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MINIREVIEW

Components of the Calcium-Calcineurin Signaling Pathway in Fungal Cells and Their Potential as Antifungal Targets

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In recent years, the emergence of fungal resistance has become frequent, partly due to the widespread clinical use of fluconazole, which is minimally toxic and effective in the prevention and treatment of *Candida albicans* infections. The limited selection of antifungal drugs for clinical fungal infection therapy has prompted us to search for new antifungal drug targets. Calcium, which acts as the second messenger in both mammals and fungi, plays a direct role in controlling the expression patterns of its signaling systems and has important roles in cell survival. In addition, calcium and some of the components, mainly calcineurin, in the fungal calcium signaling pathway mediate fungal resistance to antifungal drugs. Therefore, an overview of the components of the fungal calcium-calcineurin signaling network and their potential roles as antifungal targets is urgently needed. The calcium-calcineurin signaling pathway consists of various channels, transporters, pumps, and other proteins or enzymes. Many transcriptional profiles have indicated that mutant strains that lack some of these components are sensitized to fluconazole or other antifungal drugs. In addition, many researchers have identified efficient compounds that exhibit antifungal activity by themselves or in combination with antifungal drugs by targeting some of the components in the fungal calcium-calcineurin signaling pathway. This targeting disrupts Ca^{2+} homeostasis, which suggests that this pathway contains potential targets for the development of new antifungal drugs.

Invasive fungal infections have become frequent in severely immunocompromised individuals, such as transplant, cancer chemotherapy, and HIV-infected patients (1, 2). *Candida* spp., *Aspergillus* spp., and *Cryptococcus* spp. are the most pervasive fungal pathogens isolated in invasive fungal infections (3–8). In contrast with bacterial infections, many of which can be treated with multiple classes of antibiotics, the therapeutic options for fungal infections are exceedingly insufficient due to the limited number of antifungal drugs available and their potential toxicity (9, 10). Azoles, especially fluconazole, have frequently been used in clinical practice due to their great efficacy in the prevention and treatment of *Candida albicans* infections and their reduced toxicity, but their use results in the emergence of drug resistance. Moreover, innately resistant species, such as non-*albicans Candida* species, are increasingly being isolated, which is a serious problem in the fight against fungal infections (11–13). Therefore, new antifungal drugs or new approaches for coping with invasive fungal infections are urgently needed (14). However, the development of brand-new antifungal drugs is time consuming and costly. Moreover, fungal cells are eukaryotic, and they share the conserved biochemical and molecular biological networks of all eukaryotes, which complicates the identification of fungal-specific targets that are essential for fungal cell growth (9). Thus, antifungal agents with novel modes of action, such as targeting the virulence, filamentation, and biofilm formation of pathogenic fungi, are urgently needed (10).

In recent years, calcium signal transduction in fungi has been the focus of extensive study due to its essential role in the survival of fungi (15–17). One of the regulators of calcium homeostasis, calcineurin (CN), has been identified as a virulence factor in filamentous fungi, and some calcium channel proteins have been found to be responsible for the filamentation of these pathogenic fungi (18–21). Moreover, calcium-mediated and calcineurin-mediated azole resistance has frequently been documented (22–24). Many findings indicate that various components of the calcium signaling pathway play important roles in fungal physiological processes, mediate stress responses, and promote virulence (22, 25). There are also many reports documenting that nonantifungal compounds, such as amiodarone, cyclosporine (CsA), tacrolimus (FK506), the estrogen receptor antagonists tamoxifen and toremifene, and some calcium channel blockers, exhibit antifungal activity alone or in combination with antifungal drugs through interference with the functions of these components. Although some of the components in fungal cells are similar to those of mammalian cells, the subtle structural differences have made them a hot area in the development of new antifungal agents or research into new approaches to resisting invasive fungal infections (26). However, the calcium channels, exchangers, pumps, and downstream signaling components involved in this complex system of fungal cells are not fully understood. Therefore, reviewing the calcium signaling pathway and its regulatory mechanisms is important. The budding yeast *Saccharomyces cerevisiae* is among the simplest eukaryotic organisms that are widely used as valuable tools for the study of basic cellular processes and pathways. Fur-

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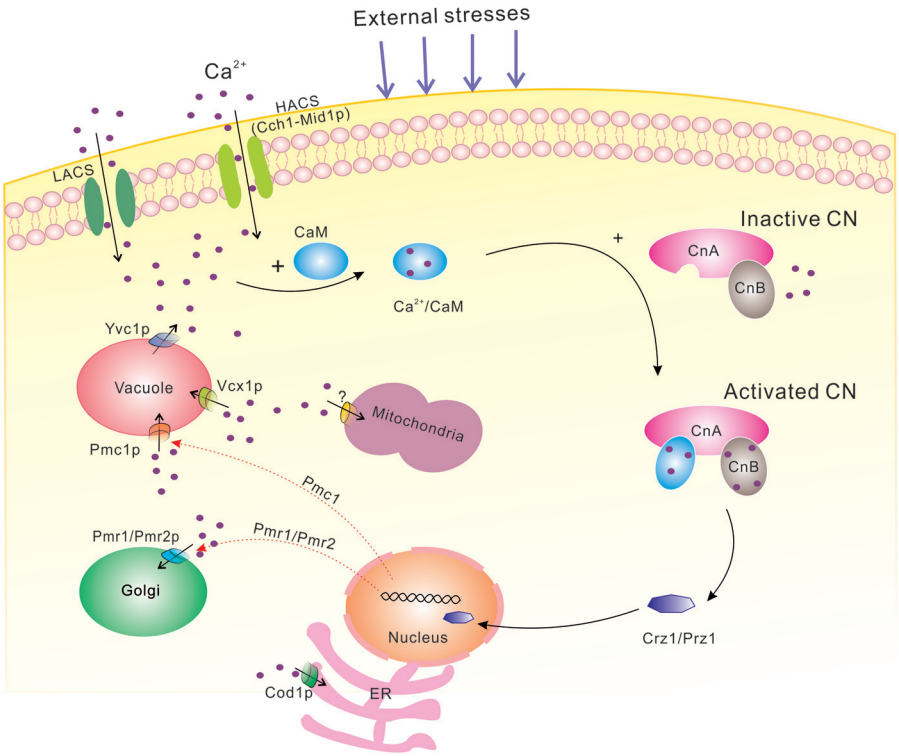

FIG 1 Description of the calcium-calcineurin signaling pathway in fungal cells. When external stresses are encountered, the plasma membrane Ca^{2+} influx system (HACS and LACS) is activated, resulting in a rapid influx of Ca^{2+}. Transient increases in intracellular Ca^{2+} concentrations may also be due to secretion from internal compartments. The increased Ca^{2+} concentrations are sensed by CaM, and three calcium ions bind to CaM; then, Ca^{2+}-calmodulin specifically binds to subunit A of CN and, simultaneously, Ca^{2+} binds to the high-affinity Ca^{2+}-binding sites on the B subunit of CN, leading to its activation. Activated CN acts on its downstream targets *CRZ1* and *PRZ1*, inducing their dephosphorylation and translocation from cytoplasm to nucleus. Calcineurin-*PRZ1/CRZ1* signaling induces the expression of a set of Ca^{2+}/CN-dependent target genes, including *PMC1*, *PMR1*, and *PMR2*. Subsequently, the intracellular Ca^{2+} concentration is reduced to basal levels, attributed to the uptake of Ca^{2+} by organelles. CaM, calmodulin; CN, calcineurin; ER, endoplasmic reticulum; LACS, low-affinity Ca^{2+} influx system; HACS, high-affinity Ca^{2+} influx system.

thermore, this yeast is an excellent organism for the identification of molecular targets and elucidation of the molecular/cellular mechanisms of sensitivity to various drugs because the major signaling pathways and processes involved in the cellular response to cytotoxic agents are conserved between yeasts and mammalian cells (27). Here, we mainly describe the latest findings concerning the genes, proteins, and enzymes involved in the calcium signaling pathway of *Saccharomyces cerevisiae*, which is the main yeast model (the calcium-calcineurin signaling pathway is depicted in Fig. 1). The relationship between calcium signaling and fungal cell survival is then analyzed, with the findings implicating a close connection between calcium signaling and fungal resistance. Next, we summarize the compounds that exhibit antifungal activity when used alone or in combination with antifungal drugs by interfering with components in the calcium signaling pathway (Fig. 2). Although some compounds with antifungal activity also show defined effects on mammalian cells, e.g., calcium channel blockers, they have safely been used in the clinic, and the study of their antifungal mechanisms could provide new clues for the identification of drugs with greater fungal specificity or new antifungal targets.

CALCIUM SIGNALING PATHWAY IN FUNGAL CELLS

Intracellular calcium ions (Ca^{2+}) are important second messengers in all organisms. The concentrations of cytosolic Ca^{2+} are very low at resting states, ranging from 50 to 200 nM in fungal cells when the environmental Ca^{2+} concentrations range from <1 μM to >100 mM (26, 28, 29). The calcium homeostasis system, which consists of various calcium channels and pumps, as well as many related proteins and enzymes, plays an important role in maintaining the optimal Ca^{2+} concentrations in the cytosol and intracellular compartments, such as the vacuole, endoplasmic reticulum (ER), and Golgi apparatus (29–31). In general, the plasma membrane Ca^{2+} influx system is activated to result in a rapid influx of Ca^{2+} ions in response to various external stresses, such as store-operated stress, hyperosmotic stress, alkaline stress, cold stress, thermal stress, oxidative stress, and ethanol stress (32–37). The transient increases in the intracellular Ca^{2+} concentrations may also be elevated by secreting Ca^{2+} from internal compartments (38). An increased Ca^{2+} concentration in yeast and filamentous fungal cells affects a wide range of cellular processes, such as cell cycle progression, sporulation, spore germination, oriented hyphal tip growth, hyphal branching, gene expression, and circadian rhythms. This increased concentration also modulates signaling cascades and activates the calcineurin pathway to reduce the Ca^{2+} concentration to the basal level (36, 39). However, the decrease of the intracellular Ca^{2+} concentration due to the inhibition of the Ca^{2+} influx system or efflux of Ca^{2+} from intracellular compartments to the extracellular space is less well documented. Therefore, we mainly discuss the Ca^{2+} influx system, the

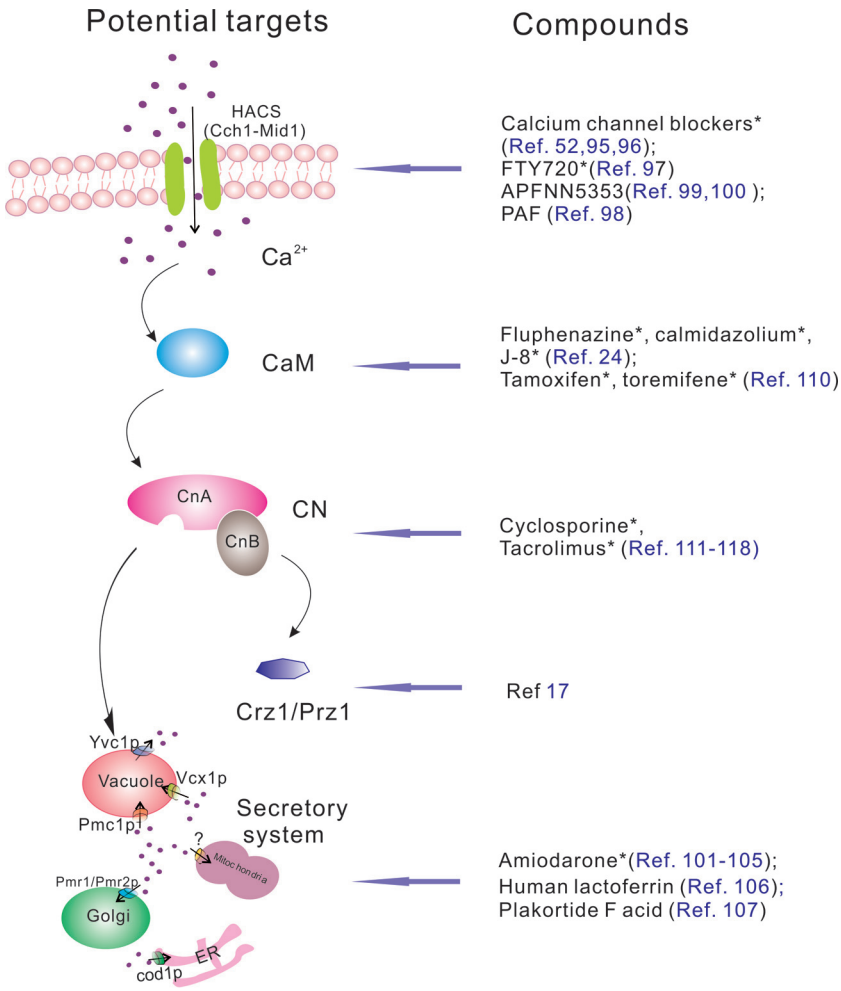

FIG 2 Potential targets in the calcium-calcineurin signaling pathway and some compounds that exhibit antifungal activity by themselves or in combination with antifungal drugs through interference with these potential targets. CaM, calmodulin; CN, calcineurin; ER, endoplasmic reticulum; *, compound is in clinical therapeutic use.

secretory Ca^{2+} system, and the calcineurin cascades of calcium signaling transduction to characterize the calcium signaling pathway.

CALCIUM INFLUX SYSTEM ON CELL MEMBRANE

Most fungal plasma membranes contain at least two different Ca^{2+} influx systems, the high-affinity Ca^{2+} influx system (HACS) and the low-affinity Ca^{2+} influx system (LACS) (40). The HACS consists of two putative proteins, Cch1p and Mid1p, which are expressed and colocalize to the plasma membrane in a variety of fungi, such as the saprophytes *Schizosaccharomyces pombe* and *Neurospora crassa* (41, 42), the animal-pathogenic fungi *Candida albicans* and *Cryptococcus neoformans* (43–45), and the plant-pathogenic fungi *Gibberella zeae*, *Claviceps purpurea*, and *Uromyces appendiculatus* (46–49). Notably, *Aspergillus* species (50, 51) express the putative Ca^{2+} channel homologs of *CCH1* and *MID1*, namely, *cchA* and *midA*, whose topology is similar to the overall topology of Ca^{2+} voltage-gated channels in higher eukaryotes. These channels play unique and complex roles in low-calcium environments. In *Saccharomyces cerevisiae*, the sequence and topological structure of Cch1p are similar to those of the pore-forming *a*1 subunit of mammalian L-type voltage-gated Ca^{2+} channels (VGCCs) (52), and Mid1p was suggested to be analogous to the *a*2δ subunit of animal VGCCs because of structural features like N glycosylation, a cysteine-rich domain, and a putative N-terminal signal peptide (53). These two essential subunits form a stable complex that is activated in response to sudden stimulation, allowing the influx of Ca^{2+} from the extracellular space (20). More importantly, both proteins have been shown to be indispensable for the uptake of extracellular Ca^{2+} in cells that respond to mating pheromones (54, 55). HACS was found to be regulated by Ecm7p, a member of the PMP-22/EMP/MP20/Claudin superfamily of transmembrane proteins that includes the λ subunits of VGCCs (53). *ECM7* is stabilized by *MID1*, and *MID1* is stabilized by *CCH1* in nonsignaling conditions, suggesting that all of these proteins interact. Moreover, the *ecm7*Δ/Δ mutants of *Candida albicans* were shown to be sensitive to oxidative stress, which resulted in a defect in hyphal development and attenuated the ability of yeast cells to invade and diffuse in mouse kidneys compared with the phenotype of the wild-type strain (20). LACS, whose main important component or regulator is Fig1p (22), displays a 16-fold-lower affinity for Ca^{2+} than HACS does (40). Many other factors

related to polarized morphogenesis and cell fusion, such as Fus1p, Fus2p, Rvs161p, Bni1p, Spa2p, and Pea2p, were also found to be necessary for LACS activity (22). LACS can reportedly produce robust calcium signals in response to pheromones via *cch1* and *mid1* double mutants that lack HACS. However, LACS and HACS are required for hyphal orientation in response to electric fields and surface topography (21). LACS appears to function only in rich media, such as yeast extract-peptone-dextrose (YPD) or synthetic complete (SC) media, and is insensitive to calcineurin, while HACS is almost undetectable in rich media due to feedback inhibition by calcineurin (56).

Ca^{2+} SECRETORY SYSTEM ON ENDOMEMBRANE

Like animal cells, fungal cells employ a compartmentalized secretory system that contains numerous Ca^{2+}-dependent proteins and enzymes, such as channels, transporters, or pumps, on the vacuole, Golgi apparatus, mitochondria, and endoplasmic reticulum (ER). The vacuole, an important compartment in fungi, is crucial for differentiation, adaptation to stress, endocytosis, autophagy, and pathogenesis (57). Moreover, this compartment, not the ER, is the major site of intracellular calcium storage in fungal cells (58, 59). Vacuolar calcium channels, which are similar to IP3 or ryanodine receptors in the mammalian ER membrane, are undoubtedly involved in cellular calcium homeostasis and cell response to an environmental stimulus.

Yvc1p, the transient receptor potential (TRP) homolog, has been identified as a vacuole membrane-localized calcium channel protein in some eukaryotic cells (15, 60, 61). Just like the *CCH1-MID1* complex, *YVC1* also mediates calcium transport and contributes to cytoplasmic calcium fluctuation by releasing calcium from the vacuole into the cytoplasm as a response to an alkaline stimulus (15). However, a sustained increase in the cytosolic Ca^{2+} concentration is detrimental to fungal cells. Numerous enzymes that catalyze the folding, modification, processing, and trafficking of secretory proteins are activated in response to stress, which results in Ca^{2+} sequestration in secretory organelles or the initiation of other restorative pathways. Specifically, the Ca^{2+}-ATPase Pmc1p and H^+/Ca^{2+} exchanger Vcx1p, which are located on the vacuole membrane, and the Ca^{2+} pumps Pmr1p, Cod1p, and Eca1p, which are predominantly located on the Golgi apparatus and ER, are activated to direct the cytosolic Ca^{2+} to secretory organelles, such as the vacuole, Golgi apparatus, and ER (31, 58, 62–65). However, Vcx1p was identified as the protein complex that is predominantly responsible for restoring cytosolic Ca^{2+} concentrations after a brief challenge with high extracellular Ca^{2+} concentrations, while Pmc1p appears to be critical for long-term Ca^{2+} tolerance (59). Furthermore, most cells express Ca^{2+} release channels in the endoplasmic reticulum that can be activated by secondary messengers during responses to extracellular stimuli. Rapid Ca^{2+} release lowers the Ca^{2+} concentration in the endoplasmic reticulum and elevates the free Ca^{2+} concentrations in the cytosol, which then can activate various signaling transduction pathways. Because Ca^{2+} pumps in the plasma membrane compete with secretory organelle pumps for substrates, the intracellular Ca^{2+} concentration can return to basal levels prior to the refilling of secretory compartments. Thus, in the absence of Ca^{2+} influx into the cell, the repetitive or continuous activation of Ca^{2+} release channels will only transiently elevate the intracellular Ca^{2+} and result in the sustained depletion of the secretory Ca^{2+} reservoir.

CALCIUM-REGULATING PROTEINS IN THE CYTOPLASM

Calcineurin (CN) is a Ca^{2+}/calmodulin (CaM)-activated protein phosphatase that is highly conserved from fungi to mammals (66) and transmits Ca^{2+} signals to elicit downstream responses mainly by regulating various transcriptional factors, such as *CRZ1* and *PRZ1* (19, 67, 68). CN consists of two subunits, a catalytic subunit A (encoded by *CNA1* and *CNA2*) (66, 69) and a regulatory subunit B (encoded by *CNB1*) (70). The A subunit contains a central calmodulin-binding domain, and the B subunit is identified as a dumbbell-shaped protein with four EF hands, which serve as the high-affinity Ca^{2+}-binding site (71, 72). These two subunits are tightly associated via hydrophobic interactions at a 1:1 ratio in an inactive state (71). However, the Ca^{2+} sensor protein calmodulin (CaM) detects increases in the intracellular Ca^{2+} concentrations and binds cytosolic Ca^{2+} ions at EF-hand motifs of CaM to subsequently activate several Ca^{2+}/CaM-dependent enzymes, such as the phosphatase calcineurin (73). Activated calcineurin acts on its downstream targets *CRZ1*/*TCN1*, *PRZ1*, and other *CRZ1* orthologues, which are C_2H_2-type zinc finger transcription factors, inducing their dephosphorylation and translocation from the cytoplasm to the nucleus (67, 74, 75). Calcineurin-*PRZ1/CRZ1* signaling then induces the expression of a set of Ca^{2+}/CN-dependent target genes, including the Ca^{2+}-ATPase genes *PMC1*, *PMR1*, and *PMR2* (encoding Ca^{2+}-pumping ATPases in the vacuole and Golgi complex) and the glucan synthase gene *FKS2*, by binding to CN-dependent responsive elements. This signaling also strongly inhibits the function of Vcx1p (74, 76–79). Subsequently, the intracellular Ca^{2+} level is reduced to the basal level due to the uptake of Ca^{2+} by organelles and the inhibition of Ca^{2+} release from the vacuole. While this set of calcineurin targets generally seems to be coordinately regulated, the authors of another report (80) demonstrate that a deletion mutation of any of the components *SNF7*, *SNF8*, *STP22*, *VPS20*, *VPS25*, *VPS28*, and *VPS36* of the endosomal sorting complex required for transport (ESCRT) complex activates Ca^{2+}/CN signaling in yeast cells but, surprisingly, reduces the expression of the ER/Golgi calcium pump gene *PMR1* by nearly half, independent of calcium stress. Although this finding seems to contradict the well-known fact that $Ca2^+$/CN signaling positively regulates Pmr1, it is consistent with the important role of *PMR1*, together with *PMC1*, in preventing the lethal activation of calcineurin under standard (low-Ca^{2+}) conditions (81).

THE ROLES OF CALCIUM SIGNALING IN FUNGAL CELL SURVIVAL

The survival of all organisms depends critically on their interactions with their environment, which are mediated largely by the actions of small molecules, such as reprogramming of gene expression, dephosphorylation of calcineurin, unfolded protein response (UPR), etc. (82, 83). A calcium cell survival (CCS) pathway may be involved in the survival of cells subjected to a variety of cellular stresses, because the activation of a variety of Ca^{2+} channels, calmodulin, calcineurin, and other factors is necessary for the long-term survival of cells undergoing ER stress, and the genes involved in this pathway are known to be essential in many cell biological processes; these essential components of the whole pathway would likely make good targets for antifungal therapy (19, 56, 84–86).

The major calcium influx system components *CCH1* and *MID1* have been identified as important factors in the survival of

many fungi (45, 48, 50, 87). Previous reports have demonstrated that *CCH1* and *MID1* are responsible for the resistance of *Candida albicans* to azoles, as the deletion of the *CCH1* and *MID1* genes attenuated the strain's resistance to fluconazole and itraconazole, and the *cch1*Δ/Δ or *mid1*Δ/Δ mouse models displayed attenuated virulence (85). Moreover, hypha formation and maintenance defects, as well as sensitivity to oxidant agents, were identified in the mutant strains, which demonstrates that *CCH1* and *MID1* play important roles in morphogenesis, the oxidative stress response, and virulence in *Candida albicans* (86, 87). *CCH1* also plays a role in mediating the virulence of *C. neoformans* and is required for the growth of *C. neoformans* at low extracellular Ca^{2+} concentrations, especially at mammalian body temperatures (45). In aspergilli, the homologs of *CCH1* and *MID1*, *cchA* and *midA*, not only have the functional benefits of fast growth but also play important roles in calcium homeostasis and virulence (50, 51).

The components of the Ca^{2+} secretory system in fungal cells also play critical roles in fungal survival, virulence, and infections. Many reports have demonstrated that *VCX1*, *YVC1*, *PMC1*, and *PMR1* are involved in the tolerance and virulence of a variety of fungi, such as *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus* (51, 88, 89).

Moreover, calcineurin has been demonstrated to be essential for the survival of *Candida* spp. and required for virulence and stress responses in many other major fungi (18, 84, 90, 91). Zhang et al. found that calcineurin and its downstream target *CRZ1* were responsible for *Candida lusitaniae*'s pseudohyphal growth, cell wall integrity, ER stress response, optimal growth in serum, virulence in a murine systemic infection model, and antifungal drug tolerance (19). Another study also demonstrated that the activation of the Ca^{2+} channel, calmodulin, calcineurin, and other factors was necessary for the long-term survival of cells undergoing ER stress (92). When treated with tunicamycin (TM), an inhibitor of N glycosylation in the ER, yeast strains lacking Cch1p, Mid1p, Ca^{2+}/calmodulin (Cmd1-6 mutants), Ca^{2+}/calmodulin-dependent protein kinases (*CMK1 CMK2* double mutants), and calcineurin (*CNB1* mutants) died rapidly, whereas mutants lacking the calcineurin-dependent transcription factor Tcn1p behaved similarly to wild-type cells, remaining fully viable for long periods of time in survival assays. Moreover, the calcineurin-binding protein CBP1 and calmodulin direct the morphogenesis and high-temperature growth of *Cryptococcus neoformans* (36, 93). These findings demonstrated that calcium factors such as Cch1p, Mid1p, calmodulin, and calcineurin promote the long-term survival of cells that suffer ER stress. Furthermore, the authors also demonstrated that the CCS pathway is responsible for the resistance to azole antifungal drugs and operates in pathogenic fungi, such as *C. albicans* and *Candida glabrata*.

The CCS pathway is so closely related to the survival of fungal cells that its presence in fungi may provide new opportunities for the treatment of fungal infections.

POTENTIAL ANTIFUNGAL TARGETS IN FUNGAL CALCIUM SIGNALING PATHWAY

Calcium is a highly versatile intracellular signal that can regulate many different cellular functions, such as cell differentiation, division, cell-cell fusion, endocytosis, and mating morphogenesis (94). Therefore, the balance of the flows between the extracellular and intracellular stores that constitute the cytoplasmic concentration of Ca^{2+} must be maintained at close to 0.1 mM to ensure normal intracellular signaling transduction. Small increases in the intracellular calcium concentration can trigger a variety of cellular responses, such as the activation of pathways that control ion channel activity, secretion, and gene transcription. However, larger, sustained increases can be deleterious to cells and may cause cell death, which highlights the Ca^{2+}-mediated cell death pathway as a promising approach to antifungal drug development. Many of these findings suggest that specific inhibitors of fungal Ca^{2+} channels, such calmodulin, calcineurin, or other as-yet-unknown components of the CCS pathway, could greatly improve the efficacy of existing antifungal therapies.

TARGETS IN FUNGAL MEMBRANE SYSTEM

The concentration of calcium in fungal cells may increase in response to external or internal stresses, leading to a variety of intracellular responses, such as the opening of calcium channels and exchangers on the plasma membrane or endomembrane system. These calcium channels or exchangers and their genes contribute significantly to the cytosolic calcium concentration fluctuation, and the deletion of some calcium signaling components is detrimental to fungal survival. Thus, interfering with the influx or uptake of calcium through the channels or transporters to disturb the calcium homeostasis may benefit fungicidal activity.

INTERFERENCE WITH CALCIUM INFLUX SYSTEM ON CELL MEMBRANE

Calcium channel blockers, which exert their functions by inhibiting the VGCC on the plasma membrane of mammalian cells, have been the intensive focus of research that examines genes related to the fungal Ca^{2+} influx system because of the homology of calcium channels in fungi and mammals. For example, the *CCH1-MID1* complex is similar to the VGCC of mammals in structure, and reports confirm that it is sensitive to the L-type VGCC blockers nifedipine and verapamil, which decrease the Ca^{2+} concentration. However, another L-type VGCC blocker, diltiazem, activates Ca^{2+} entry (52). Fewer reports have documented the antifungal effect of calcium channel blockers used alone, whereas verapamil has been shown to inhibit *Candida albicans*' hyphal development, adhesion, and gastrointestinal tract colonization, which is related to decreased expression and abnormal transport of the proteins required for morphogenesis (95). Verapamil and fluconazole or tunicamycin have also been observed to exert combined effects, and the results of these previous studies demonstrate synergistic effects on the inhibition of the formation of *Candida albicans* biofilm. Furthermore, verapamil alone or in combination with fluconazole or tunicamycin significantly decreased the transcriptional level of ALS3, which is essential for biofilm development (96). Because verapamil exerts its inhibitory effects on the plasma membrane calcium channel CCH1 in *Saccharomyces cerevisiae* (52), calcium channel blockers may be attractive targets for the prevention or eradication of *Candida albicans* biofilm. Therefore, further studies to observe the effects of other calcium channel blockers used alone or in combination with azole antifungal drugs are needed.

Hagihara et al. (97) provided insight into the molecular mechanisms of fingolimod hydrochloride (FTY720), which is a novel sphingosine 1-phosphate (S1P) receptor modulator that acts on Ca^{2+} signaling in fission yeast. FTY720 induced a dose-dependent increase in the cytoplasmic Ca^{2+} levels immediately after the addition of FTY720. This effect was due to an influx of Ca^{2+} across

the Ca^{2+} machinery on the plasma membrane that is involved in Ca^{2+} entry, because the addition of EGTA (extracellular Ca^{2+} chelator) inhibited the peak responses and increased the cytoplasmic Ca^{2+} levels via FTY720. In addition to the agents discussed above, some secreted antifungal proteins have been reported to disrupt Ca^{2+} homeostasis by increasing the intracellular Ca^{2+} concentration. These agents inhibited the growth of a broad range of filamentous fungi (98). PAF is secreted from *Penicillium chrysogenum*, and it belongs to a family of antifungal peptides. Its elevation of the intracellular Ca^{2+} resting level within the conidial germ is primarily due to the influx of extracellular Ca^{2+}. Because Ca^{2+} is a selective chelator, BAPTA [bis-(aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] ameliorated the PAF toxicity in growth inhibition assays and counteracted the PAF-induced perturbation of Ca^{2+} homeostasis (98). In contrast, the effects of the L-type Ca^{2+} channel blocker diltiazem on the response of cells were analyzed, and the results demonstrate that diltiazem and PAF disrupt Ca^{2+} homeostasis in a similar manner. Moreover, combining diltiazem and PAF had an additive effect on the growth inhibition and change in Ca^{2+} signatures in response to external stimuli. However, experiments with an aequorin-expressing Δ*cch1* deletion strain of *N. crassa* indicated that the L-type Ca^{2+} channel CCH1 was not responsible for the observed PAF-induced elevation of the intracellular Ca^{2+} resting level. Thus, the specific mechanism of PAF in Ca^{2+} homeostasis disruption requires more research. Another antifungal protein, AFP_{NN5353}, which is a defensinlike protein of *Aspergillus giganteus*, has been examined (99, 100). This protein mediates the germination and growth of filamentous ascomycetes, including important human and plant pathogens, as well as the model organisms *Aspergillus nidulans* and *Aspergillus niger*, by inducing the rapid influx of extracellular Ca^{2+}. This influx eventually results in a loss of intracellular Ca^{2+} homeostasis, because the Ca^{2+}-selective, membrane-impermeable chelator BAPTA did not influence the resting level of intracellular Ca^{2+} in 12-h-old *A. niger* cultures, whereas a pretreatment of the samples with 10 mM BAPTA prior to the addition of AFP_{NN5353} inhibited the protein-specific increase in the intracellular Ca^{2+} resting level. These findings demonstrate that calcium signaling plays important roles in the mechanistic function of antifungal agents.

INTERFERENCE WITH THE CALCIUM SECRETORY SYSTEM ON THE ENDOMEMBRANE

The calcium secretory system also plays important roles in maintaining normal cytosol Ca^{2+} concentrations by releasing or sequestrating Ca^{2+} in a secretory Ca^{2+} reservoir. Therefore, agents that interfere with the secretory system may impair fungal cells. The antiarrhythmic drug amiodarone (AMD) has been shown to display potent fungicidal activity against not only *Saccharomyces* but also pathogenic yeasts, such as *Candida*, *Cryptococcus*, *Fusarium*, and *Aspergillus* species, by interfering with the channel proteins in the calcium secretory system (101–103). Mutants that lack key regulators of calcium homeostasis, including the secretory pathway Ca^{2+} pump Pmrlp, vacuolar H^+-ATPase, and Ca^{2+}/calmodulin-activated protein phosphatase calcineurin, were shown to be hypersensitive to amiodarone, which underlines the important role of Ca^{2+} in the cellular mechanism of amiodarone toxicity (104, 105). One report (106) indicates that Ca^{2+} uptake by the mitochondria and Ca^{2+} release from intracellular stores, such as vacuoles, are crucial in the candidacidal activity of human lactoferrin (hLF), which is an antimicrobial protein. Oxalate, which inhibits Ca^{2+} release from intracellular stores in various cell types, partially inhibited and a high Ca^{2+} level completely blocked the hLF-induced killing of *Candida albicans*. Moreover, ruthenium red interferes with the mitochondrial Ca^{2+} uniporter to inhibit mitochondrial Ca^{2+} uptake and block the peptide-induced killing of *Candida albicans*. However, the specific secretory stores have not been identified. In addition, ruthenium red, oxalate, high extracellular $CaCl_2$, and EGTA completely blocked the hLF-induced change in mitochondrial rhodamine 123 staining, suggesting that mitochondrial Ca^{2+} uptake and Ca^{2+} release from intracellular stores are essential for the hLF-induced changes in the mitochondrial membrane potential. Another marine-derived polyketide, endoperoxide plakortide F acid (PFA), was found to elicit a transcriptomic response indicative of a Ca^{2+} imbalance. This response affected the expression of genes known to be responsive to altered cellular calcium levels and strongly inhibited the opportunistic fungal pathogens *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (107). The authors showed that calcium transporters, including those with *pmr1/pmr1* and *pmc1/pmc1* mutations, were sensitive to PFA, and this finding agreed with the transcriptional response to PFA, which appears to be indicative of Ca^{2+} overload-related stress. In addition, *cch1*/*cch1*, *mid1*/*mid1*, *cna1*/*cna1*, *cna2*/*cna2*, *cnb1*/*cnb1*, and *crz1*/*crz1* mutants were all hypersensitive to PFA; Ca^{2+} deprivation in these mutants may result in a compensatory induction of the intracellular Ca^{2+} levels, and the Ca^{2+} regulation function deficiency of calcineurin mutants prevented a recovery to normal Ca^{2+} concentrations, which caused PFA to be more toxic under these conditions.

TARGETS IN FUNGAL CYTOPLASM SYSTEM

The calcium signaling transduction system includes enzymes and proteins, such as calmodulin, calcineurin, and the transcription factors encoded by *CRZl*/*TNC1* and *PRZ1*, that have been shown to be nonessential for normal growth but critical in mediating cell survival in response to stress (19, 84). Calcineurin-mediated resistance has been considered one of the important factors in the failure of clinical treatment of mycoses (18, 19, 23), and its activation is evoked by the calcium-binding protein calmodulin (73). Therefore, the inhibition of calmodulin and calcineurin activity in order to reverse antifungal resistance and increase the antifungal activity of existing antifungal drugs has been extensively studied.

INTERFERENCE WITH CALMODULIN

Calmodulin is a small calcium-binding protein that participates in the transduction of calcium ions to its effector proteins (108). An increase in the calcium concentration to approximately 10^{-5} M results in the binding of three calcium ions to fungal calmodulin (109). Ca^{2+}-calmodulin then specifically binds to calcineurin, leading to its activation to regulate the stress response (73). Therefore, preventing calmodulin from exerting its function may perturb Ca^{2+} homeostasis. Fortunately, Edlind et al. (24) verified this assumption. The authors observed the antifungal effects of three structurally distinct compounds known to be inhibitors of the Ca^{2+}-binding regulatory protein calmodulin: fluphenazine, calmidazolium, and J-8 (W-7 analogue). These three compounds exhibited little or no inhibitory activity of their own, but they all enhanced the activities of azole drugs (miconazole, itraconazole, and terbinafine), and this enhancement varied from 1.6- to >11-fold. To further confirm that these inhibitors are truly specific for

calmodulin, strains with calmodulin site-directed mutagenesis were constructed. The results demonstrated that calmodulin mutants showed increased sensitivity to miconazole, terbinafine, or itraconazole compared with the sensitivities of the parent strains. Recently, another report demonstrated that the estrogen receptor antagonists tamoxifen and toremifene exerted their anticryptococcal activity alone or in combination with fluconazole and amphotericin B by directly binding to the essential EF-hand protein calmodulin, which prevented calmodulin from binding to its well-characterized substrate calcineurin and blocked calcineurin activation (110). This finding indicated that calmodulin antagonism contributes to the antifungal activity of this scaffold. More studies that inhibit the function of calmodulin are needed to identify its influence on fungal cell survival in order to discover more efficient antifungal agents.

INTERFERENCE WITH CALCINEURIN

Calcineurin is a major protein phosphatase that is responsible for maintaining calcium homeostasis by activating downstream events, and calcineurin-mediated fungal resistance to fungicides constitutes cause for concern. Therefore, many studies have searched for antifungal agents by inhibiting the activation of calcineurin. An early study demonstrated that the immunosuppressants cyclosporine (CsA) and tacrolimus (FK506) could bind to the receptor cyclopilin (CyP) and FK506 binding protein (FKBP), respectively, in fungal cells and then interact with the regulatory subunit B of calcineurin (111) to exhibit antifungal activity against *Cryptococcus neoformans* (112, 113), which identified calcineurin as a novel antifungal drug target. Thus, screening for calcineurin inhibitors via different methods may be a new approach for the development of antifungal agents. Uesugi et al. (114) found that inhibiting the calcineurin pathway, such as via the addition of FK506 and CsA to the growth medium or the disruption of the *CNB1* and *CRZ1* genes in *S. cerevisiae*, confers tolerance to high-temperature stress on cells with a ubiquitin deletion mutation. Therefore, the authors screened approximately 800 methanol extracts from natural resources for compounds that could restore the growth inhibition of ubiquitin deletion mutant strains following high-temperature treatment and found that some diterpenoid compounds inhibited calcineurin, while their specific antifungal activities and mechanisms require further research. The calcineurin inhibitors FK506 and CsA not only showed antifungal effects when used alone, their combination with antifungal drugs like fluconazole, posaconazole, and itraconazole has also been proposed to treat calcineurin-mediated azole resistance due to their synergistic antifungal effects (115–118).

This fungicidal synergistic interaction deserves further study, as it may be a useful adjunct therapeutic strategy for mycoses. However, FK506 and cyclosporine are not only active *in vitro* against fungal cells but are also immunosuppressive in the host, which may limit their clinical therapeutic application. Thus, research on the differences between the calcineurins of fungal and mammalian cells is urgently needed to develop antifungal-specific drugs. Fortunately, Juvvadi et al. (119) have found a novel serine-proline-rich region (SPRR) that is evolutionarily conserved and unique to filamentous fungi but completely absent in human calcineurin. The SPRR appears to be required for the phosphorylation of calcineurin that enables it to be active and function well. This finding provides a clue for the development of innovative drugs to fight invasive fungi by harnessing this unique SPRR. In addition, some reports have demonstrated that *RTA2*, a potential stress-related gene that likely encodes a phospholipid translocase, is responsible for the emergence of calcineurin-mediated azole resistance and sphingoid long-chain base release in *Candida albicans* (23, 120). The sensitivity of *Candida albicans* to fluconazole was significantly reduced because calcium-activated calcineurin blocked the impairment of the plasma membrane by fluconazole via Rta2p. Thus, Rta2p may serve as the direct target of antifungal agents.

DISCUSSION AND CONCLUSION

Signaling molecules commonly play critical roles in mediating the cellular stress responses of fungal pathogens. Adverse stimuli activate cellular signaling that prompts fungal cells to respond and adapt to the environment. Calcium, which acts as a secondary messenger molecule, operates over a wide temperature range to regulate many different cellular processes in both fungi and mammals. Similar to mammalian cells, fungal calcium signaling tool kits consist of various signaling molecules that include sensors, such as calmodulin, effectors, such as calcineurin, and the downstream targets of calcineurin, such as *CRZ1* and *PRZ1*. Reports have demonstrated that calcium and calcineurin can mediate the drug resistance of invasive fungal strains. Thus, harnessing these stress responses via the pharmacological inhibition of signaling pathways may provide the foundation for new therapies that could enhance the efficacy of our limited clinically useful antifungal drugs or impede the evolution of antifungal resistance.

Many reports have documented compounds that exhibit antifungal activity alone or in conjunction with antifungal drugs by interfering with the functions of components in the calcium signaling pathway. Although some of these compounds and some combinations have been demonstrated not to be effective in clinical application, their mechanisms of action may provide clues for the search for fungal-specific targets from the calcium signaling pathway. To date, the regulation of Ca^{2+} homeostasis has not been well studied in all fungal cells, and further searches for safe fungal-specific calcium channel blockers are warranted. The development of biotechnology has allowed transcriptional profiling experiments coupled with genetic and biochemical analyses to be employed to gain insight into the mechanism of action of various antifungal agents, which will delineate the calcium signaling pathway.

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