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Minireview

Calcium, calmodulin and cell cycle regulation

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Abstract

Calcium and its ubiquitous intracellular receptor calmodulin are required for cell proliferation. Studies in a variety of model systems are beginning to identify components of the calcium/calmodulin cascade required for movement of quiescent cells into the cell cycle as well as for proliferating cells to move from G_1 to S, G_2 to M and through mitosis. Two calcium/calmodulin-dependent enzymes, the multifunctional calcium/calmodulin-dependent protein kinase and the protein phosphatase 2B (calcineurin) as well as a spindle pole body protein that binds calmodulin in the absence of calcium have been shown to be essential at specific phases of the cell cycle. In addition, the status of the intracellular calcium pools is critical for normal traverse of the cell cycle.

Key words: Calcium; Calmodulin; Cell cycle; Protein kinase; Protein phosphatase

As one of the predominant intracellular second messengers, it is little wonder that Ca^{2+} is involved in the regulation of cell proliferation. This ion and its primary receptor protein calmodulin are essential for cells to survive and grow. Calcium and calmodulin have been implicated in the re-entry of quiescent cells into the proliferative cycle as well as for traversing the G_1/S, G_2/M and metaphase/anaphase boundaries of the cell cycle [1, 2]. However, because of the ubiquitous nature of this dynamic duo, and the plethora of cellular events they regulate, it has been difficult to ascribe to them anything more than a supporting or housekeeping role in mammalian cell cycle regulation. In stark contrast, Ca^{2+} is both the necessary and sufficient signal for resumption of meiosis in quiescent marine oocytes and eggs awaiting fertilization [2]. The frog egg, sea urchin egg and surf clam oocyte are arrested in meiotic metaphase, G_1 (before START) and G_2, respectively. In these cases, a transient increase in intracellular Ca^{2+} is the physiological trigger used to initiate a cascade of events resulting in completion of meiosis and initiation of subsequent mitotic divisions.

Perhaps most is known regarding the role of Ca^{2+} and calmodulin in *Xenopus* fertilization. The fully grown frog oocytes are arrested in prophase of meiosis I. Progesterone results in a release of this block and cells progress through meiosis I to metaphase of meiosis II where they again arrest. Sperm entry normally awakens these quiescent eggs and the primary regulatory event is the release of Ca^{2+} from intracellular stores. The original block in prophase of meiosis I is due to formation of a protein complex called Cytostatic Factor or CSF. Paradoxically this complex includes the protooncogene c-*mos*, a protein kinase that is involved earlier in the pathway in the activation of Maturation Promoting Factor (MPF). MPF is composed of the $p34^{cdc2}$ protein kinase and its regulatory cyclin B_1 subunit and is required for the breakdown of the germinal vesicle membrane (GVBD). Following GVBD, the MPF is also required for the progression of meiosis and, upon arrest at metaphase of the second meiotic division, both MPF and CSF are active. Thus fertilization and the requisite Ca^{2+} increase, must inactivate both CSF and MPF in order for meiosis to resume.

A variety of microinjection experiments have been used to evaluate the temporal sequence of events set into play by the Ca^{2+} transients. Microinjection of Ca^{2+}, inositol-1,4,5-trisphosphate (IP_3), and Ca^{2+}/calmodulin were shown to activate whereas anticalmodulin drugs, calmodulin binding peptides and c-*mos* were found to be inhibitors of the Ca^{2+} signal [2]. Subsequent to the Ca^{2+} signal and formation of Ca^{2+}/calmodulin, various investigators showed destruction of cyclic B_1 and c-*mos*, as well as inactivation of CSF measured by the ability of an oocyte extract to induce meiotic arrest [3–5]. Since it was clear that a Ca^{2+}/calmodulin pathway was involved, the question was to identify the relevant Ca^{2+}/calmodulin target protein. This was accomplished by Lorca et al. [6] who took advantage of reagents made possible due to the molecular understanding of the anatomy of calmodulin-dependent protein kinases.

Calmodulin-dependent kinases composed of a single polypeptide chain are inactive in the absence of Ca^{2+}/calmodulin. This inhibition is due to a portion of the protein assuming a conformation that prevents access of

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the protein substrate and has been termed intrasteric inhibition [7,8]. This autoinhibitory region is contiguous or overlapping with the Ca^{2+}/calmodulin binding region. Binding of Ca^{2+}/calmodulin results in relief of this autoinhibition and allows phosphorylation of substrate proteins. Because of this anatomy, it has been possible to create three biological peptidic reagents each with a unique property. These three reagents are: (i) a Ca^{2+}/calmodulin binding peptide [9]; (ii) a constitutively active enzyme created by deletion of the autoregulatory domain [10]; and (iii) a peptide analog of the autoinhibitory region that does not bind Ca^{2+}/calmodulin but will serve as a competitive inhibitor of the enzyme [11,12]. Lorca et al. [5] first showed that a Ca^{2+}/calmodulin binding peptide based on the corresponding region of smooth muscle myosin light chain kinase would prevent the Ca^{2+} induced activation of *Xenopus* eggs. This arrest could be overcome by the co-injection of a constitutively active form of a Ca^{2+}/calmodulin-dependent protein kinase IIα (CaM kinase IIα) [6]. Finally the effect of this fragment of CaM kinase IIα could be prevented by co-injection of the synthetic peptide based on the autoinhibitory sequence of this enzyme. Confirmation that a CaM kinase II-like enzyme represents the relevant Ca^{2+}/calmodulin target in vivo came from the demonstration that Ca^{2+} caused the rapid activation of a kinase in the egg that: (a) phosphorylated a known peptide substrate of CaM kinase II; (b) was inhibited by the Ca^{2+}/calmodulin binding peptide; and (c) was inhibited by the autoinhibitory peptide but not by another peptide based on the autoinhibitory region of protein kinase C [6].

Even though Lorca et al. [6] presented compelling evidence that CaM kinase mediates the Ca^{2+}/calmodulin stimulation of the resumption of meiosis in *Xenopus* eggs, the nature of the immediate downstream target of this kinase is unclear. Inactivation of CSF seems to be secondary to the destruction of cyclin B_1 which also signals the inactivation of MPF [3]. Cyclin B_1 is rapidly proteolyzed following ubiquitination [13]. Thus a logical target for CaM kinase II would be a rate limiting component of the ATP-dependent ubiquitin protein cascade. Surely the frog egg system is a good one in which to evaluate this possibility.

Additional evidence in a variety of experimental systems also suggests a role for a CaM kinase in G_2/M progression. Baitinger et al. [14] have reported that this enzyme mediates the Ca^{2+}/calmodulin requirement for nuclear envelope breakdown that is required for resumption of meiosis following fertilization of sea urchin eggs. Planas-Silva and Means [15] revealed that inducible expression of a constitutively active form of CaM kinase IIα in a mouse cell line resulted in a G_2 arrest. These observations imply that not only is a CaM kinase-dependent phosphorylation event(s) required for G_2/M progression but this substrate(s) must also be dephosphorylated for entry into mitosis to occur.

Creation of a strain of the filamentous fungus *Aspergillus nidulans* that is conditional for the expression of calmodulin, not only was used to show that the unique calmodulin gene was essential, but also that Ca^{2+} and calmodulin are required for spores to re-enter the proliferative pathway (G_0 reentry), G_1/S, G_2/M and metaphase of mitosis [16]. The latter two arrest points are consistent with the results in the marine egg systems described previously (i.e. *Xenopus* and sea urchin) and raised the question of whether CaM kinase might also mediate the progression into and exit from mitosis in *Aspergillus*. The *Aspergillus* CaM kinase was first purified by Bartelt et al. [17] and the cDNA was cloned and sequenced by the same laboratory [18]. Although a monomer and only 29% identical to mammalian CaM kinase IIα, it phosphorylates many of the same substrates in vitro. We have found this gene to be unique and essential and overexpression of a constitutively active form in exponentially growing cells arrests cells in G_2. At the G_2 arrest point caused when calmodulin (or Ca^{2+}) levels are held low, two protein kinases that are essential for the G_2/M transition in *Aspergillus* are inactive [19]. These kinases are $p34^{cdc2}$ which remains tyrosine phosphorylated and NIMA, a Ser/Thr specific protein kinase that requires phosphorylation on Ser/Thr residues to exhibit activity [20], and is now known to have a homolog in mammalian cells [21]. Preliminary experiments in our laboratory suggest that CaM kinase may be involved in activation of both enzymes. The *Aspergillus* CaM kinase will phosphorylate and restore some activity in vitro to bacterially expressed, dephosphorylated and inactive NIMA. Calmodulin kinase will also phosphorylate NIMT which is the homolog of cdc25, the tyrosine phosphatase that dephosphorylates and thereby activates $p34^{cdc2}$. Patel et al. [22] have also reported that mammalian CaM kinase IIα will phosphorylate the human cdc25 homolog ($p54^{cdc25H}$) and that this phosphorylation increases phosphatase activity by several fold. Thus CaM kinase may be a primary mediator of the requirement for Ca^{2+}/calmodulin both for entry into and exit from mitosis.

Whereas CaM kinase is likely to be important in mediating the Ca^{2+}/calmodulin requirement for the G_2/M transition and the metaphase anaphase transition, other targets for calmodulin clearly exist. In the budding yeast *Saccharomyces cerevisiae*, the calmodulin gene CMD1 is also unique and essential [23]. However, at least two CaM kinase genes exist in this organism and even when both genes are deleted, no obvious phenotype was observed [24,25]. At least two of the essential functions of calmodulin in yeast are chromosome segregation and the polarized growth required for bud formation [26,27]. The unexpected finding made by Geise et al. [28] was that these essential functions of calmodulin were independent of Ca^{2+} binding. In an elegant series of experiments Geiser et al. [29] identified the essential mitotic target of calmodulin as the 100-kDa component of the

spindle pole body called Nuf1p or SPC110. This interaction does not require Ca^{2+} and calmodulin/Nuf1p are co-localized to the spindle pole body in yeast cells. Recently Sprang et al. [30] have identified the product of the CDC31 gene also to be a component of the spindle pole body in yeast. This gene is also essential and encodes a 161 amino acid Ca^{2+} binding protein with a striking homeology to calmodulin. Since the spindle pole body in budding yeast is homologous to the centromere of mammalian cells and since calmodulin is localized to the spindle poles throughout mitosis in mammalian cell [31], it is probable that essential mitotic functions for calmodulin exist in other species that are also independent of Ca^{2+} and presumably not related to a enzyme regulatory role. Examination of this possibility in other genetically tractable systems in which calmodulin is essential should prove illuminating.

On the other hand other essential functions for calmodulin in yeast remain to be discovered. Ohya and Botstein [32] have individually mutated the phenylalanine residues in the CMD1 gene and selected temperature sensitive phenotypes for evaluation. The mutations were classified into four complementation groups affecting actin or calmodulin organization, nuclear division or bud emergence. Interestingly all mutations affecting calmodulin localization and bud emergence were located in the NH_2-terminal half of the protein, whereas actin organization or bud emergence phenotypes tracked to the COOH-terminal half. Whether these functions of calmodulin require Ca^{2+} binding and identification of the respective targets, remain to be determined.

Calcium and calmodulin are also required for quiescent cells to re-enter the cell cycle in response to a mitogen and at the G_1/S boundary where the concentration of calmodulin is controlled [1]. Considerably less is known regarding relevant target proteins for Ca^{2+}/calmodulin at these critical progression control points. Calmodulin kinase has been implicated in the re-entry pathway as it will phosphorylate the CREB transcription factor and regulate expression of immediate early response genes such as the protooncogene c-*fos* [33]. It has also been reported that the quite selective pharmacological inhibitor of CaM kinase, KN-93 [34], will prevent quiescent ws-1 human fibroblasts from reaching S phase upon serum stimulation [35]. Since some cellular events that occur in G_0 are unique to this phase of the cell cycle, there is no reason to exclude CaM kinase as a component of the regulatory cascade that signals G_0. However, it is possible that the multifunctional CaM kinases may not prove to be as critical for the progression from mitosis to DNA synthesis. Preliminary data suggest that addition of KN-93 will specifically arrest exponentially growing HeLa cells in G_2 (C. Rasmussen, personal communication). In *Aspergillus*, CaM kinase appears to be required for spores to initiate DNA synthesis upon germination and for G_2 progression but not for the movement of cells through G_1. In the fungal system, it seems probable that one relevant Ca^{2+}/CaM target required for the G_1/S transition is the protein Ser/Thr phosphatase 2B known as calcineurin. The catalytic subunit gene of this enzyme has been cloned shown to be unique and essential [36]. In the absence of calcineurin A, cells arrest early in the cell cycle, most likely in G_1. This observation is consistent with studies in other systems in which calcineurin is required for growth and could act early in the cell cycle. Three genes encoding calcineurin subunits, 2A and 1B, have been cloned from budding yeast. Whereas deletion of all three genes in the same strain was not lethal, such cells cannot survive α factor, which normally arrests cell before START [37]. It has also been reported that calcineurin A mediates inhibition of recovery from α factor arrest resulting from treatment of cells with the immunosuppressive drugs FK-506 and cyclosporin A [38]. As calcineurin is the primary intracellular target for these drugs when associated with their respective binding proteins, it follows that this phosphatase is required for resumption of growth in yeast. Finally, calcineurin is known to be involved in the cascade of events triggered by activation of the T-cell receptor on quiescent lymphocytes [39]. This enzyme is normally responsible for dephosphorylation of a component of the NFAT transcription factor. NFAT and other transcription factors are required to activate genes encoding lymphokines such as IL-2 which are involved in initiation of cell cycle progression. Thus, in organisms as diverse as budding yeast, filamentous fungus and mammalian cells, a case can be made for a calcineurin requirement in G_0/G_1.

It should be mentioned that Ca^{2+} and intracellular Ca^{2+} pools may also play important roles in cell proliferation independent of calmodulin. For example depletion of the IP_3 sensitive intracellular Ca^{2+} pool with pharmacological inhibitors such as thapsigargin, results in cell cycle arrest [40]. We have shown that G_0/G_1 and S phases are sensitive to pool depletion whereas G_2 and M are not. These growth inhibitory effects are independent of intracellular free Ca^{2+} levels and remarkably, pool depletion at any sensitive portion of the cell cycle resets the cell cycle to a state very similar to G_0. Pool depletion also activates a capacitive Ca^{2+} entry pathway through a subset of plasma membrane associated Ca^{2+} channels [41], about which nothing is known biochemically. Preventing Ca^{2+} entry via this pathway arrests cells in G_2 or M even though such cells are not affected by intracellular pool depletion. This entire area is only now becoming appreciated and will be an important component of Ca^{2+} research in the future. These few observations illustrate how complicated the Ca^{2+} field has become but also how important unraveling these mechanism will be to gain a solid appreciation of the multiple roles played by Ca^{2+}, calmodulin and the components that control intracellular Ca^{2+} homeostasis in regulation of cell proliferation.

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