

Differential Regulation of Two Ca²⁺ Influx Systems by Pheromone Signaling in *Saccharomyces cerevisiae*

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ABSTRACT

The budding yeast *Saccharomyces cerevisiae* generates calcium signals during the response to mating pheromones that promote survival of unmated cells. A Ca²⁺ channel composed of Cch1p and Mid1p was previously shown to be necessary for the production of these calcium signals. However, we find that the Cch1p-Mid1p high-affinity Ca²⁺ influx system (HACS) contributes very little to signaling or survival after treatment with α -factor in rich media. HACS activity was much greater after calcineurin inactivation or inhibition, suggesting the Cch1p-Mid1p Ca²⁺ channel is subject to direct or indirect regulation by calcineurin. Instead a distinct low-affinity Ca²⁺ influx system (LACS) was stimulated by pheromone signaling in rich medium. LACS activity was insensitive to calcineurin activity, independent of Cch1p and Mid1p, and sufficient to elevate cytosolic free Ca²⁺ concentrations ([Ca²⁺]_c) in spite of its 16-fold lower affinity for Ca²⁺. Overexpression of Ste12p or constitutive activation of this transcription factor in *dig1 dig2* double mutants had no effect on LACS activity but stimulated HACS activity when calcineurin was also inactivated. Ste12p activation had no effect on Cch1p or Mid1p abundance, suggesting the involvement of another target of Ste12p in HACS stimulation. LACS activation required treatment with mating pheromone even in *dig1 dig2* double mutants and also required *FAR1*, *SPA2*, and *BNL1*, which are necessary for proper cell cycle arrest and polarized morphogenesis. These results show that distinct branches of the pheromone-signaling pathway independently regulate HACS and LACS activities, either of which can promote survival during long-term responses.

INTRACELLULAR calcium signals can be defined as transient increases in cytosolic free calcium concentrations ([Ca²⁺]_c) that are potentially translated into cellular responses. All eukaryotic cells employ calcium signaling to regulate a wide variety of functions, including gene expression, exocytosis, cytoskeletal rearrangement, and cell physiology. For example, the process of fertilization in mammalian cells is regulated by several different calcium signals. One such signal is initiated by the binding of sperm cell receptors to glycoproteins on the surface of eggs, which triggers a calcium signal that is necessary and sufficient for initiating the acrosome reaction (DARSZON *et al.* 1999; WASSARMAN 1999). Binding and fusion of sperm also induce calcium signals within the egg that are important for release from cell cycle arrest, exocytosis of cortical granules, and metabolic activation (SWANN and PARRINGTON 1999). The Ca²⁺ channels, their regulators, and the downstream signaling factors involved in these complex reactions are not fully understood.

The yeast *Saccharomyces cerevisiae* also undergoes a mating cycle where two haploid cells of opposite mating type (\mathbf{a} or α) fuse to form a diploid zygote. This process

is initiated by the binding of secreted peptide pheromones (\mathbf{a} -factor or α -factor) to a seven-transmembrane receptor linked to a heterotrimeric G-protein and subsequent activation of the pheromone-signaling cascade (SPRAGUE and THORNER 1992; POSAS *et al.* 1998). Approximately 45 min after initiation of pheromone signaling, the rate of Ca²⁺ influx increases and [Ca²⁺]_c becomes elevated, leading to activation of calcium signaling pathways (OHSUMI and ANRAKU 1985; IIDA *et al.* 1990). The precise role of calcium signaling during the response to mating pheromones is not known. Depletion of extracellular Ca²⁺ or mutations that inactivate the Ca²⁺ influx proteins (Mid1p and Cch1p), calmodulin (Cmd1p), calmodulin-dependent protein kinases (Cmk1p and Cmk2p), or the calmodulin-dependent protein phosphatase calcineurin (Cna1p-Cnb1p and Cna2p-Cnb1p) have little or no effect on mating efficiency. However, influx of extracellular Ca²⁺ and activity of all the calcium signaling factors are essential for maximum survival during long-term treatment with pheromone in the absence of a mating partner (IIDA *et al.* 1990, 1994; CYERT *et al.* 1991; CYERT and THORNER 1992; MOSER *et al.* 1996; FISCHER *et al.* 1997; PAIDHUNGAT and GARRETT 1997; WITHEE *et al.* 1997). How the pheromone-signaling pathway promotes death and how the Ca²⁺ signaling pathway prevents it have not yet been determined.

The mechanism by which pheromone signaling stimu-

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lates Ca^{2+} influx in yeast is also not fully understood. Downstream of the cell type-specific pheromones and receptors, the pheromone-signaling cascade in both α and β cells is identical. The $G_{\beta\gamma}$ subunits of the heterotrimeric G-protein stimulate a Rho-type small GTPase, a p21-activated protein kinase (PAK), and a mitogen-activated protein (MAP) kinase signaling module (SPRAGUE and THORNER 1992; POSAS *et al.* 1998). Activation of the MAP kinase Fus3p induces the expression of many genes by directly phosphorylating and inactivating the related proteins Dig1p and Dig2p, which bind and inhibit the transcription factor Ste12p in nonsignaling conditions (COOK *et al.* 1996; TEDFORD *et al.* 1997). Fus3p also phosphorylates and activates Far1p, which then binds and inhibits the G1-specific cyclin-dependent protein kinase Cdc28p, resulting in cell cycle arrest (CHANG and HERSKOWITZ 1990; ELION *et al.* 1993; PETER *et al.* 1993; TYERS and FUTCHER 1993; CHERKASOVA *et al.* 1999). Far1p together with Spa2p, Bni1p, and other factors promotes polarized cell growth (shmooing) in the direction of the pheromone gradient (VALTZ *et al.* 1995; BLONDEL *et al.* 1999; MILLER *et al.* 1999; SHIMADA *et al.* 2000). Ca^{2+} influx and calcium signaling factors were not required for induction of mating-specific genes, cell cycle arrest, or the initial stages of polarized morphogenesis. Whether any of these processes specifically trigger Ca^{2+} influx is the subject of this study.

Mutants lacking both Dig1p and Dig2p exhibit constitutive Ste12p activity and high expression of mating-specific genes even in the absence of mating pheromone, cell cycle arrest, or shmooing (COOK *et al.* 1996; TEDFORD *et al.* 1997). Here we show that activation of the Ste12p transcription factor is sufficient to stimulate the activity of the Cch1p-Mid1p high-affinity Ca^{2+} influx system (HACS) but has no obvious effect on the cellular levels of these proteins. However, HACS is seldom utilized due to regulation by calcineurin and the stimulation of a distinct low-affinity Ca^{2+} influx system (LACS) by a separate branch of the pheromone-signaling cascade, which leads to polarized morphogenesis. The data suggest two Ca^{2+} influx pathways can be independently and differentially regulated during the mating response of yeast.

MATERIALS AND METHODS

Media, yeast strains, and plasmids: Synthetic complete (SC), synthetic minimal (SD), and complex (YPD) media were prepared and supplemented with 2% glucose as described previously (SHERMAN *et al.* 1986), using reagents from Difco (Detroit) and Sigma Chemical Company (St. Louis). Synthetic media lacking calcium were prepared similarly using reagents obtained from BIO 101 (Vista, CA). Where indicated YPD was buffered to pH 5.5 with 5 mM succinic acid and supplemented with CaCl_2 . The synthetic mating pheromone α -factor was obtained from Star Biochemicals (Torrance, CA). FK506 was provided by Fujisawa USA (Tokyo, Japan).

Table 1 lists all yeast strains employed in this study, which were derived from either strains BY4741 (*MATa his3-1 leu2-2 met15-0 ura3-0*) or from W303-1a (*MATa ade2-1 can1-100 his3-1 leu2-3,112 trp1-1 ura3-1*; WALLIS *et al.* 1989), using standard

methods of transformation and/or crossing. The *ste12::URA3* disruption was created by transformation of strain MT1153 with a 3-kbp *Clal* fragment of pNC163 (COMPANY *et al.* 1988) to create strain EMY149. The *sst1::URA3* disruption was created by transformation of W303-1A with the 5.1-kbp fragment of pJGsst1 (ELION *et al.* 1993) produced by digestion with *EcoRI* plus *SaII*. Other *sst1* strains were derived by isogenic crosses with K410. The *far1::URA3* disruption was constructed by transformation of W303-1A with pFC13 (CHANG and HERSKOWITZ 1990) after digestion with *NotI*. All knockouts were confirmed by examining phenotype and/or molecular genotype.

The plasmid pKC147 [2μ *URA3 PMAI-aequorin*] was constructed by subcloning a 1.1-kbp fragment of the *PMAI* promoter region (from nucleotides -936 to +171 relative to codon 1) plus a 0.7-kbp *BclI-KpnI* fragment of pRSV-AQ (TANAHASHI *et al.* 1990) into the *HindIII* plus *SaII* and *BamHI* plus *KpnI* sites of YE356R (MYERS *et al.* 1986), respectively. In some aequorin experiments, plasmid pEVP11/AEQ89 [2μ *LEU2 ADH-aequorin*] (BATIZA *et al.* 1996) was utilized. The 4X-*CDRE-LacZ* reporter construct pAMS366 was described previously (STATHOPOULOS and CYERT 1997). Plasmid pNC252 [2μ *URA3 GAL-STE12*] was provided by B. Errede.

Methylene blue viability assays: Cells grown to logarithmic phase in YPD or SD-100 media at 30° were diluted to a concentration of 10^6 cells/ml and exposed to 20 μM α -factor (Star Biochemicals) in the appropriate medium. At each time point indicated an aliquot of cells was harvested by centrifugation and resuspended in fresh media containing 100 $\mu\text{g}/\text{ml}$ methylene blue (Sigma). The number of methylene blue-positive and -negative cells was determined immediately by bright field microscopy. A minimum of 200 cells was counted for each strain. The number of viable cells at time 0, for each respective strain, was set to 100%.

$^{45}\text{Ca}^{2+}$ accumulation assays: Accumulation of $^{45}\text{Ca}^{2+}$ into yeast cells growing in various culture media was measured as described previously (CUNNINGHAM and FINK 1996). In a standard experiment, log-phase cells were harvested by centrifugation, resuspended in fresh medium, treated with 20 μM α -factor and/or 2.0 $\mu\text{g}/\text{ml}$ FK506, labeled with tracer amounts of $^{45}\text{CaCl}_2$ (Amersham Life Sciences, Arlington Heights, IL) for 4 hr at 30°, collected by filtration onto GFF filters (Whatman), washed three times with 5 ml ice-cold buffer A (5 mM Na-HEPES at pH 6.5, 10 mM CaCl_2), dried, and processed for liquid scintillation counting. The specific activity of the culture medium was determined in each experiment and used to convert counts per minute into nanomoles of Ca^{2+} . Cell number was determined by measurements of optical density at 600 nm.

β -Galactosidase assays: Yeast strains carrying reporter genes were grown at 30° in SC minus uracil medium to log phase, harvested, and resuspended in fresh YPD (pH 5.5) supplemented with 40 μM α -factor, 100 mM CaCl_2 , or 2 $\mu\text{g}/\text{ml}$ FK506; and shaken for 4 hr at 30° in 24-well flat-bottomed dishes. β -Galactosidase assays were performed at room temperature using chloroform/SDS-permeabilized cells and colorimetric substrate as described previously (GUARENTE and MASON 1983).

Aequorin luminescence assays: Cells were grown to log phase in SC media lacking either uracil or leucine, harvested by centrifugation, resuspended in fresh medium to ~ 10 OD₆₀₀ units, and loaded with 25 $\mu\text{g}/\text{ml}$ coelenterazine (Molecular Probes, Eugene, OR) for 20 min at room temperature. Loaded cells were raised in the indicated media to an OD₆₀₀ of 0.250 and treated with 20 μM α -factor and/or 2.0 μM FK506. Luminescence was monitored in a LB9507 luminometer (EG&G Wallac) and expressed as relative luminescence units (RLU). This procedure resulted in equivalent loading of different strains as judged by measuring total RLU after cell lysis with digitonin.

Western blots: Cells were grown to log phase in SC media lacking uracil or leucine, harvested, and extracted for mem-

TABLE 1
Yeast strains used in this study

Strain	Genotype	Reference
W303-1A	See MATERIALS AND METHODS	WALLIS <i>et al.</i> (1989)
ELY117	<i>cch1::TRP1</i>	LOCKE <i>et al.</i> (2000)
JGY41	<i>cmd1-3</i>	GEISER <i>et al.</i> (1991)
EMY170	<i>cch1::TRP1 cmd1-3</i>	This study
ELY138	<i>mid1::LEU2</i>	LOCKE <i>et al.</i> (2000)
ELY151	<i>cch1::TRP1 mid1::LEU2</i>	LOCKE <i>et al.</i> (2000)
K410	<i>sst1::URA3</i>	ELION <i>et al.</i> (1993)
EMY113	<i>cch1::TRP1 sst1::URA3</i>	This study
EMY119	<i>mid1::LEU2 sst1::URA3</i>	This study
EMY120	<i>cch1::TRP1 mid1::LEU2 sst1::URA3</i>	This study
MT1153	<i>dig1::TRP1 dig2::HIS3</i>	TEDFORD <i>et al.</i> (1997)
EMY188	<i>dig1::TRP1 dig2::HIS3 ste12::URA3</i>	This study
EMY134	<i>dig1::TRP1 dig2::HIS3 cch1::TRP1</i>	This study
EMY138	<i>dig1::TRP1 dig2::HIS3 mid1::LEU2</i>	This study
EMY124	<i>dig1::TRP1 dig2::HIS3 cch1::TRP1 mid1::LEU2</i>	This study
EMY001	<i>dig1::TRP1 dig2::HIS3 cnb1::LEU2</i>	This study
EMY071	<i>dig1::TRP1 dig2::HIS3 vcx1::URA3</i>	This study
EMY096	<i>dig1::TRP1 dig2::HIS3 pmc1::TRP1</i>	This study
K1133	<i>dig1::TRP1 dig2::HIS3 tcn1::G418</i>	This study
EMY090	<i>dig1::TRP1 dig2::HIS3 pmc1::TRP1 vcx1::URA3</i>	This study
EMY127	<i>CCH1-MYC::TRP1 sst1::URA3</i>	This study
K1119	<i>dig1::TRP1 dig2::HIS3 sst1::URA3</i>	This study
EMY146	<i>dig1::TRP1 dig2::HIS3 cch1::TRP1 sst1::URA3</i>	This study
EMY022	<i>dig1::TRP1 dig2::HIS3 mid1::LEU2 sst1::URA3</i>	This study
EMY147	<i>dig1::TRP1 dig2::HIS3 cch1::TRP1 mid1::LEU2 sst1::URA3</i>	This study
EMY185	<i>bni1::G418 cch1::TRP1</i>	This study
EY707	<i>ste12::URA3</i>	ELION <i>et al.</i> (1993)
K436	<i>far1::URA3</i>	CHANG and HERSKOWITZ (1990)
BY4741	See MATERIALS AND METHODS	Research Genetics
RG01173	<i>bni1::G418</i>	Research Genetics
RG01509	<i>spa2::G418</i>	Research Genetics

brane proteins as described (KATZMANN *et al.* 1999). Briefly, 2 OD₆₀₀ units of log phase cells pretreated for 4 hr at 30° with 1 μM α-factor and/or 2.0 μg/ml FK506 were lysed in breaking buffer [0.3 M sorbitol, 0.1 M NaCl₂, 5 mM MgCl₂, 10 mM Tris-Cl (pH 7.6) plus protease inhibitor cocktail with BAME, TAME, TLCK, AEBSE, leupeptin, and pepstatin (Sigma)] with glass beads by vortexing at high speed for 30 sec followed by incubation on ice for 30 sec a total of four times. Extracted proteins were solubilized in sample buffer [40 mM Tris-Cl (pH 6.8), 8 M urea, 15% SDS, 0.1 mM EDTA, 1% β-mercaptoethanol, 0.01% bromophenol blue, and protease inhibitor cocktail], heated at 37° for 1 min, centrifuged, fractionated by 8% SDS-PAGE, transferred to polyvinylidene difluoride (Millipore, Bedford, MA), and probed with either 12CA5 (Boehringer Mannheim, Indianapolis) or 9E10 (Santa Cruz Biotechnologies) monoclonal antibodies specific for Mid1p-HA (IDA *et al.* 1994) and Cch1p-MYC (LOCKE *et al.* 2000) tagged proteins, respectively. Cross-reacting proteins were then detected, using an ECL kit (Amersham).

RESULTS

Two independent Ca²⁺ influx pathways are stimulated by pheromone signaling: Ca²⁺/calmodulin-dependent activation of protein kinases (Cmk1p, Cmk2p) and phosphatases (calcineurin) has been shown to be important for long-term survival of a cells treated with α-factor

(MOSER *et al.* 1996; WITHEE *et al.* 1997). Cch1p and Mid1p, putative components of a HACS (LOCKE *et al.* 2000), have also been shown to be important for survival in SD-100 medium, a synthetic minimal growth medium containing 100 μM Ca²⁺ (IDA *et al.* 1994; FISCHER *et al.* 1997; PAIDHUNGAT and GARRETT 1997). To confirm that HACS and Ca²⁺/calmodulin function in the same pathway, we compared the survival rates of *cch1* null mutants, *cmd1-3* mutants, and *cch1 cmd1-3* double mutants at various times after treatment with α-factor. After treatment with α-factor in SD-100 medium, survival of *cch1* mutants was diminished relative to an isogenic wild-type strain (Figure 1A). A *cmd1-3* mutant, which carries six point mutations in calmodulin that abolish all high-affinity binding sites for Ca²⁺ (GEISER *et al.* 1991), also exhibited decreased survival after α-factor treatment (Figure 1A). Survival of a *cch1 cmd1-3* double mutant was not significantly different from that of the single mutants, suggesting HACS and calmodulin do indeed function nonadditively within a common pathway.

Surprisingly, different results were obtained using YPD growth medium, a complex rich medium containing 140 μM Ca²⁺. In YPD medium, *cch1* mutants showed no decrease in survival relative to wild type

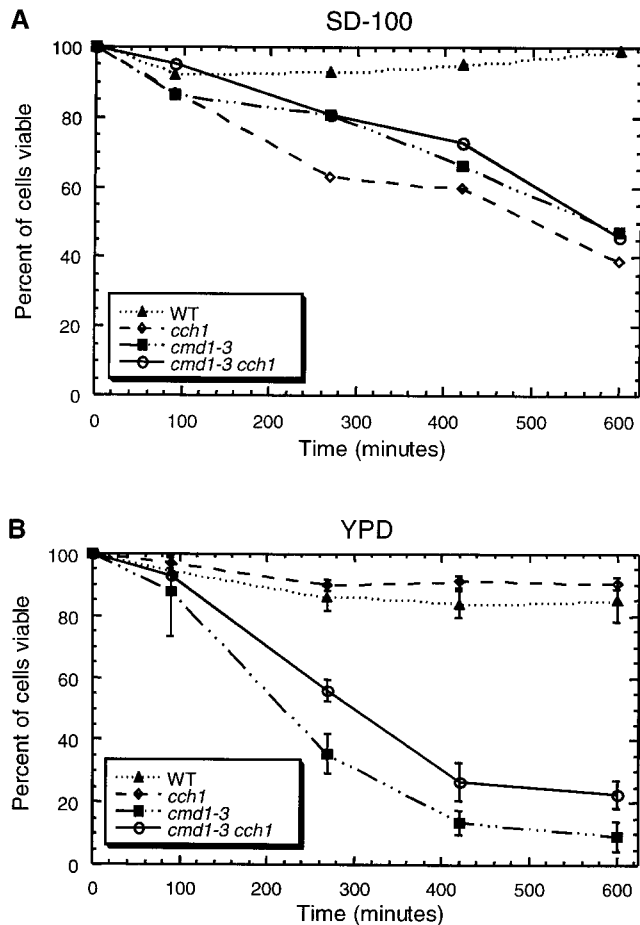


FIGURE 1.—Cch1p is required for Ca^{2+} /calmodulin-dependent survival of **a** cells treated with α -factor in minimal SD-100 media but not in rich YPD media. Log-phase cultures of wild-type, *cch1*, *cmd1-3*, and *cch1 cmd1-3* mutant strains growing in either (A) minimal SD-100 medium or (B) rich YPD medium were assayed for viability at times after treatment with $20 \mu\text{M}$ α -factor. A minimum of 200 cells were scored at each time point and the percentage of viable cells was plotted (error bars indicate the average \pm SD from three independent experiments).

whereas *cmd1-3* mutants and *cch1 cmd1-3* double mutants continued to exhibit decreased survival (Figure 1B) much like the *cmd1-6* mutant used in previous studies (MOSER *et al.* 1996). Therefore, in rich YPD medium functional Ca^{2+} /calmodulin promotes survival independent of the HACS protein Cch1p. These results suggest that a mechanism distinct from HACS may supply Ca^{2+} to calmodulin under these conditions.

To test whether cytosolic Ca^{2+} signals were generated independent of HACS in rich YPD medium, expression of the calcineurin-dependent reporter gene *CDRE-lacZ* was measured after treatment of **a** cells with α -factor. In wild-type cells, treatment with α -factor strongly induced *CDRE-lacZ* expression up to $\sim 50\%$ maximal levels obtained by adding 100 mM CaCl_2 extracellularly (Figure 2A) and this induction was totally blocked by addition of FK506, a potent inhibitor of calcineurin (SCHREIBER

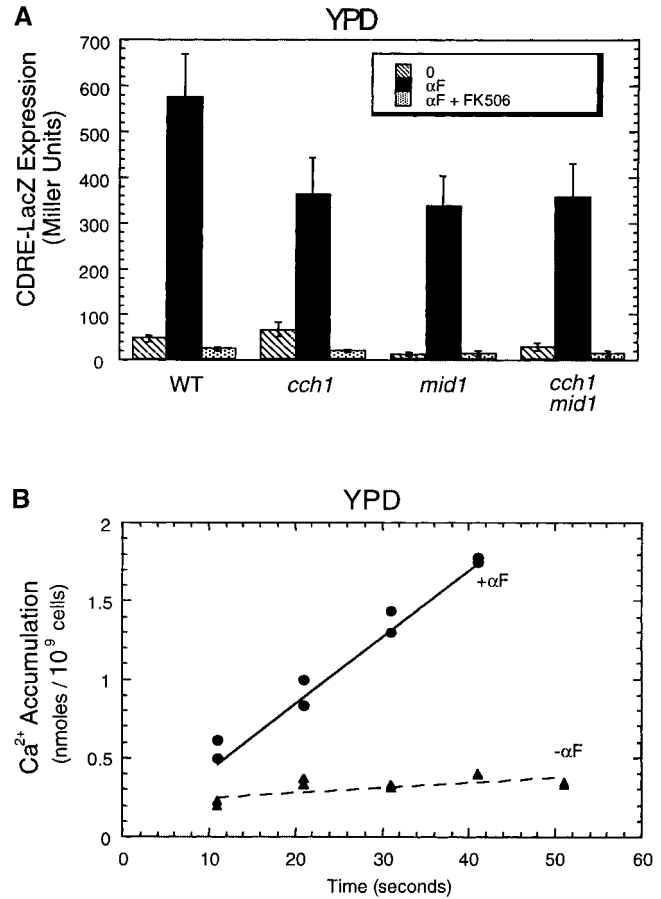


FIGURE 2.—In rich YPD medium, α -factor treatment stimulates Ca^{2+} influx and signaling independent of Cch1p and Mid1p (HACS). (A) Expression of the calcineurin-dependent reporter gene *CDRE-lacZ* from pAMS366 in wild-type, *cch1*, *mid1*, and *cch1 mid1* strains was measured after treatment with α -factor and/or FK506. Bars represent mean of three independent transformants (\pm SD). Treatment with 100 mM CaCl_2 instead of α -factor resulted in equivalent maximal expression in all four strains (1220 ± 280 , 1530 ± 220 , 1210 ± 160 , and 1140 ± 160 units \pm SD, respectively). (B) Total cellular $^{45}\text{Ca}^{2+}$ accumulation from the medium was determined over a short labeling period (< 60 sec) as described in MATERIALS AND METHODS, using a *cch1 mid1 sst1* triple mutant growing in rich YPD medium at 30° with or without 90 min pretreatment with $20 \mu\text{M}$ α -factor. The initial rate of Ca^{2+} influx was stimulated approximately sevenfold in pheromone-treated cells (circles) relative to untreated cells (triangles), suggesting the activation of a novel Ca^{2+} influx system.

and CRABTREE 1992). Calcineurin-dependent induction of the reporter gene in *cch1*, *mid1*, and *cch1 mid1* double mutants was diminished only slightly under these conditions (Figure 2A). Thus, in rich YPD medium, Ca^{2+} signals were produced independent of HACS in response to treatment with α -factor.

The HACS-independent Ca^{2+} signals described above might be generated through a variety of mechanisms, such as decreased Ca^{2+} efflux from the cytoplasm or increased Ca^{2+} influx from either intracellular stores or extracellular spaces. To help discriminate between these

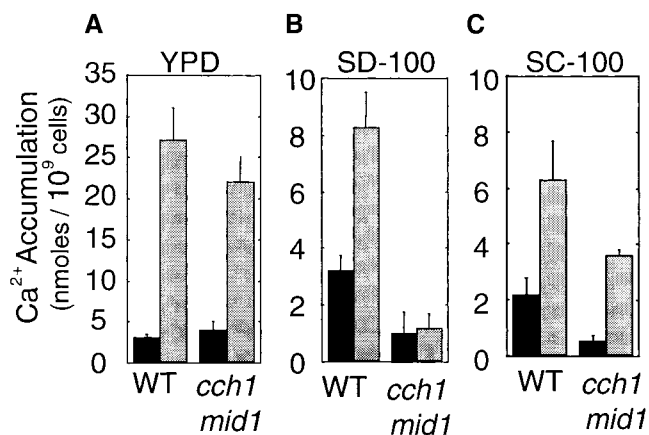


FIGURE 3.—Effects of rich YPD medium, minimal SD-100 medium, and complete SC-100 medium (SD-100 medium supplemented with amino acids) on long-term Ca²⁺ accumulation. Wild-type and *cch1 mid1* double mutants were pregrown to midlog phase in the indicated media and transferred to fresh media containing tracer ⁴⁵Ca²⁺ with (shaded bars) or without (solid bars) 20 μ M α -factor. Total cellular Ca²⁺ was determined after incubation for 4 hr at 30°. Bars represent the mean of three separate experiments (\pm SD). Results show that a novel Ca²⁺ accumulation system that is independent of Cch1p and Mid1p was stimulated by α -factor treatment in rich YPD medium and complete SC-100 medium but not minimal SD-100 medium.

possibilities, we determined the initial rates of Ca²⁺ influx into cells from the culture medium using ⁴⁵Ca²⁺ as a tracer (see MATERIALS AND METHODS). A *cch1 mid1 sst1* triple mutant, lacking the secreted protease Sst1p that degrades α -factor (CHAN and OTTE 1982), was grown in fresh YPD medium for 90 min with or without excess α -factor before addition of ⁴⁵Ca²⁺ tracer. In duplicate experiments, ⁴⁵Ca²⁺ accumulation was found to be linear with time over 60 sec (Figure 2B) and therefore useful to calculate the initial rates of Ca²⁺ influx. Cells pretreated with α -factor exhibited a sevenfold higher initial rate of Ca²⁺ influx than the untreated cells, suggesting that a novel HACS-independent Ca²⁺ influx system can be stimulated by pheromone signaling in cells grown in rich YPD medium. In contrast, cells grown in minimal SD-100 medium exhibited little or no stimulation of a HACS-independent Ca²⁺ influx system (IIDA *et al.* 1994; FISCHER *et al.* 1997; PAIDHUNGAT and GARRETT 1997).

Long-term ⁴⁵Ca²⁺ accumulation experiments represent a composite of Ca²⁺ influx and efflux rates. When ⁴⁵Ca²⁺ accumulation was measured over the entire 4-hr period of α -factor treatment (Figure 3), the results correlated well with the short-term measurements of Ca²⁺ influx (Figure 2B). In YPD medium, for example, α -factor treatment stimulated Ca²⁺ accumulation to similar degrees in wild-type and *cch1 mid1* double mutant strains (Figure 3A). In minimal SD-100 medium, however, α -factor treatment stimulated Ca²⁺ accumulation in wild type but had no significant effect on the *cch1 mid1* dou-

ble mutant (Figure 3B). Thus, a HACS-independent Ca²⁺ accumulation system was stimulated by α -factor treatment in YPD medium but not SD-100 medium. Minimal SD-100 medium lacks amino acids and many of the other nutrients present in complex YPD medium. To determine the physiological inducer of the HACS-independent system, we began by adding back nutrients to SD-100 media. Supplementing SD-100 medium with all 20 amino acids plus adenine and uracil (termed synthetic complete SC-100 medium) restored the HACS-independent ⁴⁵Ca²⁺ accumulation activity in *cch1 mid1* double mutants to near wild-type levels (Figure 3C). These results indicate that culture media rich in amino acids are necessary for stimulation of the HACS-independent Ca²⁺ influx activity and that this activity, like HACS, can be detected in long-term Ca²⁺ accumulation experiments.

Interestingly, a DNA microarray experiment revealed that expression of many calcineurin-dependent genes was induced in wild-type cells after treatment with α -factor but not in *bni1* mutants (ROBERTS *et al.* 2000). These results suggest that Bni1p, a protein required for polarized morphogenesis but not cell cycle arrest or induction of mating-specific genes, might be required for the generation of Ca²⁺ signals after α -factor treatment. To test this possibility directly, induction of the calcineurin-dependent *CDRE-lacZ* reporter gene was measured in *bni1* null mutants growing in YPD medium. After 4 hr of treatment with α -factor, induction of *CDRE-lacZ* expression was almost completely absent in the *bni1* mutant although treatment with 100 mM CaCl₂ resulted in wild-type levels of induction (Figure 4A). Moreover, α -factor treatment completely failed to stimulate ⁴⁵Ca²⁺ accumulation in *bni1* mutants relative to wild type (Figure 4B). Similar results were obtained with another mutant (*spa2*) that fails to undergo polarized morphogenesis in response to α -factor (Figure 4). Thus, factors specifically involved in polarized morphogenesis after α -factor treatment were essential for the stimulation of the HACS-independent system even in YPD medium.

To estimate the affinity of the novel influx system for Ca²⁺ we varied extracellular Ca²⁺ concentrations in SC media and monitored ⁴⁵Ca²⁺ accumulation. To do this, *cch1 mid1 sst1* triple mutant cells growing in SC-100 medium were washed in the same medium but lacking Ca²⁺ (SC-0 medium), aliquoted, and labeled with increasing concentrations of ⁴⁵CaCl₂ for 3 hr with or without α -factor treatment. In both cases, ⁴⁵Ca²⁺ accumulation was saturable and fit to the Michaelis-Menten equation by nonlinear regression. In mutants lacking a HACS (Figure 5A), α -factor treatment stimulated the appearance of a LACS with apparent *K_m* for Ca²⁺ estimated at \sim 3 mM. Data in earlier studies have indicated that calcineurin may negatively regulate Ca²⁺ accumulation stimulated by treatment with α -factor (MATHEOS *et al.* 1997; WITHEE *et al.* 1997). However, addition of FK506 during treatment with α -factor increased the *V_{max}* of

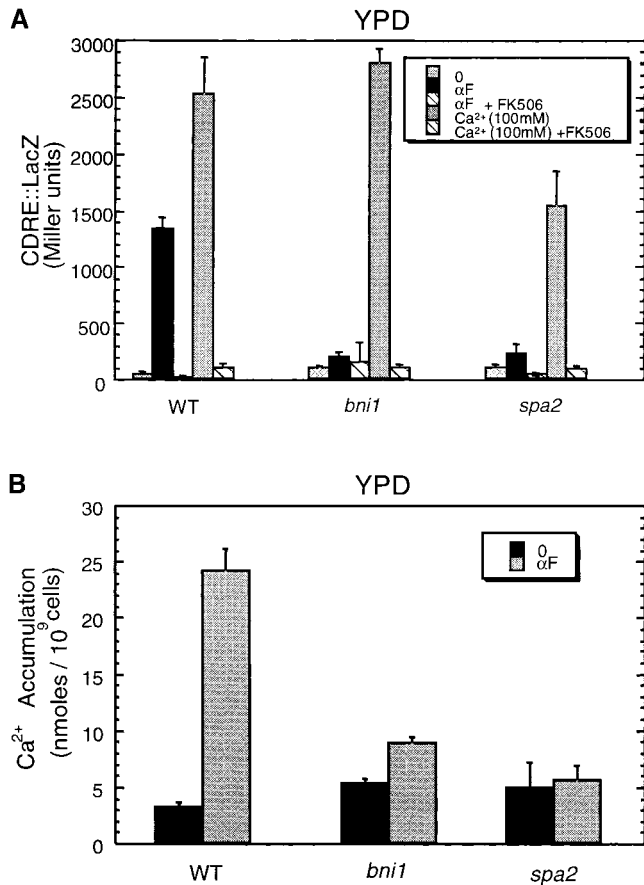


FIGURE 4.—Morphogenesis factors Bni1p and Spa2p are required for stimulation of the HACS-independent Ca²⁺ accumulation and signaling system. (A) Induction of the *CDRE-lacZ* reporter gene requires Bni1p and Spa2p. Strains transformed with plasmid pAMS366 (*CDRE-lacZ*) were grown for 4 hr at 30° in pH 5.5 YPD medium supplemented with 40 μM α-factor, 100 mM CaCl₂, and/or 2.0 μg/ml FK506 as indicated and then assayed for β-galactosidase activity as in Figure 2. (B) ⁴⁵Ca²⁺ accumulation was monitored in wild-type, *bni1*, and *spa2* mutants grown for 4 hr at 30° in YPD medium under standard conditions. The mean of three independent experiments (±SD) is shown. The results show α-factor-stimulated accumulation of Ca²⁺ that is dependent on Bni1p and Spa2p.

⁴⁵Ca²⁺ accumulation by only 1.7-fold with no significant effect on the apparent K_m (Figure 5A).

To summarize these results, a novel LACS was strongly stimulated in cells undergoing polarized morphogenesis in response to α-factor treatment and perhaps weakly modulated by calcineurin. This response was evident in cells growing in either rich YPD medium or synthetic complete SC-100 medium but not in synthetic minimal SD-100 medium. LACS activation can account for the HACS-independent cell survival and *CDRE-lacZ* expression we observed under these conditions.

Regulation of HACS by calcineurin and Ste12p activation independent of polarized morphogenesis: The above experiments revealed little or no contribution of HACS in YPD medium after treatment with α-factor. In complete SC medium containing a wide range of Ca²⁺

concentrations, treatment of *sst1* mutants with α-factor stimulated Ca²⁺ accumulation to a level that was only slightly higher than that of *cch1 mid1 sst1* triple mutants (Figure 5B, compare solid curves to dashed curves reproduced from Figure 5A). The difference between the two strains, representing HACS, was fit to the Michaelis-Menten equation but large statistical error prevented accurate estimation of K_m and V_{max} . Addition of FK506 during the α-factor treatment caused a striking difference between the two strains. After subtracting LACS activity and fitting the data to the Michaelis-Menten equation, the residual HACS activity exhibited a K_m for Ca²⁺ that was estimated at 0.19 mM, ~16-fold lower than that of LACS. Thus, α-factor treatment strongly stimulates HACS activity only in the absence of calcineurin activity.

HACS activity requires at least Cch1p and Mid1p (FISCHER *et al.* 1997; PAIDHUNGAT and GARRETT 1997; LOCKE *et al.* 2000) although it has been suggested that Mid1p may function as an ion channel in the absence of Cch1p (KANZAKI *et al.* 1999). To test whether these proteins might function independently after calcineurin-dependent regulation is removed, we performed long-term ⁴⁵Ca²⁺ accumulation experiments on *cch1* mutants and *mid1* mutants in addition to the double mutant. In SD-100 medium (Figure 6A) where LACS function is undetectable, addition of α-factor alone, FK506 alone, or FK506 plus α-factor strongly stimulated ⁴⁵Ca²⁺ accumulation into wild-type cells but had no effect on cells lacking Cch1p, Mid1p, or both. In YPD medium (Figure 6B), α-factor treatment stimulated ⁴⁵Ca²⁺ accumulation in all strains (LACS activity); but simultaneous addition of FK506 strongly increased ⁴⁵Ca²⁺ accumulation in the wild-type cells (~3.0-fold) but did so very weakly in *cch1* mutants, *mid1* mutants, and *cch1 mid1* double mutants (1.1- to 1.3-fold). Thus, there was no evidence that Cch1p and Mid1p independently function in Ca²⁺ accumulation under any conditions tested in yeast. In YPD medium, LACS appears to be the primary Ca²⁺ influx system due to (direct or indirect) inhibition of HACS activity by calcineurin.

We next investigated which branches of the pheromone-signaling pathway stimulate HACS activity. In the presence of FK506, α-factor treatment strongly stimulated ⁴⁵Ca²⁺ accumulation in *bni1* mutants and was abrogated only by further deletion of *cch1* (Figure 6C), indicating that, in contrast to LACS, HACS stimulation still occurred in the absence of polarized morphogenesis. A *far1* mutant, which fails to arrest in the cell cycle or undergo normal polarized morphogenesis (ELION *et al.* 1993; PETER *et al.* 1993; TYERS and FUTCHER 1993; CHERKASOVA *et al.* 1999), closely resembled the ⁴⁵Ca²⁺ accumulation profile of the *bni1* mutants (Figure 6C). However, a *ste12* mutant, which fails to induce any genes in response to α-factor, exhibited no response to α-factor with or without FK506. HACS stimulation therefore re-

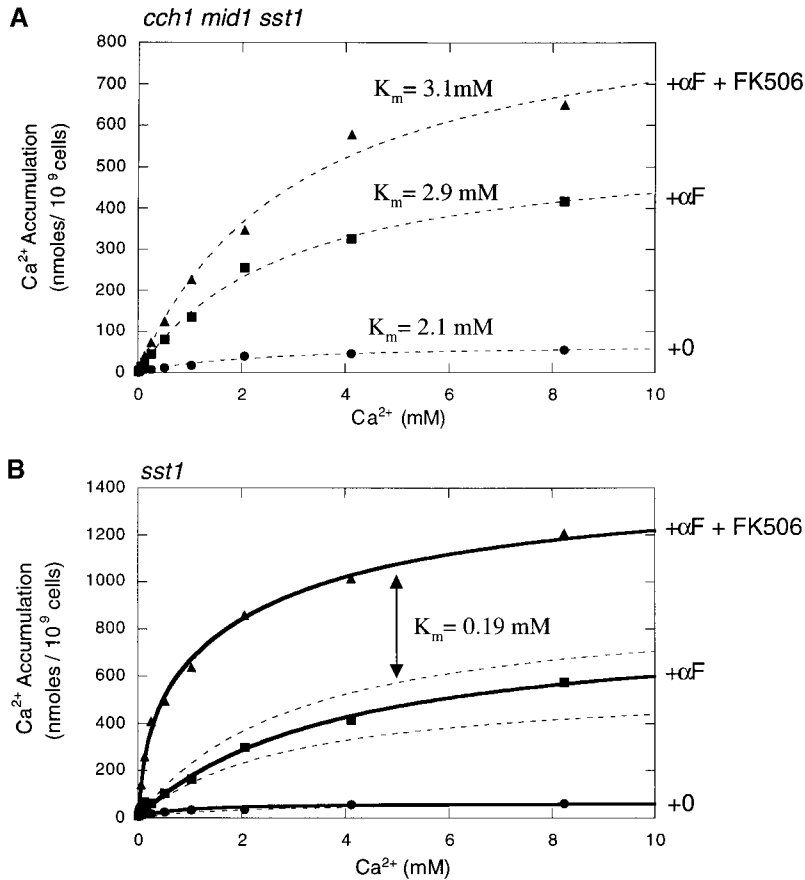


FIGURE 5.—Long-term ⁴⁵Ca²⁺ accumulation experiments in complete SC medium containing varying concentrations of Ca²⁺. In this experiment, the indicated strains were pregrown to log phase in SC-100 medium, collected, washed, and resuspended in Ca²⁺-free SC medium supplemented with 16 nM to 8 mM ⁴⁵CaCl₂, 20 μ M α -factor, and FK506 as indicated. After incubation for 4 hr at 30°, total cellular Ca²⁺ accumulation was determined for (A) a *cch1 mid1 sst1* triple mutant that lacks HACS and (B) a *sst1* mutant that retains HACS. The data in A were fit by nonlinear regression to the simple Michaelis-Menten equation (dashed curves). The data fit well with a single low-affinity Ca²⁺ accumulation system (LACS) operating in *cch1 mid1 sst1* triple mutants with an apparent K_m for Ca²⁺ \sim 3 mM. V_{max} of LACS was stimulated 1.7-fold by addition of FK506. The data in B were similarly fit to the Michaelis-Menten equation (solid curves) after subtraction of the curves shown in A (dashed curves reproduced in B for clarity). A high-affinity Ca²⁺ accumulation system (HACS) was prominent only after treatment of *sst1* mutants with α -factor plus FK506. Without FK506, HACS exhibited very low activity or very low affinity for Ca²⁺, which could not be accurately quantified above the high LACS activity.

quired induction of Ste12p-dependent genes but not cell cycle arrest or polarized morphogenesis.

If activation of the mating transcription factor Ste12p is sufficient for stimulation of HACS or LACS, cells bearing constitutively activated Ste12p would be expected to exhibit high levels of ⁴⁵Ca²⁺ accumulation independent of added α -factor. To test this hypothesis, we first examined the properties of a *dig1 dig2* double mutant, which constitutively expresses Ste12p-dependent genes (COOK *et al.* 1996; TEDFORD *et al.* 1997). ⁴⁵Ca²⁺ accumulation into *dig1 dig2* double mutants was similar to wild type in nonsignaling conditions, but, unlike wild-type cells, was also strongly stimulated after addition of FK506 (Figure 7A). FK506 addition failed to stimulate ⁴⁵Ca²⁺ accumulation in *dig1 dig2* strains that also lacked Ste12p, Mid1p, or Cch1p (Figure 7A). Alternatively, overexpression of Ste12p from the galactose promoter was capable of stimulating ⁴⁵Ca²⁺ accumulation in the presence of FK506 (Figure 7A). These results suggest constitutively active Ste12p is sufficient to activate HACS but unable to overcome the inhibitory effects of calcineurin. Introduction of the *cnb1* mutation to a *dig1 dig2* strain stimulated ⁴⁵Ca²⁺ accumulation to high levels with or without FK506 addition (Figure 7B). This finding confirms that the Ca²⁺ influx activity of Cch1p and Mid1p is regulated by calcineurin activity as opposed to a side effect of the drug.

As calcineurin also modulates the activities of vacuolar Ca²⁺ transporters Vcx1p and Pmc1p (the latter mediated by the transcription factor Tcn1p), we tested whether any of these factors were required for the effect of FK506 on *dig1 dig2* strains. ⁴⁵Ca²⁺ accumulation into *dig1 dig2 vcx1* and *dig1 dig2 tcn1* triple mutants was stimulated by FK506 to the same degree as in *dig1 dig2* double mutants (Figure 7B), demonstrating that Vcx1p and Tcn1p were not required for the effect. The *dig1 dig2 pmc1* triple mutant also responded to FK506; however, the levels of ⁴⁵Ca²⁺ accumulation were lower due to the decreased Ca²⁺ sequestration into the vacuoles of *pmc1* mutants (CUNNINGHAM and FINK 1994). FK506 addition also stimulated luminescence of *dig1 dig2 pmc1 vcx1* quadruple mutants expressing cytoplasmic aequorin but not of *pmc1 vcx1* double mutants (Figure 7C). Thus, Ste12p activation alone in the absence of signaling by upstream factors was sufficient to stimulate the Ca²⁺ influx activity of Cch1p and Mid1p through a process that was sensitive to calcineurin but independent of several known calcineurin targets (Pmc1p, Tcn1p, and Vcx1p).

To determine whether the expression of Cch1p and/or Mid1p themselves was stimulated by Ste12p activation, the abundance of these proteins was measured by Western blot analysis after treatments with α -factor and/or FK506 for 4 hr. Expression of an epitope-tagged Mid1p-HA fusion protein from a low-dosage plasmid in

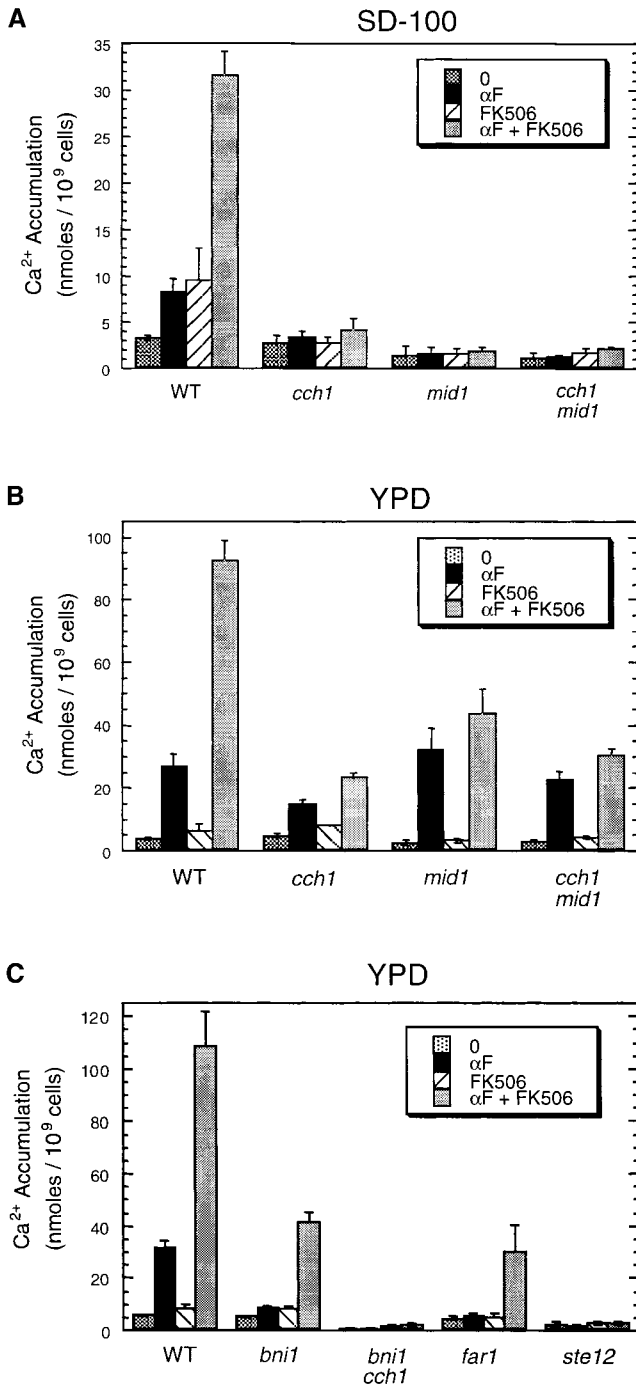


FIGURE 6.—Regulation of HACS activity by calcineurin. Long-term $^{45}\text{Ca}^{2+}$ accumulation experiments were performed on the indicated strains growing in either minimal SD-100 medium (A) or rich YPD medium (B and C) with or without treatment with α -factor and FK506. Mutants lacking Cch1p, Mid1p, or both were not significantly distinct from one another in any condition, suggesting that these proteins are jointly required for HACS activity.

an *sst1* mutant was not significantly affected by addition of α -factor, FK506, or both (Figure 8A). Similarly, expression of an epitope-tagged Cch1p-MYC fusion protein from the chromosomal locus was also unaffected

by treatments with α -factor and/or FK506 (Figure 8B). The findings suggest Ste12p induces expression of an unknown activator of HACS rather than stimulating expression of Cch1p and Mid1p themselves.

DISCUSSION

The results presented here demonstrate the existence of two Ca^{2+} influx systems in yeast that can be independently activated in response to the pheromone-signaling cascade. The previously described system (HACS) exhibited relatively high affinity for Ca^{2+} , required Cch1p and Mid1p function and Ste12p activation, and was activated strongly only when calcineurin was inactivated. The previously undescribed system (LACS) was characterized by a relatively low affinity for Ca^{2+} , little or no sensitivity to calcineurin, independence of Cch1p or Mid1p, and dependence on Bni1p and Spa2p. LACS was detected in rich YPD medium and synthetic complete SC-100 medium but not in the minimal SD-100 medium lacking amino acid supplements. Either LACS or HACS was sufficient to activate Ca^{2+} /calmodulin-dependent signaling pathways. These findings extend earlier studies that indicate survival in SD-100 medium requires Cch1p and Mid1p (IDA *et al.* 1994; FISCHER *et al.* 1997; PAIDHUNGAT and GARRETT 1997) and afford a more careful characterization of the mechanisms that coordinate HACS and LACS activities.

Regulation of LACS: A surprising finding was that *cch1 mid1* double mutants lacking HACS were still capable of producing robust calcium signals in response to pheromone. In these cells, α -factor stimulated $^{45}\text{Ca}^{2+}$ influx and accumulation, elevated $[\text{Ca}^{2+}]_c$, and induced calcineurin-dependent genes to nearly the same levels as those seen in wild-type cells. This activity has not been described in previous reports and is characterized by a relatively low affinity for Ca^{2+} , insensitivity to calcineurin, and dependence on the functions of Far1p, Bni1p, and Spa2p, in addition to treatment with α -factor. Far1p, Bni1p, Spa2p, and numerous interacting proteins become localized to the morphological projections in cells responding to α -factor (DORER *et al.* 1995; VALTZ *et al.* 1995; BUTTY *et al.* 1998; BLONDEL *et al.* 1999; MILLER *et al.* 1999; NERN and ARKOWITZ 1999; SHIMADA *et al.* 2000). Mutants lacking Bni1p or Spa2p form abnormal projections and also fail to induce a set of genes governed by the cell integrity signaling pathway, which includes the MAP-kinase Mpk1p (ROBERTS *et al.* 2000). LACS activation does not require Mpk1p because pheromone signaling stimulated LACS activity to indistinguishable levels in *mpk1* mutants and wild type (data not shown). Therefore, it is possible that normal polarized morphogenesis and/or membrane stretch, which occurs in wild-type cells but not in *bni1* mutants, stimulate LACS. Finally, LACS function appears only in rich media such as YPD or synthetic complete SC media. Because the only difference between our SC-100 and SD-100 was

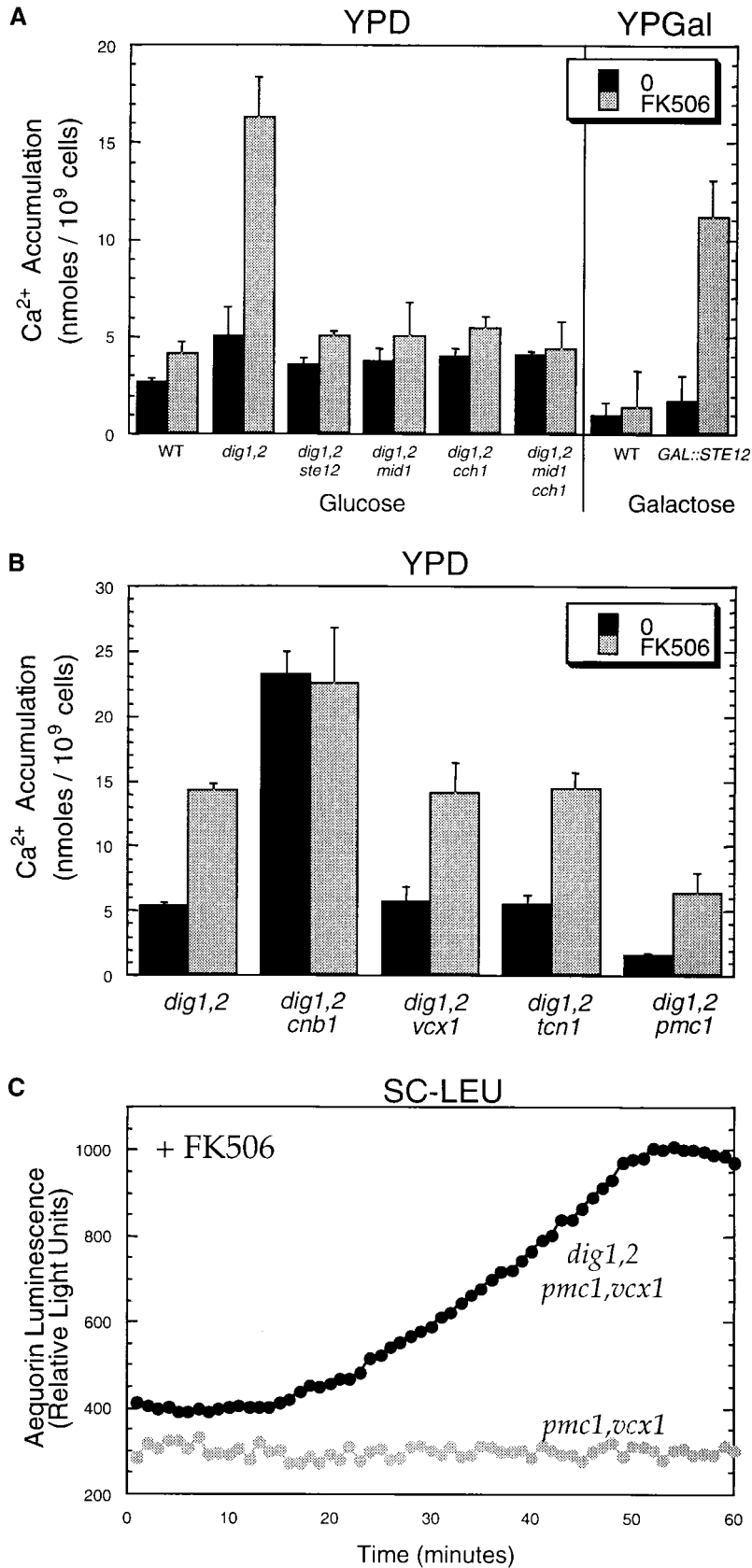


FIGURE 7.—Regulation of HACS activity by Ste12p activation. (A and B) ⁴⁵Ca²⁺ accumulation into strains containing or lacking constitutive Ste12p was measured during 4 hr growth at 30° in YPD medium without (solid bars) or with (shaded bars) added FK506. Constitutive activation of Ste12p was achieved in the absence of α -factor by either Ste12p overexpression in wild-type cells using plasmid pNC252 (A, right) or deletion of its inhibitors Dig1p and Dig2p (*dig1 dig2* double mutants). Other null mutations (*ste12*, *mid1*, *cch1*, *vcx1*, *tcn1*, and *pmc1*) were introduced and tested as indicated. (C) Cytosolic free Ca²⁺ concentrations were monitored at times after FK506 addition by measuring luminescence of cytoplasmic aequorin (expressed from plasmid pEVP11/AEQ89) in either *pmc1 vcx1* double mutants (shaded circles) or *dig1 dig2 pmc1 vcx1* quadruple mutants (solid circles).

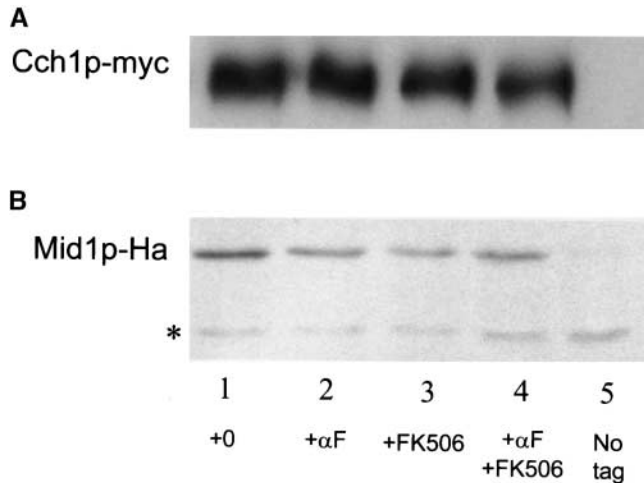


FIGURE 8.—Western blot analysis of Cch1p-Myc and Mid1p-HA after treatment with α -factor and/or FK506 in YPD medium. A *sst1* mutant strain bearing epitope-tagged Cch1p (A) or Mid1p (B) was treated with α -factor with or without FK506 in YPD medium. After incubation for 3 hr, total cell extracts were prepared, fractionated by SDS-PAGE, and analyzed by Western blotting using monoclonal antibodies.

the presence of additional amino acids and nutrients, it seems likely that nitrogen deficiency of SD-100 prevents LACS activation or expression. Further dissection of the regulatory mechanism that activates LACS would be facilitated by identification of the membrane proteins that compose this ion transporter or channel.

Regulation of HACS activity: The studies of Iida and others identified Mid1p and Cch1p as factors required for Ca^{2+} influx and survival of cells responding to α -factor (IIDA *et al.* 1994; FISCHER *et al.* 1997; PAIDHUNGAT and GARRETT 1997). We have confirmed these results and demonstrated that both proteins function in the same pathway as Ca^{2+} /calmodulin in the minimal SD-100 medium (Figure 1). Cch1p/Mid1p (HACS) activity was strongly stimulated by the response to α -factor plus FK506 after a lag time of ~ 45 min (Figures 5 and 6 and data not shown). The abundance of Cch1p and Mid1p was unchanged in these conditions (Figure 8), consistent with prior analysis of the transcripts from the *CCH1* and *MID1* genes (IIDA *et al.* 1994; ROBERTS *et al.* 2000). Nevertheless, we determined that activation of the Ste12p transcription factor was necessary and sufficient to stimulate HACS activity as long as calcineurin was inhibited or inactivated by mutation. For example, *dig1 dig2* double mutants responded rapidly to FK506 with increased $^{45}\text{Ca}^{2+}$ accumulation through a process dependent on Ste12p, Cch1p, and Mid1p (Figure 7).

The targets of Ste12p involved in HACS activation have not yet been identified. On the basis of the hypothesis that calcineurin inhibits some step in HACS activation, we speculated that a protein kinase may be the critical target of Ste12p. Of the 124 genes in yeast predicted to encode protein kinases, only *FUS3* and *KSSI*

were significantly induced in *dig1 dig2* double mutants (ROBERTS *et al.* 2000). However, we found that FK506 stimulated $^{45}\text{Ca}^{2+}$ accumulation in *dig1 dig2 fus3 kss1* quadruple mutants normally (data not shown), so these kinases are not likely to oppose calcineurin in the regulation of HACS. This experiment also reaffirms other data shown here that factors upstream of Ste12p in the pheromone-signaling cascade are not required for HACS stimulation as long as Ste12p is active. Additional studies will be required to determine the mechanisms by which Ste12p and calcineurin regulate HACS.

Expression of Mid1p alone in human embryonic kidney cells resulted in the appearance of Ca^{2+} -permeable stretch-activated channel activity (KANZAKI *et al.* 1999), suggesting Mid1p might function alone as an ion channel. Mid1p and Cch1p colocalize to the plasma membrane in yeast cells and can be co-immunoprecipitated from solubilized cell extracts (KANZAKI *et al.* 1999; LOCKE *et al.* 2000). Additionally, overexpression of Mid1p in yeast did not alter the rates of Ca^{2+} accumulation with or without α -factor (IIDA *et al.* 1994). Because *cch1* and *mid1* single mutants exhibit phenotypes quantitatively identical to *cch1 mid1* double mutants in a variety of contexts (FISCHER *et al.* 1997; PAIDHUNGAT and GARRETT 1997; LOCKE *et al.* 2000), we suggest that the two proteins comprise two essential subunits of HACS that independently have no significant Ca^{2+} transport activity in yeast.

The role of Ca^{2+} in mating and the pheromone response: Our findings support a model where yeast cells growing in complete or rich media primarily employ LACS to generate calcium signals during the pheromone response. LACS activation was sufficient to stimulate calcineurin-dependent gene expression and presumably calcineurin-dependent inhibition of HACS. If LACS activity were insufficient to activate calcineurin, HACS activity might become more obvious and more significant physiologically. HACS activity alone in *dig1 dig2* double mutants treated with FK506 was sufficient to elevate $[\text{Ca}^{2+}]_c$ (Figure 7) and presumably to stimulate calcium signaling pathways. Therefore, LACS and HACS may simply represent a means of activating calcium signaling pathways over a broader range of environmental conditions. Alternatively, HACS and LACS activation may be spatially or temporally separated in the responding cell leading to distinct types of responses. Cch1p and Mid1p localize uniformly to the plasma membrane of vegetative and shmooing cells (KANZAKI *et al.* 1999; LOCKE *et al.* 2000) and therefore may contribute to calcium signaling processes in both the cell body as well as the morphological projections of shmooing cells. The differential regulation of LACS and HACS by distinct branches of the pheromone-signaling pathway may lead to subtle differences in their physiological contributions. However, LACS and HACS activation after α -factor treatment exhibited identical kinetics and sensitivity to α -factor doses (data not shown). Finally, the distinct

properties of the two Ca²⁺ influx activities may cause different effects, as observed previously in neurons (BADING *et al.* 1993; HARDINGHAM *et al.* 1998) and sperm cells (DARSZON *et al.* 1999; WASSARMAN 1999). That yeast cells employ two separate Ca²⁺ influx activities during the pheromone response suggests an important role for calcium signaling in controlling downstream events.

What are the roles of calcium signaling during the pheromone response? Yeast cells responding to α -factor tend to survive for longer periods of time when sufficient Ca²⁺ is present in the environment and when Cch1p, Mid1p, calmodulin, calmodulin-dependent protein kinases, and calcineurin are all functional (IIDA *et al.* 1990, 1994; MOSER *et al.* 1996; WITHEE *et al.* 1997). However, the critical targets of the calmodulin-regulated enzymes responsible for promoting cell survival have not been completely defined. Calcineurin activates the transcription factor Tcn1p (also known as Crz1p and Hal8p), which then induces expression of a number of genes involved in ion homeostasis and cell wall biogenesis (MATHEOS *et al.* 1997; STATHOPOULOS and CYERT 1997; MENDIZABAL *et al.* 1998). Tcn1p overexpression partially suppresses the survival defect of calcineurin-deficient mutants and *tcn1* mutants exhibit a slight survival defect (STATHOPOULOS and CYERT 1997), suggesting that targets of Tcn1p at least partially promote survival. Many of these targets of Tcn1p were also induced following α -factor treatment, particularly in a later wave of transcription that was absent in *bmi1* mutants (ROBERTS *et al.* 2000). It is not yet clear which of these targets are important for survival during the pheromone response.

A number of new questions are raised by this study. What are the targets of Ste12p and how do they activate HACS? How does calcineurin regulate HACS activity? What are the components of LACS and how are they regulated by cell morphogenesis factors? The complete sequence of the yeast genome does not reveal any gene products with obvious similarity to the presently known families of Ca²⁺ channels, except for Cch1p, the yeast homolog of voltage-gated Ca²⁺ channels. However, it seems likely that not all families of Ca²⁺ influx channels have been identified. Answers to these questions may define a new family of Ca²⁺ channels conserved in other species and produce new insight into mechanisms of ion channel regulation.

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