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Ca^{2+} TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

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Summary

Cytosolic free Ca^{2+} is maintained at submicromolar levels in budding yeast by the activity of Ca^{2+} pumps and antiporters. We have recently identified the structural genes for two Ca^{2+} pumps, *PCM1* and *PMR1*, which are required for Ca^{2+} sequestration into the vacuole and secretory organelles, respectively. The function of either Ca^{2+} pump is sufficient for yeast viability, but deletion of both genes is lethal because of elevation of cytosolic [Ca^{2+}] and activation of calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase. Calcineurin activation decreases Ca^{2+} sequestration in the vacuole by a putative Ca^{2+} antiporter and may also increase Ca^{2+} pump activity. These regulatory processes can affect the ability of yeast strains to tolerate high extracellular [Ca^{2+}]. We propose a model in which the cellular response to changes in the environmental levels of Ca^{2+} is mediated by calmodulin and calcineurin which, in turn, modulate the various types of Ca^{2+} transporters.

Introduction

The budding yeast *Saccharomyces cerevisiae*, like other eukaryotes, actively maintains cytosolic free Ca^{2+} concentrations $[Ca^{2+}]_i$ at extremely low levels in spite of very steep gradients of this ion across the plasma membrane and across intracellular membranes. It is generally believed that this asymmetric distribution avoids aggregation of Ca^{2+} with phosphate-containing molecules in the cytosol while still providing various organelles with sufficient Ca^{2+} for their proper function. Superimposed on the need for low $[Ca^{2+}]_i$ is the requirement for Ca^{2+} as a second messenger in signal transduction. Transient increases in $[Ca^{2+}]_i$ regulate a wide variety of cellular processes in other species and there is now good evidence that Ca^{2+} signaling is important in yeast as well. Furthermore, yeast expresses the same repertoire of signaling molecules as that used in animal cells (calmodulin and calmodulin-dependent protein kinases and phosphatases). Because of this similarity, the origin of Ca^{2+} signals and the individual roles of these effector molecules in yeast has become a burgeoning field (for a review, see Davis, 1994).

At the heart of this complex and highly regulated process are a battery of Ca^{2+} channels, antiporters and pumps, which are primarily responsible for maintaining and

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altering the Ca^{2+} levels in the various compartments. Progress in understanding the individual roles of these transporters has increased dramatically with the recent cloning and molecular characterization of several key components. The ability to manipulate genetically all of the individual Ca^{2+} transporters in conjunction with the downstream Ca^{2+} signaling factors in yeast provides a powerful new perspective on the ubiquitous problem of cellular Ca^{2+} homeostasis and signaling. This article summarizes many of the recent advances in our understanding of Ca^{2+} transporters and Ca^{2+} flow in yeast.

Ca^{2+} channels

In eukaryotic cells, Ca^{2+} signals are usually initiated by the triggered opening of Ca^{2+} channels in the plasma membrane and certain organellar membranes, which allows a rapid influx of Ca^{2+} into the cytosol down its concentration gradient. The massive influx of Ca^{2+}, which typically increases $[Ca^{2+}]_i$ 10- to 100-fold over the basal level of approximately $0.1\,\mu mol\,l^{-1}$, is soon followed by channel closure and active removal of Ca^{2+} from the cytosol by the Ca^{2+} antiporters and pumps. Transient spikes and oscillations in $[Ca^{2+}]_i$ generated by this coordinated process are known to regulate a wide variety of processes in non-excitable cells; for example, exocytosis, gene expression and cell-cycle progression. In yeast, Ca^{2+} signals may regulate similar processes (for reviews, see Anraku *et al.* 1991; Davis, 1994; Youatt, 1993). Progress in understanding Ca^{2+} signaling has been slow because of limitations in the direct measurement of $[Ca^{2+}]_i$ and difficulties in quantifying Ca^{2+} channel activity in yeast.

Though Ca^{2+} channels have not been isolated or cloned from yeast, there is good evidence that these transporters exist. The patch-clamp technique has revealed a stretch-activated or mechanosensitive ion channel in the plasma membrane that passes many ions including Ca^{2+} (Gustin *et al.* 1988). Additionally, increased rates of Ca^{2+} influx into living yeast cells have been observed during the G1/S transition in the cell division cycle, during cell cycle arrest in late G1 caused by either mating pheromones (Ohsumi and Anraku, 1985) or certain temperature-sensitive mutations (Anand and Prasad, 1987; Prasad and Rosoff, 1992), and during the response to nutrient feeding (Eilam and Othman, 1990; Eilam *et al.* 1990; Nakajima *et al.* 1991). Another possible Ca^{2+} channel has been detected in purified membrane vesicles derived from the yeast vacuole (Belde *et al.* 1993). These vesicles accumulate Ca^{2+} *in vitro* and release a small portion in response to added inositol-1,4,5-trisphosphate (InsP_3), suggesting a similarity to the InsP_3 receptor in the endoplasmic reticulum of animal cells. At present, it is unclear when and how the channel activities are triggered and what processes might be affected by their opening.

Vacuolar H^+/Ca^{2+} antiport

Biochemical experiments indicate that the yeast vacuolar membrane actively transports Ca^{2+} *via* H^+/Ca^{2+} antiport (Dunn *et al.* 1994; Ohsumi and Anraku, 1983; Okorokov *et al.* 1985). Ca^{2+} uptake activity into purified vacuoles and vacuole membrane vesicles is completely dependent on the transmembrane pH gradient ΔpH (interior acid) that is normally produced by the vacuolar H^+ V-ATPase, though some uptake still occurs in the

absence of ATP if the ΔpH is generated by chemical ion diffusion gradients (Dunn *et al.* 1994). A potential difference ΔΨ (interior positive) did not promote Ca^{2+} uptake. Uptake into isolated vacuoles is saturable by cytosolic Ca^{2+} and displays an apparent K_m for Ca^{2+} at 25–50 μmol l^{-1}, which is much higher than the $[Ca^{2+}]_i$ observed in living cells (approximately 0.15 μmol l^{-1}). The antiporter has not been isolated, its structural gene has not been cloned and no mutants are available to address its specific functions and roles in yeast. Recently though, we have isolated a yeast gene whose predicted product is homologous to the retinal $Na^+/Ca^{2+},K^+$ and cardiac Na^+/Ca^{2+} exchangers from mammals and appears to be required for optimal Ca^{2+} sequestration into the vacuole *in vivo* (K. W. Cunningham and G. R. Fink, in preparation). Therefore, it is possible that the cloned gene encodes the previously characterized low-affinity H^+/Ca^{2+} antiporter or possibly some other type of Ca^{2+} transporter.

Pmc1p: a vacuolar Ca^{2+} pump

The yeast vacuole membrane also contains a putative high-affinity Ca^{2+} pump, Pmc1p, which is the product of the *PMC1* gene (Cunningham and Fink, 1994). Pmc1p is approximately 40 % identical to plasma membrane Ca^{2+}-ATPases (PMCAs) and is much less similar to other P-type ion pumps (Fig. 1). Pmc1p apparently lacks the calmodulin-binding domain at the C terminus, but otherwise appears to be a functional ion pump localized predominantly to the vacuole membrane. By analogy to the animal enzyme,

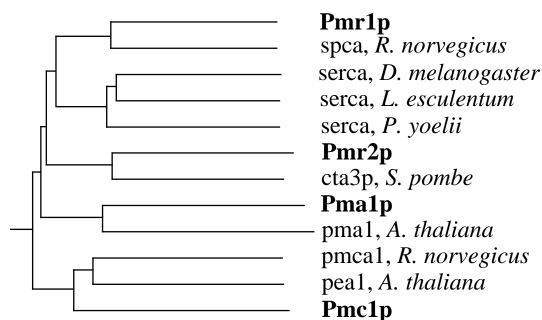

Fig. 1. Phylogenetic tree of selected P-type ATPases from *Saccharomyces cerevisiae* and other species. The weighted tree was drawn by Megalign (DNASTAR, Inc.) using the Clustal method for multiple alignment using Drs2p (Ripmaster *et al.* 1993) as an outgroup (not shown). The protein sequences Pmr1p, Pmr2p, Pma1p and Pmc1p were obtained from translation of *Saccharomyces cerevisiae* genomic DNA (Cunningham and Fink, 1994; Rudolph *et al.* 1989; Serrano *et al.* 1986). Animal sequences for the secretory pathway Ca^{2+}-ATPase (spca1), sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (serca2b) and plasma membrane Ca^{2+}-ATPase (pmca1a) were obtained from the rat *Rattus norvegicus* (Gunteski-Hamblin *et al.* 1992; Shull and Greeb, 1988) and fruit fly *Drosophila melanogaster* (Magyar and Varadi, 1990). Higher plant sequences were from *Arabidopsis thaliana* (Huang *et al.* 1993; Pardo and Serrano, 1989) and the tomato *Lycopersicon esculentum* (Wimmers *et al.* 1992). Sequences from the fission yeast *Schizosaccharomyces pombe* (Ghislain *et al.* 1990) and the protozoan *Plasmodium yoelii* (Murakami *et al.* 1990) have also been included.

Pmc1p is expected to catalyze the high-affinity (K_m approximately 1 μmol l^{-1}) and ATP-dependent transport of Ca^{2+} into the vacuole. Such an activity was not observed in previous experiments using isolated vacuole membrane vesicles (Dunn *et al.* 1994; Ohsumi and Anraku, 1983). It is possible that the Ca^{2+} transport activity of Pmc1p was obscured by the much greater H^+/Ca^{2+} antiport activity in these experiments. Alternatively, Pmc1p may have unexpected properties, such as instability, low Ca^{2+} affinity, sensitivity to protonophores, or other characteristics that prevented its earlier detection. In support of its function as a Ca^{2+} transporter in living yeast cells, mutants lacking Pmc1p accumulate Ca^{2+} in the vacuole at less than 20 % of the wild-type rate during growth in standard medium (Cunningham and Fink, 1994). The *pmc1* null mutants also display a severe sensitivity to Ca^{2+} supplements in the growth medium. Although it is likely that Pmc1p transports Ca^{2+} into the vacuole, further biochemical experiments are necessary to demonstrate its activities. The biochemical properties of Pmc1p should be resolved through studies of isolated Pmc1p or of vacuole membrane vesicles prepared from mutants specifically lacking the H^+/Ca^{2+} antiport activity.

Pmr1p: a secretory Ca^{2+} pump

Another putative Ca^{2+} pump localizes to the Golgi complex or related secretory compartments and is encoded by the *PMR1* gene (Antebi and Fink, 1992; Rudolph *et al.* 1989). Pmr1p is approximately 50 % identical to a P-type ion pump of unknown function that is expressed in many animal tissues (Gunteski-Hamblin *et al.* 1992), approximately 30 % identical to members of the SERCA sub-family, which are Ca^{2+}-ATPases found in the sarcoplasmic/endoplasmic reticulum of animal cells, and less than 25 % identical to other ion pumps (Fig. 1). The biochemical activities of Pmr1p have not been investigated, but several lines of genetic evidence suggest that it functions as a primary Ca^{2+} transporter supplying Ca^{2+} to compartments in the secretory pathway (Antebi and Fink, 1992; Rudolph *et al.* 1989). Mutants lacking Pmr1p function secrete abnormal proteins that have not been proteolytically cleaved by a Ca^{2+}-dependent protease located in a late Golgi compartment, though this defect and others can be remedied by supplementing the growth medium with Ca^{2+} concentrations greater than 10 mmol l^{-1}. Decreasing extracellular Ca^{2+} to below 1 μmol l^{-1} causes a severe growth defect in *pmr1* null mutants (Antebi and Fink, 1992; Rudolph *et al.* 1989). These and other results (see below) strongly suggest that Pmr1p functions as a primary Ca^{2+} transporter that supplies the Golgi with the Ca^{2+} required for specific secretory functions.

Ca^{2+} transport in the endoplasmic reticulum

Ca^{2+} is generally thought to play important roles in protein traffic in the endoplasmic reticulum (ER) and related secretory compartments (Sambrook, 1990). To date, there is no evidence in yeast for the existence of authentic SERCA-type Ca^{2+} pumps. With the possible exception of Pmr1p, the known P-type ATPases in yeast are not homologous to the SERCA family members from plants and animals and have functions unrelated to Ca^{2+} transport. The *PMA1* gene encodes the major P-type H^+-ATPase of the plasma

membrane (Serrano *et al.* 1986) and *PMA2* encodes a transporter of unknown function that is 90 % identical to the *PMA1* gene product (Schlesser *et al.* 1988). Pmr2p, which is encoded by at least four tandemly repeated genes, is likely to be a plasma membrane ion pump involved in Na^+ and Li^+ efflux but not in Ca^{2+} transport (Garciadeblas *et al.* 1993; Haro *et al.* 1991; Rudolph *et al.* 1989). The predicted products of *PMR2* genes are more than 97 % identical to each other and have high degree of similarity to the *CTA3* gene product of the fission yeast *Schizosaccharomyces pombe* (Fig. 1) that has been implicated in Ca^{2+} metabolism (Ghislain *et al.* 1990; Halachmi *et al.* 1992). Finally, the *DRS2* gene is expected to encode a highly divergent P-type ion pump of unknown catalytic function (Ripmaster *et al.* 1993). Whether any of these proteins (Pma1p, Pma2p, Pmr2p or Drs2p) is involved in Ca^{2+} transport into the ER or other membrane compartments is not known, but unidentified Ca^{2+} transporters have been measured in some membrane preparations (Hiraga *et al.* 1991; Okorokov *et al.* 1993).

Ca^{2+}-sensitive mutants

A genetic approach towards identifying important factors in Ca^{2+} metabolism has been to isolate yeast mutants with altered responses to Ca^{2+} in the growth medium (Ohya *et al.* 1984, 1986). Wild-type yeast strains can grow in media containing more than 100 mmol l^{-1} Ca^{2+}, but recessive mutations in at least 18 genes abolish growth under these conditions (Ohya *et al.* 1986). Many of these genes appear to be necessary for maintaining the proper structure or function of the vacuole. Mutations that inactivate subunits of the vacuolar H^+ V-ATPase or other factors necessary for acidification of the vacuole lumen cause extreme sensitivity to added $CaCl_2$ and cause about a sixfold elevation in $[Ca^{2+}]_i$ in standard media as measured in single cells using the fluorescent indicator Fura-2 (Ohya *et al.* 1991). Deactivation of the vacuolar H^+/Ca^{2+} antiporter has been proposed to explain these effects, but other indirect mechanisms are also possible.

A second search for mutants specifically sensitive to Ca^{2+} revealed the *CSG2* gene (Beeler *et al.* 1994). In response to elevated external $[Ca^{2+}]$, *csg2* mutants accumulate Ca^{2+} into an exchangeable pool rather than into the non-exchangeable (vacuolar) pool. Therefore, Csg2p may normally function to promote Ca^{2+} efflux from this unidentified compartment, to inhibit Pmr1p or another non-vacuolar Ca^{2+} transporter, or to influence cellular Ca^{2+} flow by a more indirect mechanism (Beeler *et al.* 1994). *CSG2* is identical to *CLS2* identified in the screen for Ca^{2+}-sensitive mutants described above (Y. Takita, Y. Ohya and Y. Anraku, in preparation). The predicted product of *CSG2/CLS2* has no significant similarity to other protein sequences, but contains multiple membrane-spanning domains and is localized to the ER (Y. Takita, Y. Ohya and Y. Anraku, in preparation). Further biochemical and genetic analyses may clarify the function of this interesting protein and define the roles of the other *CLS* genes in Ca^{2+} tolerance.

Ca^{2+} flow and dynamics

A working model of Ca^{2+} metabolism that takes into account the new findings is depicted in Fig. 2. Yeast cells growing in standard media (approximately 0.2 mmol l^{-1}

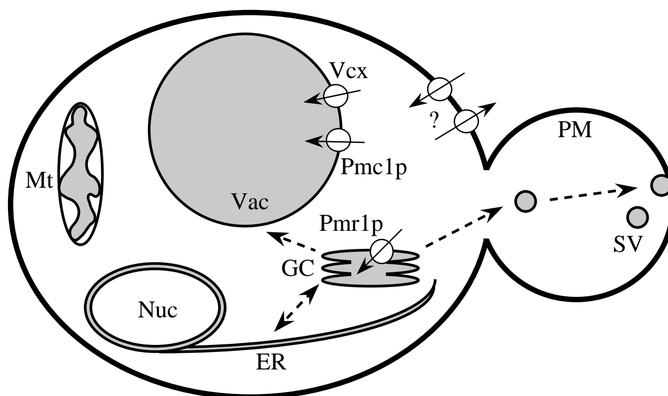

Fig. 2. Working model for Ca^{2+} flow in growing yeast cells. Solid arrows indicate Ca^{2+} movement through membranes catalyzed by putative channels (?), pumps (Pmc1p and Pmr1p) and the vacuolar H^+/Ca^{2+} antiporter (Vcx). Dashed arrows represent vesicle-mediated trafficking of proteins and presumed paths of Ca^{2+} flow. Vac, vacuole; Mt, mitochondria; Nuc, nucleus; ER, endoplasmic reticulum; GC, Golgi complex; SV, secretory vesicles; PM, plasma membrane.

Ca^{2+}) would take up Ca^{2+} from the medium *via* the opening of unidentified Ca^{2+} channels in the plasma membrane or through other unspecified mechanisms. The steady-state $[Ca^{2+}]_i$ of about 0.1 μmol l^{-1} is maintained through the combined action of Pmr1p in the Golgi complex, Pmc1p in the vacuole, the H^+/Ca^{2+} antiporter in the vacuole and possibly unidentified transporters in other organelles such as the plasma membrane. It also seems likely that vesicle-mediated transport processes ultimately contribute to the sequestration of Ca^{2+} in the vacuole and export of Ca^{2+} from the cell concomitant with protein targeting. Since Pmr1p and other Ca^{2+} transporters are presumably synthesized in the ER and then sorted to their final destinations, their operation within secretory compartments might significantly affect the overall flow of Ca^{2+} in the cell (Fig. 2). Yeast mitochondria accumulate little Ca^{2+} and their role in Ca^{2+} metabolism is poorly understood (Carafoli *et al.* 1970; Uribe *et al.* 1992).

Pulse-chase experiments using $^{45}Ca^{2+}$ have revealed two major intracellular ‘pools’ that accumulate Ca^{2+}. The smaller pool is highly exchangeable with external Ca^{2+} with a half-time of approximately 2 min (Cunningham and Fink, 1994; Eilam, 1982*a*,*b*) and probably reflects the portion of Ca^{2+} in the cytosol or in secretory organelles that can be exported from the cell. More than 90 % of the total cell-associated Ca^{2+} accumulates in a non-exchangeable pool in growing cells and this pool is largely confined to the vacuole (Eilam *et al.* 1985; Ohsumi *et al.* 1988). Estimates of vacuolar Ca^{2+} content range from 1 to 4 mmol l^{-1} in wild-type cells grown in standard media, but it is likely that intravacuolar free Ca^{2+} concentrations $[Ca^{2+}]_v$ are effectively much lower due to buffering by soluble inorganic polyphosphates (Dunn *et al.* 1994). Ca^{2+} can be completely released from isolated vacuoles or from whole cells using the ionophores A23187 or ionomycin, suggesting that the non-exchangeable pool of Ca^{2+} is soluble. As expected for vacuolar Ca^{2+} transporters, accumulation of Ca^{2+} into the non-exchangeable pool is decreased fivefold in *pmc1* null mutants relative to *PMC1* strains (Cunningham and Fink, 1994).

Conversely, the non-exchangeable pool is significantly increased in *pmr1* null mutants, presumably as a consequence of decreased Ca^{2+} accumulation in the secretory pathway and export (K. W. Cunningham and G. R. Fink, in preparation). Together, these findings suggest that the vacuole is a major Ca^{2+} sink in yeast.

Yeast cells grow very well at a wide range of environmental Ca^{2+} concentrations from less than $1\,\mu mol\,l^{-1}$ to more than $100\,mmol\,l^{-1}$ and can adapt to large and rapid fluctuations in extracellular [Ca^{2+}] (Anraku *et al.* 1991). Exponentially growing cells arrest transiently in the G1 phase of the cell division cycle after addition of A23187 plus EGTA (a chelator of Ca^{2+} and other ions) to the medium (Iida *et al.* 1990*a*). In response to increasing extracellular [Ca^{2+}], wild-type cells dramatically increase the non-exchangeable pool of Ca^{2+} (Beeler *et al.* 1994; Dunn *et al.* 1994), which probably reflects an increased rate of Ca^{2+} sequestration due to elevated levels of $[Ca^{2+}]_i$ (Halachmi and Eilam, 1993). Mutants lacking *PMC1* grow poorly in media containing a high [Ca^{2+}], although growth can be restored by overexpression of *PMR1* or the cloned antiporter gene, which implies that the rate of Ca^{2+} sequestration is growth-limiting under these conditions (Cunningham and Fink, 1994; K. W. Cunningham and G. R. Fink, in preparation). At Ca^{2+} concentrations below $10\,\mu mol\,l^{-1}$, mutants lacking *PMR1* fail to grow (Rudolph *et al.* 1989), but growth can be restored by overexpression of *PMC1*. Strains simultaneously deleted for *PMC1* and *PMR1* are inviable at all Ca^{2+} concentrations. These results are consistent with a model in which Pmc1p and Pmr1p function redundantly in Ca^{2+} sequestration, although they have distinct essential roles in response to either high or low extracellular [Ca^{2+}], respectively. The simple model of Ca^{2+} metabolism (Fig. 2) is sufficient to accomplish the cellular goals of maintaining $[Ca^{2+}]_i$ at tolerable levels and supplying Ca^{2+} to various internal compartments at a wide range of extracellular Ca^{2+} concentrations. Almost certainly, though, this model will be amended as new transporters are identified and as the modes of transporter regulation become understood.

Ca^{2+} signaling

Until recently, Ca^{2+} signals and signaling factors were thought to have only minor effects on cellular processes in yeast. Mutants expressing a defective calmodulin that is unable to bind Ca^{2+} with high affinity do not display any obvious defects in growth, mating, sporulation or various stress responses, suggesting that any signaling mediated by this factor is not required for any of these processes (Geiser *et al.* 1991). Initial reports did not identify any phenotype of mutants lacking calmodulin-dependent protein kinases (Ohya *et al.* 1991; Pausch *et al.* 1991) and identified only subtle effects of inactivating calcineurin (Cyert *et al.* 1991; Cyert and Thorner, 1992; Foor *et al.* 1992). However, clear effects of calcineurin mutations have now been observed in several new conditions. Calcineurin function appears to be necessary for growth in media containing high levels of Na^{+} and Li^{+} (Nakamura *et al.* 1993) and for the maximum induction of the *PMR2* gene in response to these conditions (Mendoza *et al.* 1994). Additionally, the Ca^{2+}- and calmodulin-dependent activation of calcineurin appears to inhibit growth of *pmc1* null mutants in high-Ca^{2+} medium (Cunningham and Fink, 1994) and to induce the expression of several other genes (K. W. Cunningham and G. R. Fink, in preparation). Identification of the targets of activated calcineurin should provide not only a useful reporter for Ca^{2+}

signaling events but also crucial information about the processes wherein Ca^{2+} signaling plays important roles.

Powerful methods of monitoring $[Ca^{2+}]_i$ in living yeast cells are now available. The fluorescent indicator Indo-1 (Halachmi and Eilam, 1989; Halachmi and Eilam, 1993) and the luminescent protein aequorin (Nakajima *et al.* 1991) have been used successfully to estimate $[Ca^{2+}]_i$ in cell suspensions, whereas Fura-2 has been employed to image Ca^{2+} in single cells (Iida *et al.* 1990*b*; Ohya *et al.* 1991). Despite the technical difficulties associated with these techniques, the ability to combine molecular genetics and cell physiology offers great promise for the future.

Future prospects

Although the key participants in Ca^{2+} transport and signaling in yeast are rapidly becoming reasonable well understood, many important questions remain to be answered. Are the yeast Ca^{2+} channels similar to those of other species? When and how are natural Ca^{2+} signals produced? What are the physiological responses to these signals? Are these processes related to Ca^{2+} metabolism and signaling in plant and animal cells? The complete understanding of the catalytic and regulatory factors that act in a coordinated manner to produce Ca^{2+} signals and control Ca^{2+} metabolism will ultimately require the concerted application of many different approaches. The genetic and molecular tools available in yeast promise to add an exciting new perspective to the basic mechanisms of Ca^{2+} transport and signaling.

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