(\alpha\) cells were incubated with \(\alpha\)-factor in a \(\text{Ca}^{2+}\)-deficient medium, SD-Ca (Fig. 3, c, d, and f). The latter result suggests that the \([\text{Ca}^{2+}]_i\); rise is generated by \(\text{Ca}^{2+}\) influx and not from release of \(\text{Ca}^{2+}\) from internal stores.

Effect of Ca\(^{2+}\) Deprivation on the Mating Pheromone Response Pathway—To investigate the possible role of \(\text{Ca}^{2+}\) influx and the rise in \([\text{Ca}^{2+}]_i\); induced by \(\alpha\)-factor, we made a \(\text{Ca}^{2+}\)-deficient medium, SD-Ca. The concentration of \(\text{Ca}^{2+}\) in this medium was 0.24 \(\mu M\), compared with 681 \(\mu M\) in SD. Vegetative growth of cells of strain RC629 in SD-Ca was very similar to that in SD (\(T_{\text{d}} = 1.8 \pm 0.1\) h in SD and \(2.0 \pm 0.1\) h in SD-Ca), even when serial reincubations into SD-Ca (seven times in succession) were performed. Similar results were reported earlier (32). We then tested whether the responses of \(\alpha\) cells to \(\alpha\)-factor were affected by incubating cells in SD-Ca. In most experiments we used the sstl (or bari) mutant that produces a defective extracellular pepsin-like protease that degrades \(\alpha\)-factor and is 10–30 times more sensitive to the factor than wild-type strains (25, 33, 34).

Fig. 4a shows that the amount of \(\text{Ca}^{2+}\) accumulated 2 h after the addition of \(\alpha\)-factor was 100-fold lower in cells in SD-Ca than those in SD (a positive control) and that the apparent initial rates of \(\text{Ca}^{2+}\) uptake of cells in SD-Ca and those in SD were 0.292 and 7.6 pmol/10^6 cells/min, respectively. The data obtained with cells grown in SD are comparable to those obtained with cells grown in YPD (22). Fig. 3, c, d, and f, shows that \([\text{Ca}^{2+}]_i\); in \(\alpha\) cells incubated with \(\alpha\)-factor did not rise during \(\alpha\)-factor treatment. Under these conditions, the calcium content of the medium had no effect on the kinetics of the responses to \(\alpha\)-factor, including growth arrest (data not shown), disappearance of budded cells (Fig. 4b), appearance of shmoos (Fig. 4b), and increase in the population of cells with G1 DNA content (data not shown). These results indicate that the \([\text{Ca}^{2+}]_i\); rise generated by \(\text{Ca}^{2+}\) influx are not responsible for progression of these early events during the mating pheromone response pathway.

However, we found differences in size and shape between shmoos incubated in SD-Ca and those in SD when the cells...
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FIG. 4. Responses of a cells growing in SD or SD-Ca medium to \(\alpha\)-factor. a, induction of Ca\(^{2+}\) influx by \(\alpha\)-factor. Strain RC629 was used as a mating type cells. Incorporation of \({}^{45}\text{Ca}\) into cells was measured as described under “Materials and Methods.” The main figure shows data obtained with \(\alpha\)-factor in SD-Ca (\(\bullet\)) and those obtained in SD; \(\Delta\), with \(\alpha\)-factor; \(\Delta\), without \(\alpha\)-factor. The figure in the inset shows a replot of the data obtained with \(\alpha\)-factor in SD-Ca (\(\bullet\)) and data obtained without \(\alpha\)-factor in SD-Ca (\(\circ\)). b, time course of morphological changes of a cells growing in SD-Ca or SD in response to \(\alpha\)-factor. Each time point represents the number of shmoos or budded cells as a percentage of the total number of cells. \(\bullet\), % shmoos in SD-Ca; \(\circ\), % shmoos in SD; \(\Delta\), % budded cells in SD-Ca; \(\Delta\), % budded cells in SD. c, decrease in viability of a cells during \(\alpha\)-factor treatment. Cells of a mating type were grown for several generations in SD-Ca and the culture was divided into two portions. Cells from one portion were incubated with 6 \(\mu\text{M}\) \(\alpha\)-factor in SD-Ca; those from the other were incubated with the same concentration of the factor and 681 \(\mu\text{M}\) CaCl\(_2\) (i.e. in SD). Aliquots of the cultures were taken at the indicated times, and cell viability was determined by the methylene blue method as described under “Materials and Methods.” Using the same cultures, colony-forming units (CFU) were also determined on YPD plates. \(\bullet\), viability in SD-Ca; \(\circ\), viability in SD; \(\Delta\), colony-forming units/ml in SD-Ca; \(\Delta\), colony-forming units/ml in SD.

were incubated for 4 h or more. In SD-Ca, the shmoos were smaller in size than in SD, and most of the shmoos in SD-Ca had only one projection, while those in SD had two or three projections. This observation suggests that cellular growth of shmoos formed in SD-Ca may be restricted or the shmoos may die due to a lack of Ca\(^{2+}\) accumulation. To test these possibilities, viability was determined by the methylene blue method (28) and by measuring colony-forming ability. Microscopic observation showed that small shmoos with one projection after 10 h of incubation in SD-Ca were all methylene blue-positive, indicating that they were inviable (Fig. 5, a–c).

The time course of the appearance of these inviable cells showed that 85% died within 5 h of the addition of \(\alpha\)-factor, while 80% of the cells remained viable when incubated in complete medium (Fig. 4c). Similar results were obtained when colony-forming units were determined (Fig. 4c). On the basis of those microscopic observations and kinetic data for viability and shmoos formation, it is obvious that this \(\alpha\)-factor-induced cell death occurred after a cells had changed into shmoos. We have noticed that this phenomenon is more severe and occurs more rapidly when a low pH medium (pH 3.5) is used, in which degradation of \(\alpha\)-factor is diminished (30). In addition, we have found that a cells also die when incubated with \(\alpha\)-factor in SD-Ca, indicating that the same phenomenon applies to both factors. The results shown in this paper were obtained with a cells incubated in media with the usual pH of 5.7.

Fig. 6 shows that \(\alpha\)-factor-induced cell death is specifically caused by the lack of Ca\(^{2+}\) in the medium. Addition of CaCl\(_2\) above 1 \(\mu\text{M}\) resulted in rescue of the cells from \(\alpha\)-factor-induced death, the most effective concentrations being 681 \(\mu\text{M}\) and 1 \(\text{mM}\) both of which gave 71% viability. MgCl\(_2\) had no effect showing this response is specific to calcium.

Fig. 7 shows the concentration dependence of \(\alpha\)-factor on the induction of cell death. The dose resulting in 50% lethality
thereby induced in galactose-based media and repressed in glucose-based medium as GPAl expression stopped and the populations of a-factor. Viability of the cells was measured as described in the legend to Fig. 4.

Aste2 (LD50) was 3 x 10^-6 M in the wild-type cells. This dose-response curve is similar to that for a-factor-induced Ca^{2+} influx observed in a complete medium YPD in which cells are alive (22). The mutant sst1 which is defective in a-factor degradation and the mutant sst2 which is defective in recovery from pheromone-induced growth arrest and is supersensitive to the pheromone (25, 35) gave LD_{50} values of 1 x 10^-7 M and 2 x 10^-6 M, respectively (Fig. 7). We have found that a-factor does not induce cell death in a cells, a/a diploid cells or a Aste2 mutant cells which lack the a-factor receptor (36, 37) (data not shown). Furthermore, we used a mutant conditionally defective in expression of the GPAI (or SCGI) gene that encodes the LY subunit of the mating factor receptor-coupled G protein (19, 20). In this mutant, the GPAI promoter is replaced by the GAL1 promoter and GPAI expression is thereby induced in galactose-based media and repressed in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. 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This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based media. This mutant behaves like mating pheromone-treated cells when incubated in glucose-based medium as GPAI expression stopped and the population of unbridged cells rose. These results indicate that cell death is a consequence of signal transduction through the mating pheromone signaling pathway including specific pheromone receptor interaction and the function of the pheromone receptor-coupled G protein under low Ca^{2+} conditions.

**Ca^{2+}-requiring Period**—To determine the Ca^{2+}-requiring period during the a-factor response pathway, we performed the following two experiments. First, 6 μM a-factor was added to cells growing exponentially in SD-Ca, and 681 μM CaCl2 was then supplemented to the medium at the times indicated in Fig. 9a after the addition of the factor. Viability of each culture was measured 10 h after the addition. The result showed that cells could escape death if CaCl2 was supplemented to the medium within 4 h of the addition of the factor. Second, cells growing exponentially in SD received 6 μM a-factor and were shifted to SD-Ca at the times indicated in Fig. 9b and viability of each sample was measured. The result showed that cells could escape death only if high Ca^{2+} was present for at least 3 h after the addition of a-factor; half-maximal viability was observed when Ca^{2+} was present for 4.2 h after the addition. After this time, cells could survive in SD-Ca (Fig. 9b). Since shmoos can be formed in the absence of extracellular Ca^{2+}, these results suggest that the period of Ca^{2+} requirement is between the time at which cells have changed into shmoos and 4.2 h after the addition of the factor.

**Mating between a and a Cells in Ca^{2+}-deficient Conditions**—We tested whether the lack of Ca^{2+} in the medium affects mating between a and a cells by mixing and incubating them in SD-Ca. Table III shows that mating efficiency, scored by counting diploids formed, was 3-fold lower in SD-Ca than in SD. Microscopic observation of the cells 5 h after mixing shows that in SD-Ca, viability of unbridged zygotes was mostly diminished (Table III and Fig. 5, g and h) and viability of unbridged cells was also slightly diminished, probably due to exposure to the mating pheromone from cells of the opposite mating type. We could not confidently distinguish between growing unbridged cells and some shmoos in the mixed
population, and therefore both were counted as unbudded cells. Table III also shows that inviable unbudded zygotes accumulate in SD-Ca, and the formation of viable budded zygotes is thereby reduced. These results account for the low mating efficiency in SD-Ca and indicate that Ca\(^{2+}\) influx followed by [Ca\(^{2+}\)] rise is physiologically important in the mating process.

**DISCUSSION**

**Mating Pheromone-induced Cell Death in Ca\(^{2+}\)-lacking Conditions**—In this report, we have presented the first evidence that Ca\(^{2+}\) influx followed by a rise in [Ca\(^{2+}\)] induced by a-factor is essential for maintaining viability of *S. cerevisiae* cells late in the mating pheromone response pathway. Prevention of the Ca\(^{2+}\) influx and the [Ca\(^{2+}\)] rise by incubating α cells in a Ca\(^{2+}\)-deficient medium correlates with induction of death of the cells that have changed into cells with one projection of the cell surface due to the action of α-factor. However, this prevention affects neither the kinetics of early responses to the factor, such as G\(_1\) arrest and morphological changes into shmoos with one projection on the cell surface nor viability of cells that have been exposed to the factor for 4.2 h or more (Fig. 9), most of which (>98%) have changed into shmoos. Thus, the Ca\(^{2+}\)-requiring stage appears to be limited between the time at which cells have changed into shmoos and 4.2 h after the addition of α-factor, and specific to shmoos with one projection.

The [Ca\(^{2+}\)], rise appeared not to necessarily take place in a synchronous manner after the addition of α-factor (Fig. 3). We think that this was not due to technical error but rather due to a mixed cell population used in this study. Exponential growing cells respond to α-factor randomly and thus the time of [Ca\(^{2+}\)], rise should be random. Another compatible explanation is that the duration that cells have high [Ca\(^{2+}\)] is considerably short.

α-Factor-induced cell death requires a high dose of α factor (LD\(_{50}\) = 3 × 10\(^{-6}\) M) in wild-type cells (Fig. 7). This result applies to a model that high affinity α-factor receptor mediates early responses such as G\(_1\) arrest and agglutinin induction, and low affinity α-factor receptor mediates projection formation and Ca\(^{2+}\) accumulation (22, 39). It is still possible to speculate that death may be an unphysiological response to a high dose of α-factor. However, this possibility is unlikely because mating between α and α cells is significantly impaired in the Ca\(^{2+}\)-deficient medium (Table III). In this condition, cells die mainly at the stage of conjugation or of unbudded cells that have been probably arrested in G\(_1\) due to the action of a mating pheromone produced by opposite mating type cells. Thus, we conclude that mobilized Ca\(^{2+}\) observed in this study is required for the late stage of the mating pheromone response pathway. A schematic model for Ca\(^{2+}\)-requiring stages during mating pheromone response pathway is presented in Fig. 10.

Some of the possible targets of mobilized Ca\(^{2+}\) may be Ca\(^{2+}\)-binding proteins. *S. cerevisiae* cells have calmodulin (39, 40), a major Ca\(^{2+}\)-binding protein, and other, putative, Ca\(^{2+}\)-binding proteins such as the CDC31 gene product (41) and the CLS4 (or CDC24) gene product (42). Among them, the CLS4 (CDC24) gene product is of particular interest. This gene product has two putative Ca\(^{2+}\)-binding regions (42) and is essential for the morphogenetic processes of the cell division cycle which involve ordered polar cell surface growth (43). At the nonpermissive temperature, the temperature-sensitive cdc24 mutant of the α mating type does not form projections in response to α-factor (44), is defective in localized secretion of acid phosphatase (44), and has low mating efficiency (45). Thus, it is possible to speculate that disordered cell surface growth may occur due to the aberrant function of Ca\(^{2+}\)-dependent processes, such as that of the CLS4 (CDC24) gene product, in Ca\(^{2+}\)-lacking conditions and this may cause serious damage leading to cell death. Although other yeast mutants defective in the function of calmodulin or the CDC31 gene product, in Ca\(^{2+}\)-transport (46) or in Ca\(^{2+}\) metabolism (47-50) have been isolated and characterized, none have been intensively tested in terms of the regulation of the mating process. The study of these mutants may uncover the molecular mechanisms underlying the mating process from the viewpoint of Ca\(^{2+}\) function.

**Changes in [Ca\(^{2+}\)] and Ca\(^{2+}\) Homeostasis**—By employing fura-2 as a Ca\(^{2+}\)-specific probe, we have shown that [Ca\(^{2+}\)] in individual, exponentially growing yeast cells is about 100 nM (Fig. 3). This concentration is comparable to that in many higher eukaryotic cells (51). In addition, we have determined...
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Mating efficiency was determined as described under "Materials and Methods." Viability of various types of cells was determined 5 h after mixing of a and a cells as described under "Materials and Methods." V, viable cells; I, inviable cells. Values in parentheses represent the percentage of viable or inviable cells of each cell type. Note that both budded cells and unbudded cells consist of haploids and diploids.

| Medium | Mating efficiency | Budded cells | Unbudded cells | Unbudded zygoites | Budded zygoites |
|--------|-------------------|--------------|----------------|-------------------|-----------------|
|        | Time h | % | no. of each cell type in a total of 1000 cells | no. of each cell type in a total of 1000 cells | no. of each cell type in a total of 1000 cells |
| SD     | 3      | 42 | 472 | V | I | V | I | 111 |
|        | 4      | 15.8 | 379 | 10 | V | I | V | I |
|        | 5      | 25.1 | (99) | 3 | (98) | (99) | (90) | (95) |
|        | 6      | 42.9 | (8) | 2 | (40) | (40) | 105 | 6 |
| SD-Ca  | 3      | 1.3 | 359 | 570 | V | I | V | I |
|        | 4      | 7.5 | 312 | 47 | V | I | V | I |
|        | 5      | 5.7 | (87) | (13) | V | I | V | I |
|        | 6      | 13.8 | (87) | (13) | V | I | V | I |

![Diagram](Fig. 10. Summary and schematic representation of Ca\textsuperscript{2+}-requiring stages in the mating pheromone response pathway. The Ca\textsuperscript{2+}-requiring stages are indicated by open arrows.)

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