Calcium signaling is involved in diverse cellular processes in fungi

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ABSTRACT
Calcium (Ca\(^{2+}\)) is a universal signalling molecule of life. The Ca\(^{2+}\) signalling is an evolutionarily conserved process from prokaryotes to eukaryotes. Ca\(^{2+}\) at high concentration is deleterious to the cell; therefore, cell maintains a low resting level of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). The resting [Ca\(^{2+}\)]\(_i\) is tightly regulated, and a transient increase of the [Ca\(^{2+}\)]\(_i\) initiates a signalling cascade in the cell. Ca\(^{2+}\) signalling plays an essential role in various processes, including growth, development, reproduction, tolerance to stress conditions, and virulence in fungi. In this review, we describe the evolutionary aspects of Ca\(^{2+}\) signalling and cell functions of major Ca\(^{2+}\) signalling proteins in different fungi.

Evolution of calcium as a unique signalling molecule

Since the evolution of life on earth, where water covers three-fourth of the surface, both calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)) have evolved as divalent cations with a difference in the ability to form a complex with water molecules. Ca\(^{2+}\) has formed when oxygen and neon fused with successive particles (Clapham 2007). Ca\(^{2+}\) has a high degree of hydration and can accommodate 6–8 water molecules (Williams 2006). Ca\(^{2+}\) binds less tightly to water than Mg\(^{2+}\) and can precipitate phosphate, which may be lethal to the cell (Clapham 2007). Ca\(^{2+}\) can easily interact with molecules of complex geometry like proteins due to its unique properties such as charge, ionic radius, polarisability, and hydration energy (Brini et al. 2013a, 2013b). Therefore, cell efficiently controls the Ca\(^{2+}\) level for its survival and signalling. Thus, Ca\(^{2+}\) has evolved as a universal signalling molecule, signifying its importance in the evolution of life that started about 3.5 billion years ago (Plattner and Verkhratsky 2013). From the time life evolved, ATP has emerged as the central molecule, which was responsible for the formation of DNA/RNA subsequently (Ponnampерuma et al. 1963; Galimov 2009); and ATP synthesis was dependent on low Ca\(^{2+}\) concentration (Verkhratsky and Parpura 2014). It is also possible that in the process of evolution, Ca\(^{2+}\) assisted in the stability of DNA or RNA molecules, which exist as primitive stable molecules for the evolution of life (Jaiswal 2001). Since the early days of bacteria and protozoan evolution, Ca\(^{2+}\) was considered as a molecule of cell signalling, much before it got established as a ubiquitous secondary messenger molecule in the eukaryotic system (Shemarova and Nesterov 2005; Case et al. 2007), and the evolution of proteins allows the messenger function (Williams 2006). Thus, Ca\(^{2+}\) has evolved as an essential signaling ion across the different forms of life (Plattner and Verkhratsky 2013). Due to its versatile role in cell signaling, Ca\(^{2+}\) is also considered as a molecule for life and death (Berridge 1998; Berridge et al. 1998).

Ca\(^{2+}\) concentration gradient is the main switch behind the Ca\(^{2+}\) signalling machinery

The Ca\(^{2+}\) concentration outside the cell is as high as 10\(^{-3}\) M (Chin and Means 2000), the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_j\)) at resting state is maintained at ~100 nM; therefore, cells maintain a more than 10,000-fold gradient across the plasma membrane (Berridge et al. 2003). Cells store excess Ca\(^{2+}\) in various intracellular stores, including endoplasmic reticulum (ER), mitochondria, and vacuoles (Cornelius and Nakashima 1987). In the endoplasmic reticulum, the Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{ER}\)) is maintained at several hundred μM. Besides, cells also need to maintain...
intracellular Ca\(^{2+}\) homoeostasis to avoid severe Ca\(^{2+}\) fluctuations and their effects (Berridge et al. 2003). Specific receptors and channels mediate the entry of Ca\(^{2+}\) across the plasma membrane in response to stimuli, including membrane depolarisation, mechanical stretch, and external agonists. The inositol 1,4,5-triphosphates receptors (IP\(_3\)R) and the ryanodine receptors (RyR) induces Ca\(^{2+}\) release from the internal stores (Mikoshiba and Hattori 2000; Zeng et al. 2003; Hamilton 2005). Several proteins, including the plasma membrane Ca\(^{2+}\)-ATPase (PMCA), the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), the Na\(^+\)/Ca\(^{2+}\) exchanger, and the mitochondrial uniporter are responsible for sequestering the excess Ca\(^{2+}\) from the cytosol by transporting Ca\(^{2+}\) either to external medium or into different cellular compartments (Berridge et al. 2003). Thus, in response to various stimuli, the Ca\(^{2+}\) signalling pathway is activated and causes expression of the specific target genes in the nucleus (Figure 1). In this review, we briefly describe major families of Ca\(^{2+}\) signalling proteins (Table 1) and their roles in cellular processes in different fungi.

**Phospholipases and their significance in Ca\(^{2+}\) signalling pathway in fungi**

Phospholipases are a diverse class of enzymes involved in hydrolysing membrane phospholipids, mainly glycerophospholipids (Köhler et al. 2006; Hong et al. 2016). Phospholipases act on membrane phospholipids to produce small lipophilic signalling molecules like free fatty...
| Organism                     | Genome Size (Mb) | Number of protein coding genes | Number of Calcium signaling genes | Subfamily                                                                 | References                                      |
|------------------------------|------------------|--------------------------------|----------------------------------|---------------------------------------------------------------------------|------------------------------------------------|
| Saccharomyces cerevisiae     | 12.1             | 5,596                          | 40                               | Ca²⁺ permeable channel (3), Ca²⁺/cation ATPases (5), Ca²⁺ exchanger (4), phospholipase C (2), CaM (1), Ca²⁺/CaM regulated proteins (25) | Zelter et al. 2004; Otero et al. 2010; Engel et al. 2014 |
| Schizosaccharomyces pombe    | 13.8             | 4,940                          | 18                               | Ca²⁺ permeable channel (8), CaM (2), Ca²⁺/cation ATPases (8), Phospholipase (1), Ca²⁺/CaM regulated proteins (4) | Takeda and Yamamoto 1987; Toda et al. 1993; Yoshida et al. 1994; Okorokova-Facanha et al. 2003; Wood et al. 2002; Cortés et al. 2004 |
| Aspergillus fumigatus        | 29.4             | 9,926                          | 13                               | Ca²⁺ permeable channel (3), Ca²⁺ exchanger (3), Ca²⁺/cation ATPases (3), Phospholipase (3), CaM (1) | Birch et al. 1996; Nieman et al. 2005; Dinamarco et al. 2012; de Castro et al. 2014 |
| Beauveria bassiana           | 33.7             | 10,366                         | 21                               | Ca²⁺ permeable channel (8), Ca²⁺/cation ATPases (6), Ca²⁺ exchanger (5), CaM (1), Ca²⁺/CaMK (2)Ca²⁺/CaM regulated proteins (4) | Xiao et al. 2012; Fan et al. 2012; Li et al. 2015 |
| Candida albicans             | 14               | 6,100                          | 11                               | Ca²⁺/cation ATPases (1), Ca²⁺ permeable channel (4), CaMK (1), CaM (1), Ca²⁺/H⁺ exchanger (1), Phospholipase (3) | Bennett et al. 1998; Andaluz et al. 2001; Jones et al. 2004; Luna-Tapia et al. 2019 |
| Cryptococcus neoformans      | 19               | 6,572                          | 40                               | Ca²⁺ permeable channel (3), Ca²⁺/cation ATPases (6), Ca²⁺ exchanger (3), phospholipase C (2), CaM (1), Ca²⁺/CaM regulated proteins (21), Ca²⁺/CaMK (4) | Odom et al. 1997; Kmetzsch et al. 2011; Bahn and Jung 2013; Lee et al. 2016 |
| Fusarium oxysporum           | 59.9             | 17,735                         | 4                                | Ca²⁺ Permeable channel (3), Phospholipase (1) | Ma et al. 2010; Su et al. 2017 |
| Magnaporthe grisea           | 40.3             | 11,109                         | 42                               | Ca²⁺ Permeable channel (3), Ca²⁺/cation ATPases (12), Ca²⁺ exchanger (6), phospholipase C (4), CaM (1), Ca²⁺/CaM regulated proteins (16) | Zelter et al. 2004; Dean et al. 2005 |
| Neurospora crassa            | 40               | 10,082                         | 48                               | Ca²⁺ Permeable channel (3), Ca²⁺/cation ATPases (9), Ca²⁺ exchanger (8), phospholipase C (4), CaM (1), Ca²⁺/CaM regulated proteins (23) | Galagan et al. 2003; Zelter et al. 2004; Tamuli et al. 2013 |
acids (FFAs), diacylglycerol (DAG), phosphatidic acid (PA), and lysophospholipids (LPLs) (Köhler et al. 2006; Hong et al. 2016). Phospholipases are of two types, acyl hydrolases, and phosphodiesterases. Phospholipases are classified, based on the specific ester linkage they cleave within phospholipid molecule, into four broad classes such as phospholipase A (PLA₁ and PLA₂), phospholipase B (PLB), phospholipase C (PLC), and phospholipase D (PLD) (Köhler et al. 2006). Both PLA and PLB are acyl hydrolases, while PLC and PLD belong to the phosphodiesterase class of phospholipases (Richmond and Smith 2011). The membrane-bound phosphoinositide-specific PLC cleaves phosphatidylinositol-4,5-bisphosphate (PIP₂) into two important second messengers, inositol 1,4,5-triphosphates (IP₃) that induce Ca²⁺ release from internal stores and diacylglycerol (DAG) that activates protein kinase C (PKC), and triggers a range of cellular activities (Cornelius and Nakashima 1987; Chae et al. 2007; Clapham 2007). The IP₃ causes release of Ca²⁺ to activate calmodulin (CaM)-dependent enzymes (Berridge 1993), IP₃ also acts as the precursor of many inositol polyphosphates like IP₄ or IP₆ (York et al. 1999). Majority of the eukaryotic PLCs contain five conserved domains, comprising of two catalytic domains X and Y, an N-terminal pleckstrin homology (PH) domain for interaction with the membrane phospholipid, a C-terminal Ca²⁺ dependent C2 domain for binding to phospholipids, and an EF-hand motif for Ca²⁺ binding and interaction of the PH domain with phospholipid (Sutton et al. 1995; Yamamoto et al. 1999). PLC is important for various cellular processes and pathogenicity in several organisms, including filamentous fungi. The budding yeast *Saccharomyces cerevisiae* contains Plc1p, a phosphatidylinositol-specific phospholipase C (PI-PLC), which shows sequence homology to mammalian PI-PLC-δ isoforms (Flick and Thorner 1993). The Plc1p is necessary for growth at nonpermissive temperatures (above 35°C), survival under osmotic stress, and utilisation of alternative carbon sources like galactose, raffinose, or glycerol at permissive temperatures (23 to 30°C) (Flick and Thorner 1993). In the fission yeast *Schizosaccharomyces pombe*, plc1-1 is a homolog of *plc1*, mutation of *plc1-1* induced by nitrosoguanidine caused sensitivity to high amounts of phosphate in the medium (Fankhauser et al. 1995). The growth defect of the *plc1-1* deletion mutant was partially restored in low inositol and low phosphate minimal media containing a high concentration of nitrogen, which suggests a potential role of *plc1-1* in ammonium sensing (Fankhauser et al. 1995). In the grey mould fungus *Botrytis cinerea*, Plc1 homolog BcPLC1 is required for vegetative growth, conidial formation, germination, and virulence (Schumacher et al. 2008). In the human-pathogenic fungus *Candida albicans*, the PLC1 homolog CaPLC1 is an essential gene and its conditional mutant showed increased sensitivity to a high concentration of sorbitol or NaCl, increased sensitivity to lower (18°C) or higher temperatures (43°C), and reduced growth in medium containing galactose, but not glucose, as the sole carbon source (Kunze et al. 2005). The CaPLC1 conditional mutant was also sensitive to nocodazole that inhibits chromosome segregation, and showed reduced growth in filamentous growth-inducing conditions and on media containing only arginine as the sole nitrogen source (Kunze et al. 2005). However, two additional PLC genes in CaPLC2 and CaPLC3 genes were non-essential for growth as loss of Caplc2 and Caplc3 did not show any visible growth defects in *C. albicans* (Knechtle et al. 2005; Kunze et al. 2005). Moreover, in a mouse systemic infection model, the CaPLC2 and CaPLC3 mutants showed survival rate similar to the wild type, suggesting that both CaPLC2 and CaPLC3 are not essential for virulence (Knechtle et al. 2005; Kunze et al. 2005). In the citrus fungal pathogen, *Alternaria alternata*, the PLC1 homolog is important for vegetative growth, conidial formation, Ca²⁺ homeostasis, and virulence (Tsai and Chung 2014). In encapsulated yeast and human pathogen *Cryptococcus neoformans*, CnPlc1, a homolog of the mammalian PI-PLC-δ is essential for cellular homoeostasis and virulence (Lev et al. 2013). The model filamentous fungus *Neurospora crassa* possesses four novel PLC-δ proteins, including PLC-1, which is highly divergent among the natural isolates (Galagan et al. 2003; Borkovich et al. 2004; Gavric et al. 2007; Barman and Tamuli 2015, 2017; Barman et al. 2018). The *plc-1* mutant, generated using repeat-induced point mutation (RIP; Selker et al. 1987), is viable, but showed reduced growth, abnormal hyphal morphology, lower turgor, increased sensitivity to low extracellular and increase intracellular Ca²⁺ concentrations, and responded differently to PLC inhibitor 3-nitrocoumarin (Gavric et al. 2007). In another study, phenotypes of the RIP-generated and knockout mutants of *plc-1* were different, and analysis of the mutant phenotypes suggested a role for *plc-1* in hyphal tip
growth in \textit{N. crassa} (Lew et al. 2015). Moreover, the \textit{plc-1} knock out mutant displays aberrant hyphal morphology in the presence of Ca$^{2+}$ ionophore A23187, accumulates an increased amount of carotenoid, and shows reduced survival during oxidative and thermal stress (Barman and Tamuli 2015; Barman et al. 2018). In the rice-blast fungus \textit{Magnaporthe oryzae}, PI-PLC-δ isoform \textit{MoPLC1} regulates intracellular Ca$^{2+}$ fluxes and plays an essential role in fungal development, appressorium formation, and pathogenicity (Rho et al. 2009).

\section*{Ca$^{2+}$ transporters and their involvement in cell processes in fungi}

In fungi, six major types of Ca$^{2+}$ transporters, including Ca$^{2+}$ pumps, Ca$^{2+}$/H$^+$ exchangers (Lange and Peiter 2020), high-affinity calcium system (HACS), low-affinity calcium system (LACS), TRP-like Ca$^{2+}$ channels, and mitochondrial Ca$^{2+}$ uniporter (MCU) have been identified (Tisi et al. 2016). The HACS and LACS are two critical Ca$^{2+}$ uptake systems that are conserved across different fungi and mediate the entry of Ca$^{2+}$ under different cellular conditions (Martin et al. 2011). In fungi, Ca$^{2+}$ release from internal stores such as Golgi bodies, endoplasmic reticulum, vacuole, and mitochondria are carried out by P-type Ca$^{2+}$ ATPases driving the energy from the synthesis of ATP to transfer Ca$^{2+}$ against the ion gradient (Li et al. 2019). In \textit{A. fumigatus}, McuA is a Ca$^{2+}$ uniporter localised to the mitochondrial membrane, and the deletion of \textit{mcuA} results in disruption of the mitochondrial Ca$^{2+}$ homeostasis, suggesting its role in Ca$^{2+}$ uptake (Song et al. 2016). In addition, the deletion of \textit{mcuA} also causes resistance to azoles and oxidative stress; however, overexpression of \textit{mcuA} restores the azole sensitivity phenotype in a deletion mutant of \textit{agcA} that encodes for a mitochondrial carrier protein in \textit{A. fumigatus} (Song et al. 2016). The knockouts of \textit{cchA}, \textit{midA}, and \textit{yvcA}, which are the homologues of the \textit{S. cerevisiae} genes encoding for voltage-gated \textit{CCH1}, stretch-activated \textit{MID1}, and vacuolar \textit{YVC1} Ca$^{2+}$ channels, respectively, were not virulent in mice model of invasive aspergillosis, suggesting that these transporters contribute to virulence in \textit{A. fumigatus} (de Castro et al. 2014). In fungi, vacuoles are one of the major Ca$^{2+}$ stores, the Ca$^{2+}$ ATPases and the Ca$^{2+}$/H$^+$ exchangers are important transporters that guide the entry of Ca$^{2+}$ into the vacuoles (Pittman 2011). The \textit{pmcA}, \textit{pmcB}, and \textit{pmcC} type Ca$^{2+}$ transporters in \textit{A. fumigatus} are homologues of the \textit{S. cerevisiae} plasma membrane Ca$^{2+}$-ATPase \textit{PMC1} (Cunningham and Fink 1994), transcribed by the calcineurin A-CrzA pathway, and necessary for growth and survival under the Ca$^{2+}$ stress condition (Dinamarco et al. 2012). In \textit{A. fumigatus}, the \textit{pmcA} conditional mutant was not virulent in the mice model of invasive aspergillosis, suggesting that \textit{pmcA} has a role in conferring virulence and pathogenicity to the fungi; \textit{pmcA} also regulates the metabolism of Ca$^{2+}$ and Mn$^{2+}$ (Dinamarco et al. 2012). There are different classes of P-type Ca$^{2+}$ ATPases such as plasma membrane Ca$^{2+}$-ATPase (PMCA), sarco (endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) and secretory pathway Ca$^{2+}$-ATPase (SPCA) are ATP-driven pumps involved in the active transport and homeostasis of Ca$^{2+}$ (Sze et al. 2000; Carafoli 2002; Ton and Rao 2004). In \textit{S. cerevisiae}, deletion of the Ca$^{2+}$-ATPase \textit{Pmr1p} results in the lack of adequate amount of Ca$^{2+}$ within the Golgi, an increase in the cytosolic Ca$^{2+}$ concentration causing vacuolar fragmentation possibly to sequester excess Ca$^{2+}$ (Kellermayer et al. 2003). In \textit{S. pombe}, \textit{Cps5p} is the \textit{Pmr1p} homolog, which plays a role in the cell wall formation, protein glycosylation, and maintains intracellular Ca$^{2+}$ homeostasis by depletion of the excessive cytosolic Ca$^{2+}$ via the interaction with a vacuolar Ca$^{2+}$-ATPase homolog, \textit{Pmc1p} (Cortés et al. 2004). In \textit{Calbicans}, the deletion of a Ca$^{2+}$-ATPase gene \textit{CaPMr1}, the homolog of \textit{PMR1} in \textit{S. cerevisiae}, results in altered glycosylation, causing a defect in the cell wall formation and loss of virulence (Bates et al. 2005). In \textit{N. crassa}, the deletion of a PMCA family of Ca$^{2+}$ ATPase NCA-2 causes restricted growth, sensitivity to increasing concentration of Ca$^{2+}$ in the media, and female sterile phenotype (Bowman et al. 2011; Deka and Tamuli 2013). In addition, the \textit{nca-2} gene plays a role in carotenoid accumulation, regulation of the circadian clock with a Ca$^{2+}$ sensor \textit{nsc-1}, and thermotolerance with a cation-ATPase \textit{trm-9} in \textit{N. crassa} (Deka and Tamuli 2013; Laxmi and Tamuli 2015).

\section*{Calmodulin is a major calcium-binding protein involved diverse cell functions in fungi}

The change in the Ca$^{2+}$ concentration inside the cell is detected by an array of Ca$^{2+}$ sensors belonging to the EF-hand family of proteins. CaM is a primary Ca$^{2+}$ sensor containing EF-hand (Lewit-Bentley and Réty 2000) motifs, and conserved from lower to higher eukaryotes (Chin and Means 2000). Because of its evolutionary conservation, CaM transduces about
300 effectors for the activation of the downstream signalling cascade in the eukaryotes (Halling et al. 2016). The S. cerevisiae CaM has three functional EF-hands; disruption of this gene has a lethal phenotype, and therefore, essential for viability (Davis et al. 1986). In S. pombe, disruption of cam1 causes growth defects and abolishes cell division (Takeda and Yamamoto 1987). The cam1 disruption results in improper chromosomal segregation, hyper condensation, uneven distribution of the chromosomal material, and absence of the spindle body proteins responsible for spore formation in S. pombe (Itadani et al. 2010). Immunofluorescence assay of the GFP-tagged cam1 protein in S. pombe indicated the localisation of the Cam1 to the spindle body fibres and also to the sites responsible for polarised cell growth (Moser et al. 1997; Itadani et al. 2010). Calmodulin also mediates protein phosphorylation via calmodulin-dependent kinase in the presence of N-acetyl-D-glucosamine (GlcNAc), which is involved in germ tube formation causing morphogenesis of C. albicans; however, calmodulin inhibitor trifluoperazine (TFP) inhibits the phosphorylation and germ tube formation (Roy and Datta 1987; Paranjape et al. 1990). In addition, calmodulin binds to an integral membrane protein Df1lp that activates mitogen-activated protein kinase (MAPK) Cek1p, which is required for invasive filamentation in C. albicans (Davis et al. 2013). In C. neoformans, CAM1 is required for the infestation in the human body at 37°C (Kraus et al. 2005). In the presence of calcineurin inhibitor FK506, the temperature-sensitive cam1-ts mutant of C. neoformans showed defects in growth and bud formation at 25°C (Kraus et al. 2005). The cam1-ts mutant also showed reduced CAM1 expression and growth defect at 37°C, which was not complemented when transformed with a calmodulin independent calcineurin A allele (CNA1-Ald) lacking the coding region for the C-terminal calmodulin-binding site and the autoinhibitory domain (Kraus et al. 2005). Therefore, CAM1 has a role in both calcineurin-dependent and independent developmental pathways in C. neoformans (Kraus et al. 2005). In M. grisea, CaM is required for early-stage appressorium formation and conidial germination (Lee and Lee 1998; Liu and Kolattukudy 1999). The cmd is an essential gene in N. crassa; therefore, knockout of the cmd gene is not viable (Tamuli et al. 2013). CaM antagonists trifluoperazine (TFP) and chlorpromazine (CPZ) caused defects in hyphae formation, reduced growth, and impaired sexual development in N. crassa (Laxmi and Tamuli 2015). In addition, the RIP-generated cmd mutants showed reduced growth, less carotenoid accumulation, decreased survival in exposure to ultraviolet (UV) irradiation, and defect in the sexual development causing female sterility (Laxmi and Tamuli 2017). CaM also interacts with Ca^{2+}/CaM-dependent kinases, including Ca^{2+}/CaMK-2 that is required for full fertility in N. crassa (Kumar and Tamuli 2014; Laxmi and Tamuli 2017).

**Neuronal calcium sensor-1 regulates growth, sexual development, and Ca^{2+} homeoeostasis in fungi**

Neuronal calcium sensor-1 (NCS-1), which belongs to the family of EF-hand containing protein, binds to Ca^{2+} and senses the change in the concentration of the Ca^{2+} inside the cell (Burgoine 2007; Tamuli et al. 2011). The NCS-1 was identified as frequenin (Frq1), enriched in the synapses of the Drosophila melanogaster nervous system, which facilitates the frequency-dependent release of neurotransmitter (Pongs et al. 1993). Additionally, Frq2, parologue of Frq1, was evolved via an unusual frequenin gene duplication in D. melanogaster (Sánchez-Gracia et al. 2010). In S. cerevisiae, the NCS-1 orthologue FRQ1 plays a role in cell growth, and regulates a phosphatidylinositol-4-OH kinase (PIK-1) that is essential for cell survival (Hendricks et al. 1999). In S. pombe, Ncs1p has a role in the sexual development by regulation of spore formation and conjugation via Ca^{2+} dependent manner (Hamasaki-Katagiri et al. 2004). In A. fumigatus, the knock-out mutaition of ncs-1 homologue ncsA causes polarity defects, confers Ca^{2+} resistance, and increased sensitivity to EGTA, sodium dodecyl sulphate (SDS), ergosterol-depletion agents voriconazole and itraconazole, and the ergosterol intercalating agent amphotericin B; however, did not affect the virulence (Mota Júnior et al. 2008). NcsA also promotes expression of the S. cerevisiae Pmc1 homologs pmcA, a P-type Ca^{2+}-ATPase, and pmcB, a Ca^{2+}-translocating P-type ATPase in A. fumigatus (Mota Júnior et al. 2008; Soriani et al. 2008). In M. grisea, null-mutant of the FRQ1/NCs-1-like gene Mg-NCs-1, showed restricted growth at high Ca^{2+} concentrations or acidic conditions (Saitoh et al. 2003). The knockout mutant of ncs-1 in N. crassa shows reduced growth and increased sensitivity to high
concentrations of Ca\(^{2+}\) and UV irradiations (Tamuli et al. 2011; Deka et al. 2011). Besides, in response to the high concentrations of Ca\(^{2+}\), transcription of ncs-1 is upregulated by the Crz-1 transcription factor in N. cressa (Gohain and Tamuli 2019). Furthermore, NCS-1 interacts with the Ca\(^{2+}\) permeable channel MID-1 in the plasma membrane, possibly to block Ca\(^{2+}\) influx, which might be critical for survival under the high concentration of Ca\(^{2+}\) (Gohain and Tamuli 2019).

**Calcineurin plays important roles in asexual and sexual developments, stress responses, and pathogenicity in fungi**

Calcineurin was first identified as an inhibitor of CaM-dependent cyclic nucleotide phosphodiesterase in a column fraction (Wang and Desai 1976). Calcineurin is the only serine/threonine phosphatase that requires both Ca\(^{2+}\) and CaM for its activity (Klee et al. 1979, 1998). The heterodimeric enzyme calcineurin has two subunits, a catalytic subunit and a regulatory subunit (Klee and Krinks 1978). Calcineurin has a critical role in fungal development, stress responses, and virulence in pathogenic fungi (Rusnak and Mertz 2000; Juvvadi et al. 2014). The S. cerevisiae MATa strains containing null-mutations in the calcineurin or protein phosphatase 2B (PP2B) subunits encoding genes CNA1 and CNA2 showed an increased sensitivity to growth arrest induced by the mating pheromone α-factor (Cyert et al. 1991). In S. cerevisiae, null mutations in both the calcineurin catalytic subunits (cnmA cmp2 or cnA1 cmp2), and the regulatory (cnb1) subunit, cause sensitivity to high concentrations of Mn\(^{2+}\) (MnCl\(_2\)), because the mutants were not able to block the entry of Mn\(^{2+}\) into the cell (Farcasanu et al. 1995). In N. crassa, the RIP-generated cnb-1 mutant showed a defect in hyphal growth and differentiation (Kothe and Free 1998). Inhibition of cnb-1 using antisense RNA and inhibitor of calcineurin FKS06, results in a loss of steep Ca\(^{2+}\) gradient at the hyphal tip in N. crassa (Prokisch et al. 1997). Furthermore, the RIP-generated Cna1 and cnb-1 mutants displayed reduced thermotolerance, increased sensitivity to osmotic stress, and defect in the asexual and sexual developments in N. crassa (Kumar et al. 2019). In M. oryzae, application of antisense RNA against the catalytic subunit of calcineurin MCNA exhibited lessening in mycelial formation, conidiation, and appressorium formation, resulting in the reduction of the fungal pathogenicity (Choi et al. 2009a). In C. neoformans, the calcineurin catalytic subunit CNA1 is required for growth at elevated temperature, survival in increased levels of CO\(_2\) and alkaline pH conditions, cation homeostasis, and virulence (Odom et al. 1997). In the entomopathogenic fungus Beauveria bassiana, the cnA1 and cnA2 genes encode for two calcineurin catalytic subunit paralogues CnA1 and CnA2, and the cnb1 gene encodes the calcineurin regulatory subunit B (Cnb1) (Li et al. 2015). The B. bassiana ΔcnA1, ΔcnA2, and Δcnb deletion mutants showed reduced growth and conidiation, decreased virulence, sensitivity to stress-inducing chemicals and the fungicide carbendazim, and reduced survival in response to heat-shock, UV-B irradiation (Li et al. 2015). In addition, the ΔcnA1 and ΔcnA2 mutants showed altered cell wall composition, and the Δcnb mutant showed sensitivity to osmotic stress induced by NaCl and KCl (Li et al. 2015). In A. fumigatus, calcineurin regulates hyphal growth, septation, and virulence (Lamoth et al. 2013; Juvvadi et al. 2014). In S. pombe, analysis of a null mutant of calcineurin-like gene ppb1\(^{+}\) showed its involvement in cytokinesis, pole body positioning, cell shape and polarity, and mating (Yoshida et al. 1994).

In addition, calcineurin plays a critical role in fungal pathogenicity. A. fumigatus plays a common life-threatening disease called aspergillosis in humans. In the Aspergillus and Candida species, the antifungal paradoxical effect is a phenomenon, where reversal of growth inhibition occurs at higher concentrations of the antifungal drug echinocandins, usually caspofungin, which inhibit β-1,3-glucan synthase (FKS1 in C. albicans, and FksA in A. fumigatus) causing damage of the fungal cell walls (Sanglard et al. 2003; Steinbach et al. 2015). The key players in the paradoxical effect are calcineurin catalytic subunit (CnaA) and the heat shock protein 90 (Hsp90) in A. fumigatus and C. albicans (Steinbach et al. 2015). Furthermore, the A. fumigatus calcineurin upregulates chitin biosynthesis, which causes increased chitin content in the cell wall, by transcriptional regulation of chitin synthase genes during paradoxical effect in response to high concentrations of caspofungin (Fortwendel et al. 2010). In A. fumigatus, caspofungin transiently increases [Ca\(^{2+}\)]\(_{c}\) concentration, and activates CaM-calcineurin signalling, which causes paradoxical effect (Juvvadi et al. 2015). The A. fumigatus calcineurin catalytic A subunit (CnaA) contains a serine-proline-rich-region (SPRR)
unique to the filamentous fungi, but absent in the human calcineurin α-catalytic subunit (Juvvadi et al. 2013). In the SPRR, phosphorylation of S406, S408, S410, and S413 residues are required for the function of calcineurin in hyphal growth and virulence of the A. fumigatus (Juvvadi et al. 2013). Identification of this critical SPRR region unique to filamentous fungi, but absent in human, is a significant step towards the development of new antifungal drugs for invasive aspergillosis (Juvvadi et al. 2013, 2014). Moreover, at paradoxical-growth concentrations of caspofungin (4 μg/ml), phosphorylation of S406, S410, and S413 residues in the SPRR of CnaA activates calcineurin, which may cause nuclear localisation of the transcription factor calcineurin responsive zinc finger 1 homologue CrzA for transcriptional activation to regulate paradoxical growth (Juvvadi et al. 2015). CrzA also has a role in chitin synthase expression in caspofungin paradoxical effect (CPE) in A. fumigatus (Ries et al. 2017). The pathways mediated by the CrzA and ZipD, which is a basic leucine zipper transcription factor and another target of calcineurin, genetically interact during Ca²⁺ stress (de Castro et al. 2019). Furthermore, in A. fumigatus, ZipD regulates cell wall composition and organisation, tolerance to osmotic stress, pathogenesis, and resistance to echinocandin antifungals, including caspofungin (de Castro et al. 2019). Therefore, targeting calcineurin CrzA and ZipD transcription factors may be potential drug targets against A. fumigatus. In addition, the role of CrzA has been investigated for differentiation and mycotoxin production in aflatoxin producing fungi A. flavus and A. parasiticus (Chang 2008; Lim et al. 2019). In M. oryzae, the calcineurin catalytic subunit A (MCNA) plays a role in mycelial growth, conidiation, formation of specialised infection structure called appressorium, and pathogenicity (Choi et al. 2009a). Moreover, in M. oryzae, the knock-down of catalytic subunit A-like gene moderately reduced appressorium formation, but the knock-down of regulatory subunit B-like gene causes a complete loss of pathogenicity against the host plants, suggesting that the catalytic and regulatory subunits play a distinct role in pathogenicity (Nguyen et al. 2008). In C. neoformans, Δcna1 deletion mutants were unable to survive at a body temperature of 37°C, alkaline pH, and CO₂ at high levels (Odom et al. 1997). The C. neoformans virulence was examined by removal of cerebral blood spinal fluid (CSF) and counting of the colony-forming units (CFU), which revealed a low number CFU in the Δcna1 mutant of C. neoformans, indicating that calcineurin is required for growth in the mammalian host (Odom et al. 1997). Similarly, disruption of cnb1 in C. neoformans results in temperature-sensitivity and reduced virulence in the murine model of cryptococcosis (Fox et al. 2001). Calcineurin in C. neoformans was identified as a novel antifungal drug target (Cruz et al. 2000). C. neoformans is sensitive to FKS06 and CsA at physiological temperature (Cruz et al. 2000). A mutational analysis using a novel 6 bp duplication in the calcineurin B gene (CNB1) inhibits the immunophilins FKB12 – FKS06 binding to Cnb1, which revealed the mechanism of drug action in C. neoformans (Fox et al. 2001). Calcineurin A is also vital for providing tolerance to antifungal agents along with some metabolic inhibitors in C. albicans (Cruz et al. 2002). In C. albicans, disruption of cna-1 results in the loss of viability in the presence of antifungal agents fluconazole, amorolfine, itraconazole, terbinafine, and voriconazole (Sanglard et al. 2003). In addition to calcineurin, Hsp90 has a role in providing resistance to echinocandins in C. albicans (Singh et al. 2009). Calcineurin inhibitors, cyclosporine and fluconazole have a synergistic effect in the prevention of biofilm formation and increase susceptibility to fluconazole in C. albicans (Jia et al. 2016).

**Calcineurin responsive zinc finger 1 (Crz1) plays a critical role in regulating cellular functions, tolerance to stress conditions, and virulence**

There are different target proteins of calcineurin found across different organisms (Li et al. 2011). The best-known target is a transcription factor called calcineurin-responsive zinc finger 1 (Crz1) in lower eukaryotes and the nuclear factor of activated T cells (NFAT) in mammals (Thewes 2014). In S. cerevisiae, the calcineurin target Crz1p, also known as Tcn1p, drives the expressions of PMC1, PMR1, PMR2A, and FKS2 genes, which play a vital role in the tolerance to high Ca²⁺, Mn²⁺, Na⁺, and cell wall damage, respectively (Matheos et al. 1997; Stathopoulos and Cyert 1997). In S. pombe, the null mutant of prz1, the crz1 homologue, did not show any defects in morphology and sexual development; however, the Δprz1 mutant was hypersensitive to Ca²⁺ (Hirayama et al. 2003). In M. grisea, the CRZ1 homologue MgCRZ1 partially complemented...
the S. cerevisiae Δcrz1 mutant and suppressed the Li⁺ sensitivity (Zhang et al. 2009). The MgCRZ1 is required for growth, development, Ca²⁺ stress tolerance, and full virulence (Zhang et al. 2009). In C. neoformans, crz1 drives the expression of the chitin synthase gene chs6, and crz1 deletion mutant showed a defect in cell wall synthesis (Lev et al. 2012). In C. albicans, the CRZ1 deletion mutant was more sensitive to Ca²⁺, Li⁺, and Mn²⁺ cations, hypersensitive to anionic detergents SDS and antifungal azoles, including fluconazole and miconazole (Santos and de Larrinoa 2005). In Aspergillus parasiticus, crzA is required for vegetative growth, asexual development, and aflatoxin production under the Ca²⁺ stress condition (Chang 2008). In A. fumigatus, knock out of crzA results in a defect in germination and polarised hyphal growth, and reduced sporulation (Cramer et al. 2008). The A. fumigatus ΔcrzA mutants also displayed sensitivity to heat shock conditions (Soriani et al. 2008). Studies in A. flavus, which produces aflatoxin, the deletion of crzA renders the strain more vulnerable to cell wall stress and osmotic pressure (Lim et al. 2019). In the A. flavus ΔcrzA mutants, conidiophore production was reduced as the head of the conidiophore become short, along with a decrease in the number of conidia (Lim et al. 2019).

Crz 1 also plays a critical role in fungal virulence. In M. grisea, when infected to onion surface or rice leaves, Δmocrz1 showed a significant reduction in the appressorial penetration rate (Choi et al. 2009b). In M. grisea, lipid droplets are transported from conidia to nascent appressorium, which fully melanised within 12–24 h (Zhang et al. 2009). However, in the M. grisea Δcrz1 mutant, the lipid droplets were undegraded even after 48 h (Zhang et al. 2009). Thus, targeting calcineurin-CRZ1 signalling cascade results in a lack of functional appressorium that failed to penetrate the host cuticle, causing loss of full virulence in M. grisea (Zhang et al. 2009). In B. bassiana, Δcrz1 mutant showed defects in growth and conidiation, suppressed growth in the presence of osmotic salts, sensitivity to stress-inducing chemicals and carbendazim and osmotic salts, decreased thermostolerance, reduced resistance to UV-B irradiation, changed cell wall composition, and longer virulence period (Li et al. 2015).

Conclusions

Ca²⁺ signalling regulates multiple cell functions ranging from growth, development, fertility, stress tolerance, and virulence in fungi. Phospholipases are enzymes that act on membrane phospholipids and classified into four types. In response to specific signals, PLC produces IP₃ and DAG, which mediate Ca²⁺ release and activation of PKC, respectively. There are also six major types of Ca²⁺ transporters that are required for Ca²⁺ homeostasis and signalling. The increase in [Ca²⁺]₉ activates various Ca²⁺ sensors such as CaM and NCS-1 that interact with specific downstream proteins as a response to a signal. Calcineurin, which consists of catalytic A and regulatory B subunits, is one of the major downstream Ca²⁺ signalling proteins required for multiple cell functions, including growth, stress tolerance, and virulence in fungi. Calcineurin is also identified as a target for antifungal drugs. Calcineurin activates the Crz 1 transcription factor to activate expressions of target genes required for growth, developments, tolerance to stress conditions, and pathogenicity. Further research on the Ca²⁺ signalling machinery will unravel its complex molecular network under different cellular conditions.

Acknowledgements

We are thankful to all researchers in the relevant field for their contributions that helped us to write this review article. We regret our inability to cite all the references, which is beyond the scope of this review. AR, AK, and DB were supported by Research Fellowships from the Ministry of Human Resource Development, Government of India. We thank IIT Guwahati for partial financial support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Ministry of Human Resource Development [Research Fellowships to AR, AK, and DB].

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