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Topical Review

Involvement of Sarco/endoplasmic Reticulum Ca^{2+} ATPases in Cell Function and the Cellular Consequences of Their Inhibition

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Introduction

Calcium serves as an important signaling molecule that modulates diverse cellular processes. Elevation of cytosolic Ca^{2+} ($Ca^{2+}{}_i$) follows the activation of many cell surface receptors as well as depolarization of neuronal and muscle tissues [4]. Such Ca^{2+} transients can regulate gene transcription in a variety of cells including, among others, those of neuronal [24, 25, 90], cardiac [65] and endocrine [105] origins. Cytosolic Ca^{2+} can also modify the function of certain classes of enzymes like the kinases, phosphatases and phospholipases [16, 71]. Cellular Ca^{2+} is intimately involved in progression through the different phases of the cell cycle [6, 13, 56, 66]. A consistent feature around mitosis is the occurence of Ca^{2+} transients [32, 55]. These Ca^{2+} transients regulate both entry of the fertilized egg to the next stage of the cell division cycle, and exit of somatic cells from mitosis [55]. Finally, a rise in $Ca^{2+}{}_i$ is a key event in initiating muscle contraction [20]. In addition to entry of extracellular Ca^{2+}, to a large extent, cytosolic Ca^{2+} signals are generated from intracellular Ca^{2+} stores that have sequestered Ca^{2+} within them. Ca^{2+} within the intracellular Ca^{2+} stores serves important biological functions in its own right [67], regulating processes like protein synthesis (for example, by modifying the phosphorylation status of initiation factor 2 [95]), protein processing (by affecting function of luminal chaperones like Bip [31]), membrane trafficking between the endoplasmic reticulum and golgi complex [42], nucleo-cytoplasmic transport [75], and regulation of the store-operated calcium currents I_{CRAC} [73].

The sarco/endoplasmic reticulum Ca^{2+} transport ATPase (SERCA) plays a fundamental role in regulating cytosolic Ca^{2+} signals, as well as Ca^{2+} within the endoplasmic reticulum (ER), the sarcoplasmic reticulum (SR) and the nuclear envelope [77], through the establishment and maintenance of intracellular Ca^{2+} stores. Its primary function is to accumulate and maintain Ca^{2+} within the intracellular stores against a steep concentration gradient via ATP-dependent transport. In muscle tissues SERCAs help reaccumulate Ca^{2+} into the SR during the relaxation phase of muscle contraction.

SERCA Function

Three principle isoenzymes of SERCA (i.e., SERCA1, 2 and 3) have been cloned and their splice variants identified [10, 28, 57, 58]. The SERCA1 gene encodes two alternatively spliced isoforms, SERCA1a (adult) and SERCA1b (fetal), which are expressed exclusively in fast twitch skeletal muscle [8, 9, 59]. The SERCA2 gene also encodes two alternatively spliced isoforms, SERCA2a and SERCA2b, which diverge in their C-termini [28, 58]. SERCA2a is the predominant isoform expressed in heart and slow twitch skeletal muscle [2], while SERCA2b is expressed in multiple tissues, including smooth muscle and most nonmuscle tissues [28, 58]. Two isoforms of SERCA3 have also been recently

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cloned and characterized [78], and are found primarily in endothelial, epithelial and hematopoietic cells [1, 10, 107].

The overall topology and mechanism of action of the various SERCA isoforms are similar [38, 60]. After two molecules of cytosolic Ca^{2+} bind cooperatively to each molecule of SERCA, utilization of ATP occurs within the cytosolic head of the enzyme, resulting in the formation of a phosphorylated intermediate (EP). Consequent conformational changes in EP cause release of bound Ca^{2+} into the lumen of the ER/SR. With respect to the binding of Ca^{2+} to SERCA, earlier mutational studies provided indirect evidence for the involvement of six amino acid residues within four transmembrane domains (M4, M5, M6, and M8) of SERCA [17]. Improvements in expression and recovery of exogenous SERCA has recently allowed a more direct demonstration of Ca^{2+} binding defects in mutant SERCAs [97], confirming earlier reports on Ca^{2+} binding. Interestingly, mutations in the cytosolic loop between transmembrane domains M6 and M7 also affect Ca^{2+} binding [21], perhaps introducing structural perturbations near the Ca^{2+} binding domain.

Transient [64] and stable [36] expression of SERCA-encoding cDNAs in mammalian cell lines have been used to study various aspects of SERCA function. More recently, adenovirus-mediated gene transfer of SERCA isoforms in cardiac myocytes has been utilized to study SR Ca^{2+} transport [26, 40]. Transgenic mouse models have also been developed to study cardiac function in vivo [30, 43]. Although the patterns of expression of the SERCA transgenes and endogenous genes can be complex [30, 43], the fact that the fast-twitch skeletal muscle SERCA1a isoform exhibits functional properties that are similar to the cardiac SERCA2a isoform when expressed within the context of a cardiac background [43] suggests that a high degree of functional homology exists between the various isoforms of SERCA. This is also reflected in studies addressing the regulation of SERCA function with phospholamban, which under normal physiological conditions modifies SERCA2a function [93]. However, in vitro as well as in vivo studies utilizing transgenic expression of phospholamban in fast-twitch skeletal muscle demonstrate that it can also regulate SERCA1 function [94].

A mouse knockout model, in which one of the two SERCA2 genes was disrupted by gene targeting, has been described [76]. Although SERCA2 null mutant mice could not be obtained, in the heterozygous mutants cardiac function was impaired, suggesting that for normal cardiac function both copies of the SERCA gene are necessary [76]. In contrast to SERCA2, null mutants with respect to the SERCA3 gene, which has a much more restricted tissue distribution pattern, can be obtained via gene targeting. Even though no overt pathophysiological abnormalities are observed in such mutants, defects in endothelial cell Ca^{2+} signaling and endothelium-dependent vascular smooth muscle relaxation are seen [53]. Since SERCA2b continues to be expressed in these endothelial cells but does not appear to provide compensation for the SERCA3 deficit [53], this underscores the fact that the various SERCA isoforms can subserve overlapping as well as distinct physiological functions. These latter observations are also consistent with the recent demonstration that distinct subcellular distribution patterns of SERCA2 and SERCA3 can exist in certain epithelial cells [49].

Recently, potential changes/disruption in SERCA function as a consequence of certain mutations within specific SERCA genes have been linked to the two human disorders Brody disease and Darrier-White disease [72, 88]. In Brody disease, which is a rare recessive skeletal muscle disorder of muscle relaxation, mutations within the SERCA1 gene are found [72]. In Darrier-White disease, which is a dominantly inherited disorder characterized by abnormal skin plaques, blisters and dyskeratosis, a variety of alterations within the SERCA2 gene (including deletions, insertions and missense mutations) have been reported [88]. Altered Ca^{2+} signaling within the epidermal cells due to loss of SERCA2 function may contribute to abnormal desmosomal attachments between cells within the epidermis, resulting in some of the clinical manifestations seen in this disease [88].

Regulation of SERCA Expression and Function

Due to a central role of SERCAs in Ca^{2+} homeostasis, it is not surprising that regulation of SERCA expression is rather complex and not completely understood. Expression of SERCA is not only developmentally regulated, but also occurs in a tissue-specific manner. For instance, during fast-twitch skeletal muscle development from fetal/neonatal to adult stage SERCA2a is replaced by SERCA1b [2], while in slow-twitch skeletal muscles and cardiac muscles SERCA2a remains the predominant isoform in both fetal and adult stages [22].

Certain hormones [85, 111] and growth factors like PDGF [61, 62] can regulate expression of SERCA. In aortic smooth muscle cells (SMC), PDGF appears to preferentially upregulate SERCA2a [61, 62]. Furthermore, this upregulation coincides with the entry of SMC from G1 into S phase of the cell cycle, and seems dependent upon the Ca^{2+} influx induced by the PDGF treatment of cells [62]. In contrast, SERCA2b is not modulated by PDGF, suggesting that SERCA2a and 2b may serve different but potentially complimentary physiologic roles in response to PDGF-induced stimulation [62]. However, it is not at all clear how such differential regulation comes about since both SERCA2a and 2b are

likely to be controlled by the same *cis*-regulatory promoter elements. Another level of complexity with respect to SERCA regulation is observed during T cell activation [48]. Mobilization of cellular Ca^{2+} and activation of protein kinase C (PKC) (i.e., conditions that activate T cells) lead to isoform-specific modulation of SERCA, with SERCA3 levels decreasing by approximately 90% and SERCA2 levels increasing twofold [48]. Although the biological significance and the molecular basis for such differential regulation is not clear, calcineurin-dependent signaling has been implicated in modulating SERCA expression in the T lymphocytes [48].

In addition to being regulated by certain hormones and growth factors, SERCA expression and function can be affected by various pathophysiological states. SERCA2a expression decreases in instances of pathologic cardiac hypertrophy such as pressure overload-induced hypertrophy, as well as in hypothyroid states [3, 11]. Physiologic cardiac hypertrophy and thyrotoxic cardiomyopathy, on the other hand, are associated with increased levels of SERCA2a mRNA [11, 84]. Thyroid hormone response elements have been identified in both the rat and rabbit SERCA2 promoters [84], and may in part mediate some of the thyroid hormone-dependent SERCA2 responses. Multiple factors are involved in the development of both physiologic and pathologic cardiac hypertrophy, with norepinephrine (NE) playing a significant role primarily in the latter [69]. Interestingly, SERCA2 expression is differentially modulated by thyroid hormone and NE in ventricular myocytes in culture, with the responses in part dependent upon the contraction state of the cells [69]. The specific signaling pathways utilized by NE and thyroid hormone may account for the differences in response of SERCA to these agonists, although it is not yet clear whether such differential effects occur at the level of transcription [69]. The Raf-MEK-ERK signaling pathway has been implicated in the development of some of the molecular changes associated with cardiac hypertrophy and failure, including downmodulation of SERCA2 expression [33]. Abnormalities in Ca^{2+} homeostasis can also modify SERCA levels. For instance, mutant "leaky" ryanodine receptors that are associated with higher basal levels of cytosolic Ca^{2+} can result in induction of SERCA2, perhaps as a compensatory response [100].

The ubiquitous second messenger nitric oxide (NO) has been implicated in diverse biological processes, including regulation of cellular Ca^{2+} homeostasis. It is produced by the action of nitric oxide synthase (NOS) on endogenous substrates like L-arginine, and subsequently modulates the various cellular effects via both cGMP-dependent and -independent pathways [81, 96]. Endothelium-dependent vascular smooth muscle vasodilation is dependent upon NO-mediated regulation of cytosolic Ca^{2+} levels. Recent studies with primary cultures of aortic smooth muscle cells suggest that NO increases SERCA-dependent uptake of $Ca^{2+}{}_i$, with the consequent refilling of the intracellular stores resulting in inhibition of agonist-induced capacitative Ca^{2+} influx [18]. Other studies have suggested that NO's effects on SERCA activity may be partly mediated by cGMP-dependent phosphorylation of phospholamban [45], although direct actions of NO on the SERCA protein itself could also contribute to its overall regulation [18]. Similar experiments in platelets demonstrate that NO stimulates SERCA-dependent refilling of intracellular Ca^{2+} stores [101]. On the other hand, SERCA1 activity of skeletal muscle is inhibited by NO, and appears to occur via direct effects on the ATPase [41]. Recently, neuronal NOS has been shown to be present in cardiac SR membranes where it can potentially modulate SERCA activity by production of endogenous NO [108]. In particular, cardiac SERCA2a-dependent Ca^{2+} uptake is inhibited by NO action [108]. Thus, it seems that positive or negative regulation of SERCA activity by mediators like NO is, to a certain extent, dependent upon the cellular context in which SERCA is expressed.

Functional Consequences of SERCA Inhibition

Specific inhibition of SERCA can be achieved with several agents [39]. Of these, thapsigargin (TG) has proven to be particularly useful due to its high specificity and affinity for SERCA [87]. It has been used extensively not only to study the structural and functional properties of SERCA, but also to analyze the cellular consequences of inhibiting SERCA-dependent Ca^{2+} transport function. TG, by inhibiting SERCA, prevents the re-uptake of cytosolic Ca^{2+} back into the Ca^{2+} storage compartments, resulting in a rise of Ca^{2+} within the cytoplasm. Many of the biological consequences of TG on cell function, including alterations in signaling, gene expression, Ca^{2+} entry, cell proliferation and apoptosis, can be attributed to a rise in cytosolic Ca^{2+} and/or depletion of intracellular Ca^{2+} pools that occur as a result of the SERCA pump inhibition.

It was shown in early studies that SERCA inhibition by TG interferes drastically with the occurrence of cytosolic Ca^{2+} transients and contractile activation in cardiac myocytes, while myolemmal electrical excitability remains unaffected [46]. These studies demonstrated clearly the prominent role of SERCA in contractile activation and relaxation of heart muscle.

Depletion of Ca^{2+} from within the Ca^{2+} storing organelles by TG-mediated inhibition of SERCA activity can activate entry of extracellular Ca^{2+} into the cytoplasm, a process termed capacitative Ca^{2+} entry [5, 80]. In excitable cells, depletion of organellar Ca^{2+} results in Ca^{2+} entry through voltage- and ligand-gated Ca^{2+} chan-

nels present within the plasma membrane, with cytosolic Ca^{2+} itself providing one potential regulatory mechanism for modulating the Ca^{2+} influx through such channels [52]. In nonexcitable cells, Ca^{2+} store depletion activates Ca^{2+} entry through store-operated Ca^{2+} channels (SOCC), the molecular identity of which have yet to be clearly defined. However, Ca^{2+} currents through SOCC have been directly measured in a number of cell lines, with some of the heterogeneity with respect to their biochemical and biophysical properties possibly reflecting the existence of different isoforms of SOCC [34, 54, 74]. The mechanisms regulating SOCC may include direct protein-protein interactions between the ER and the plasma membrane [5] and/or diffusible messengers [82]. Although the nature of such diffusible messengers have yet to be determined, recent studies suggest that these factors appear to be conserved between yeast and mammalian cells [19]. Inactivation of the SOCC currents may occur as a consequence of refilling of the internal Ca^{2+} stores and/or $Ca^{2+}{}_i$-dependent inactivation of Ca^{2+} influx [5]. In this regard, SERCA activity can modulate the Ca^{2+} dependent feedback inhibition of the SOCC current by regulating the $Ca^{2+}{}_i$ concentration at the sites of Ca^{2+} entry. It has been suggested that increased SERCA activity can occur as a consequence of store depletion, resulting in low $Ca^{2+}{}_i$ in the vicinity of Ca^{2+} influx and therefore a greater SOCC current [68]. Since SERCA activity can regulate the SOCC current, inhibition of its function by agents like TG may be less effective in stimulating the inward current than circumstances where the stores are depleted but SERCA activity is preserved (for example, with IP_3-generating agonists) [54].

Various cell signaling functions can be potentially modified by Ca^{2+} transients that are induced as a consequence of TG-mediated inhibition of SERCA, including those mediated by certain kinases, phosphatases, lipases, proteases and ion channels. For instance, TG has recently been implicated in some of the signaling pathways mediated by the mitogen-activated protein kinases (MAPKs). In particular, TG can activate the MAPK/ERK family of kinases [14]. Both Src and Rafl kinase activities appear to be necessary for the TG-dependent activation of ERK, although the Ca^{2+} influx component that occurs upon TG treatment does not seem to be required for ERK activation by TG [15]. Another member of the MAPK family, i.e., JNK, can also be activated by TG in certain cells via a nonreceptor Ca^{2+}-dependent tyrosine kinase termed CADTK/PYK2 [51].

Among the first genes shown to be modulated by TG were the immediate early genes c-fos and c-jun [89]. Their induction was shown to occur via increased transcription, and was dependent upon the rise in $Ca^{2+}{}_i$ as a result of SERCA inhibition by TG [89]. Interestingly, c-fos transcription seemed to occur via the SRE promoter element and not the Ca^{2+}/cAMP response element [89]. Both TG and cyclopiazonic acid, which is another SERCA pump inhibitor, can cause a rapid increase in IL-6 mRNA and protein levels in macrophages [7]. Although a rise in cytosolic Ca^{2+} due to SERCA inhibition most likely contributes to the increase in IL-6 message, entry of extracellular Ca^{2+} may not be obligatory for this rapid increase in IL-6 levels [7]. In contrast, TG-mediated transcription of stress-response genes like grp78 is dependent upon the gradual depletion of intracellular Ca^{2+} stores rather than a rise in $Ca^{2+}{}_i$ [50]. Unique response elements (RE) have been identified within the grp78 promoter, binding of transcription factors to which is inhibited by Ca^{2+} [50, 86] and modulated by tyrosine kinase activity [12]. Similarly, the other ER resident protein calreticulin has been shown to harbor TG-sensitive RE, and is also transcriptionally regulated by the status of intracellular Ca^{2+} pools rather than extracellular or cytosolic Ca^{2+} [104]. Transient inhibition of protein synthesis as a result of TG treatment, with the subsequent activation of stress response signaling, has been proposed as another mechanism for initiating gene transcription [63]. Upregulation of gene expression is not the only outcome of TG treatment. In human prostate cancer L_NCap cells, for example, TG causes a decrease in androgen receptor mRNA levels, although the mechanisms leading to this downmodulation are not clear [27].

Mobilization of intracellular Ca^{2+} stores by agents like TG can alter the phosphorylation status of eukaryotic initiation factors and disrupt protein processing at the level of translation initiation [79]. A human homologue of the yeast translation initiation factor eIF(SUI1), termed $eIF1^{A121/SUI1}$, has recently been cloned from a genotoxic-stress induced cDNA subtraction library, and found to be upregulated by ER Ca^{2+} pool depletion [91]. Thus, modification and/or induction of factors involved in protein synthesis and processing can occur upon inducing ER stress to cells. Although some of these changes may be part of a cell's defense response to the toxic insults, TG-mediated depletion of Ca^{2+} stores can result in cell growth arrest [92] and/or induction of apoptosis [44]. Depending upon the system under study, the different consequences of TG treatment (Ca^{2+} influx, rise in cytosolic Ca^{2+}, Ca^{2+} pool depletion) have varying degrees of effects on the induction of apoptosis. For example, in prostate cancer cells the resulting elevation in cytosolic Ca^{2+} levels upon treatment of the cells with TG appears to be necessary for inducing apoptosis [23]. In insulin-secreting MIN6 cells and hypothalamic GT1-7 cells, on the other hand, it is the depleted state of the Ca^{2+} stores rather than Ca^{2+} influx or elevated $Ca^{2+}{}_i$ that triggers TG-induced apoptosis [106, 113]. Arachidonic acid metabolites [113] and components of the caspase cascade [99] can participate in the execution of TG-mediated apoptosis, while bcl2, by potentially altering cellular Ca^{2+} homeostasis, may have a protective effect [47, 106].

Adaptive Responses to SERCA Inhibition

Highly specific and irreversible stoichiometric interactions between TG and SERCA produce a stable enzyme-inhibitor dead-end complex, resulting in global disruption of SERCA's biological functions [87]. A combination of studies have recently led to a better understanding of the TG binding topology. Since TG inhibits only SERCA, and not other cation transport ATPases, chimeric exchanges between SERCA and Na^+,K^+-ATPase were used to assess TG-SERCA interactions. In initial studies, chimeric recombinations between SERCA1 and Na^+,K^+-ATPase (which is not sensitive to TG) suggested that a 30 amino acid residue within the S3, M3 domain of SERCA maybe involved in the interaction of the enzyme with TG [70, 98]. This was also consistent with the photolabeling data using TG azido derivatives [35]. More recently, site directed mutagenesis has shown a specific role of the S3 domain in such interaction [112].

By binding to SERCA and disrupting intracellular Ca^{2+} homeostasis, TG can inhibit cell proliferation and cause cell death. However, using stepwise selection, mammalian cell lines that are highly resistant to TG inhibition can be developed [29]. In many of the TG-resistant cell lines, the multidrug resistance transporter p-glycoprotein (pgp) is overexpressed [29]. Moreover, transfection of pgp-encoding cDNAs into wild-type TG-sensitive cells can render the transfectants resistant to TG inhibition, suggesting that TG is a substrate for the multidrug transporter [29]. In addition, SERCA is overexpressed in TG-resistant cells. Interestingly, introduction of exogenous SERCA into hamster cells followed by TG selection results in overexpression of both the endogenously expressed SERCA and the transfected SERCA [29]. Thus, irrespective of whether SERCA is under the control of its natural promoter or a heterologous promoter, increased production of SERCA protein is an important adaptive response to the selective inhibition of the ATPase. The mechanisms underlying this increased production of the enzyme have been investigated in TG-resistant hamster smooth muscle cells [83]. Increased transcription of the SERCA gene can occur in the TG-resistant cells, which appears to be dependent upon the recruitment of new *cis*-regulatory elements, in addition to the known promoter elements that are normally involved in SERCA gene transcription [83]. Amplification of the SERCA gene, leading to increased SERCA message and protein, can also occur upon development of the TG-resistant phenotype [83].

The fact that TG-selected cells continue to proliferate in the presence of high levels of TG suggests that they are likely to maintain many of the Ca^{2+} store-dependent signaling functions necessary for growth and division. Such Ca^{2+} signaling pools were in fact shown to exist in the TG-resistant cells [37, 102, 103]. However, the disproportionately high concentrations of TG, relative to the amounts of recovered ATPase, required to inhibit the Ca^{2+} pumping activity within these cells suggested that a TG-resisant ATPase(s) is also likely to exist in such cells [37]. Cloning and sequencing studies identified that one particular amino acid residue within the S3 domain of SERCA, i.e., the putative TG binding site, undergoes specific mutations upon TG selection [109, 110]. That is, the Phe^{256} residue can undergo specific mutations (Phe $\rightarrow$ Leu, Phe $\rightarrow$ Ser or Phe $\rightarrow$ Val) in the TG-resistant cells, with the different substitutions conferring varying degrees of resistance to TG inhibition while maintaining functional competence of the enzyme [109, 110]. Thus, several processes can participate in the adaptive response to TG selection, including overexpression of pgp, overexpression of SERCA, and alterations in SERCA, with each contributing to the overall TG-resistant phenotype.

Conclusions

SERCAs are key enzymes involved in modulating Ca^{2+}-dependent signaling and maintaining Ca^{2+} homeostasis within cells. A combination of biochemical and genetic approaches have begun to clarify their function and regulation in normal as well as altered states of Ca^{2+} homeostasis. A particularly useful approach in this regard has been with the use of highly specific inhibitors of SERCA function. Better understanding has occurred not only with respect to the mechanisms of SERCA inhibition, but also with regards to the cellular consequences and adaptive responses to such inhibition of their function.

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