Organellar Calcium Buffers

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Ca^{2+} is an important intracellular messenger affecting many diverse processes. In eukaryotic cells, Ca^{2+} storage is achieved within specific intracellular organelles, especially the endoplasmic/sarcoplasmic reticulum, in which Ca^{2+} is buffered by specific proteins known as Ca^{2+} buffers. Ca^{2+} buffers are a diverse group of proteins, varying in their affinities and capacities for Ca^{2+} , but they typically also carry out other functions within the cell. The wide range of organelles containing Ca^{2+} and the evidence supporting cross-talk between these organelles suggest the existence of a dynamic network of organellar Ca^{2+} signaling, mediated by a variety of organellar Ca^{2+} buffers.

INTRACELLULAR Ca^{2+} DYNAMICS

Ca^{2+} serves as an intracellular messenger in various cellular processes, including muscle contraction, gene expression, and fertilization (Berridge et al. 2003). To use Ca^{2+} , the cell requires a readily mobilizable source of Ca^{2+} , the majority of which is found within the lumen of the endoplasmic reticulum (ER) and/or sarcoplasmic reticulum (SR), but is also located in the Golgi apparatus, peroxisomes, mitochondria, and endolysosomal compartments. It is not surprising that Ca^{2+} levels are of central importance in dictating the function of proteins that reside in intracellular organelles. Although some Ca^{2+} exists as free ions within these compartments, much of it is buffered by specific proteins, simply known as Ca^{2+} buffers. However, these proteins are diverse in terms of structure, oligomerization, affinity and capacity for Ca^{2+} , and physical basis for binding Ca^{2+} ions, one commonality across Ca^{2+} buffers is

that they also serve other roles within the cell. These roles include catalyzing the correct folding of other cellular proteins, regulating Ca^{2+} release and retention, and communicating information about Ca^{2+} levels within organelles to other proteins.

ENDOPLASMIC RETICULUM

The ER is a multifunctional organelle within the eukaryotic cell that serves as the single largest Ca^{2+} store inside nonstriated muscle cells. The ER is also responsible for functions as diverse as protein synthesis and posttranslational modification, lipid and steroid metabolism, and drug detoxification (Michalak and Opas 2009). Within the ER lumen, the total concentration of Ca^{2+} is approximately 1 mM, with free Ca^{2+} in the range of approximately 200 μM and the remainder buffered via ER resident proteins (Michalak and Opas 2009). Most ER Ca^{2+}

Editors: Martin D. Bootman, Michael J. Berridge, James W. Putney, and H. Llewelyn Roderick
Additional Perspectives on Calcium Signaling available at www.cshperspectives.org

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Cite this article as *Cold Spring Harb Perspect Biol* 2011;3:a004069

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buffering proteins also serve as ER chaperones or folding enzymes, responsible for correctly protein folding that are transiting through the ER.

Calreticulin

Calreticulin, a 46-kDa ER luminal resident protein, is responsible for buffering up to 50% of ER Ca^{2+} in nonmuscle cells (Nakamura et al. 2001a; Nakamura et al. 2001b). Structurally, calreticulin consists of three distinct domains: N, which is the amino-terminal and implicated (together with the P-domain) in chaperone function; P, which is central, proline-rich, and a structural backbone; and C, which is the carboxy-terminal and critical for Ca^{2+} buffering. The N-domain of calnexin, which is homologous to calreticulin, is primarily β -sheet and globular, with high homology to the structure of plant lectins, suggesting a role in the binding of monoglucosylated substrates to calreticulin as part of its chaperone role (Schrage et al. 2001). Recent work using small angle X-ray scattering (SAXS) showed that the N-domain of calreticulin itself is indeed globular and fits well onto modeled calnexin (Norgaard Toft et al. 2008). The N-domain conformation is dynamic and is stabilized by oligosaccharide binding (Saito et al. 1999; Conte et al. 2007) and the binding of Ca^{2+} at a high-affinity site (Corbett et al. 2000; Conte et al. 2007), though this binding does not affect its affinity for oligosaccharides (Conte et al. 2007).

The P-domain of calreticulin is proline-rich, which suggests that it may show conformational flexibility. Its sequence contains two pairs of repeated amino acid sequences, 1 and 2, in the order 111222 (Fliegel et al. 1989). The P-domain adopts an extended conformation with antiparallel β -sheets between the repeated amino acid sequences; the domain as a whole protrudes out from the N- and C-domains (Ellgaard et al. 2001a; Ellgaard et al. 2001b). The extended protrusion requires a β -hairpin turn at amino acid residues 238 to 241; small angle X-ray scattering (SAXS) analyses indicate that this is in a spiral-like conformation (Norgaard Toft et al. 2008). TROSY-NMR experiments

showed that the tip of the P-domain protrusion accounts for the binding site of ERp57, an oxidoreductase-folding enzyme (Frickel et al. 2002).

The C-domain of calreticulin is enriched in negatively charged amino acid residues responsible for its Ca^{2+} -buffering capabilities (Nakamura et al. 2001b). It binds Ca^{2+} with high capacity (25 mol of Ca^{2+} per mol of protein) and low affinity ($K_d = 2$ mM) (Nakamura et al. 2001b). The conformation of this region is highly dependent on variations in Ca^{2+} concentrations within a physiological range (Corbett et al. 2000). SAXS studies indicate that the C-domain of calreticulin may be globular (Norgaard Toft et al. 2008). Ca^{2+} binding stabilizes the C-domain into a more compact, α -helical conformation; the Ca^{2+} concentration required to induce this change, 400 μM , is well within the range of concentrations to which calreticulin would be exposed physiologically (Villamil Giraldo et al. 2009).

Calreticulin Gain-of-Function and Loss-of-Function

Understanding the roles calreticulin and Ca^{2+} play at cellular and organismal levels requires accounting for both its role as a chaperone and as an ER luminal Ca^{2+} buffer. Cell culture and animal models of calreticulin deficiency (loss-of-function) and overexpression (gain-of-function) have shown how tight control of protein folding and Ca^{2+} homeostasis, as exerted by calreticulin, are necessary for proper function and development.

In mice, calreticulin deficiency (loss-of-function) is lethal at embryonic day 14.5 because of impaired cardiogenesis, manifested as abnormally thin ventricular walls and improper myofibrillogenesis (Mesaeli et al. 1999). The insufficiency in this pathway can be traced to a lack of nuclear translocation of NF-AT3 (nuclear factor of activated T-cells), which is activated by calcineurin, a Ca^{2+} -dependent phosphatase. *crt*^{-/-} cells showed no cytoplasmic Ca^{2+} spike in response to the agonist bradykinin, indicating that the IP_3 pathway to stimulate release of Ca^{2+} from the ER was affected

(Mesaeli et al. 1999). Embryonic lethality could be rescued via heterologous expression of a constitutively active mutant of calcineurin in the heart, demonstrating that calreticulin's role in cardiogenesis depends on its regulation of intracellular Ca^{2+} dynamics from the ER lumen (Guo et al. 2002). Calreticulin may regulate the IP_3R in a Ca^{2+} -dependent manner (Camacho and Lechleiter 1995; Naaby-Hansen et al. 2001). Furthermore, in a cell culture model, cardiomyocytes derived from $\text{crt}^{-/-}$ embryonic stem cells showed lower rates of myofibrillar development (Li et al. 2002), thought to involve transcriptional pathways regulated by Ca^{2+} (Lynch et al. 2006). The critical role of Ca^{2+} signaling is underscored by the observation that myofibrillar development could be rescued in $\text{crt}^{-/-}$ cells by transient ionomycin treatment (Li et al. 2002). Taken together, these investigations show that the absence of calreticulin severely affects Ca^{2+} -regulated pathways, via both calreticulin's Ca^{2+} buffering and its regulation of protein folding.

Calreticulin overexpression (gain-of-function) is also detrimental to the maintenance of Ca^{2+} homeostasis and the molecular pathways it regulates. Overexpressing calreticulin within the lumen of the ER increases the amount of Ca^{2+} that can be released after inhibition of SERCA via thapsigargin, showing that calreticulin does in fact buffer readily mobilizable Ca^{2+} within the ER (Mery et al. 1996). Furthermore, overexpression of calreticulin delays the process of store-operated Ca^{2+} entry if ER luminal depletion is incomplete but not when depletion is complete, again suggesting that calreticulin buffers a significant amount of Ca^{2+} within the lumen (Fasolato et al. 1998). At a cellular level, augmented calreticulin levels increase the susceptibility of SERCA2a to oxidative stress (Ihara et al. 2005). In the murine heart, targeted overexpression of calreticulin and concomitant increased ER Ca^{2+} capacity results in impaired gap junctions, aberrant Ca^{2+} handling, and arrhythmia, culminating in heart block and death (Nakamura et al. 2001a; Hattori et al. 2007). Interestingly, calreticulin overexpression also results in impaired synthesis of MEF2C (myocyte enhancer factor 2C), a transcription factor

implicated in cardiac development, suggesting that calreticulin may play multiple roles in controlling downstream gene transcription (Hattori et al. 2007).

Immunoglobulin Binding Protein BiP/GRP78

BiP (immunoglobulin binding protein), also known as GRP78, similarly to calreticulin, is an ER luminal resident protein known to play an important role in binding to unfolded proteins and assisting in the attainment of the correct conformations. Its most prominent roles are as a regulator of the unfolded protein response, a player in ER stress, and a crucial component of the protein translocation machinery (Dudek et al. 2009). BiP/GRP78 deficiency (loss-of-function) is extremely detrimental and is lethal at embryonic day 3.5 in mice (Luo et al. 2006). In addition to its protein binding functions, BiP/GRP78 serves as an important luminal Ca^{2+} buffer, likely responsible for buffering approximately 25% of the total ER Ca^{2+} load (Lievremont et al. 1997). As BiP/GRP78 is expressed at a higher level than is calreticulin, its Ca^{2+} binding should be considered low capacity, approximately 1–2 mol of Ca^{2+} per mol of protein, and low affinity (Lievremont et al. 1997; Lamb et al. 2006). BiP/GRP78 contains an ATPase domain through which it harnesses energy to fold its client proteins (Dudek et al. 2009). Intriguingly, the affinity of BiP/GRP78 for Ca^{2+} is altered by its binding to ATP or ADP, suggesting interplay between ER Ca^{2+} filling and the chaperone activity of BiP/GRP78 (Lamb et al. 2006). Indeed, prolonged diminishment of ER Ca^{2+} stores can abrogate interactions between BiP/GRP78 and its client proteins (Suzuki et al. 1991) and its ATPase activity is increased when Ca^{2+} levels are low (Kassenbrock and Kelly 1989). BiP/GRP78 also plays a role in the protein translocation machinery, both in binding to unfolded proteins to maintain and prevent their misfolding (Dudek et al. 2009) and, importantly, in closing the Sec61 channel to maintain the ER Ca^{2+} permeability barrier both before and after translocation (Haigh and Johnson 2002; Alder et al.

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2005). BiP/GRP78 is also a critical component of the unfolded protein response (Rutkowski and Kaufman 2004) and regulates ER associated degradation (ERAD) (Hebert et al. 2009). In summary, BiP/GRP78 is a crucial ER Ca^{2+} buffer, accounting for about one quarter of the ER's buffering capacity, and also shows Ca^{2+} -dependent regulation of chaperone activity.

GRP94

GRP94 (glucose regulated protein of 94-kDa, endoplasmic, CaBP4) is an ER resident protein with a Ca^{2+} buffering role (Macer and Koch 1988); (Van et al. 1989). It binds Ca^{2+} with high capacity (15 to 28 mol of Ca^{2+} per mol of protein) (Macer and Koch 1988; Van et al. 1989). Ca^{2+} binding can be further divided into four sites of higher affinity ($K_d = 2.0 \mu\text{M}$) and eleven sites of lower affinity ($K_d = 600 \mu\text{M}$) (Van et al. 1989; Ying and Flatmark 2006). Structurally, GRP94 undergoes a conformational change to be less α -helical in the presence of $100 \mu\text{M}$ Ca^{2+} (Van et al. 1989; Ying and Flatmark 2006). GRP94 binds to ER luminal peptides and this binding is increased in the absence of Ca^{2+} , consistent with its role as a response to ER stress (Ying and Flatmark 2006; Biswas et al. 2007). Moreover, GRP94 binding to ATP induces a conformational change and subsequent dimerization. This enables recognition of an immature client protein, whereas attainment of the correct folding by the client protein leads to yet another shape change in GRP94 causing release of the substrate (Immormino et al. 2004; Rosser et al. 2004). Intriguingly, GRP94 is protective in cardiac pathologies, reducing cardiomyocyte necrosis in response to artificially induced Ca^{2+} overload or simulated ischemic insult (Vitadello et al. 2003). Furthermore, GRP94 expression is increased in hearts undergoing prolonged atrial fibrillation, hypothesized to have a protective role against injury (Vitadello et al. 2001). GRP94 also plays a role in apoptosis, particularly when related to perturbations in Ca^{2+} homeostasis. The hepatitis C virus E2 protein blocks apoptosis by inducing overexpression of GRP94: artificially increasing GRP94 levels

blocks apoptosis, whereas siRNA knockdown of GRP94 eliminates the antiapoptotic effect of HCV E2 (Lee et al. 2008). In pancreatic cancer patients, heightened expression of GRP94 correlated with a worsened prognosis because of GRP94's antiapoptotic effect (Pan et al. 2009). Hypoxic conditions are known to up-regulate GRP94, underscoring its importance during tumor development (Paris et al. 2005). GRP94 has been shown to regulate apoptosis via stabilization of Ca^{2+} homeostasis (Bando et al. 2004), suggesting that its antiapoptotic effects are a consequence of its Ca^{2+} buffering rather than its peptide-binding activity. GRP94 also protects against cell death induced by ischemia/reperfusion injuries (Bando et al. 2003).

Protein Disulfide Isomerase

Protein disulfide isomerase (PDI) is an ER luminal protein that is capable of isomerizing disulfide bonds on proteins transiting through the ER. It has long been known to bind Ca^{2+} (Macer and Koch 1988) with high capacity (19 mol of Ca^{2+} per mol of protein) (Lebeche et al. 1994); (Lucero et al. 1994). Heterologous expression of PDI in Chinese hamster ovary cells increased the Ca^{2+} stored within ER microsomes (Lucero et al. 1998). Structurally, the carboxy-terminal region of PDI is enriched in paired acidic residues; removal of these residues significantly reduces the amount of Ca^{2+} that PDI can bind (Lucero and Kaminer 1999). In general, PDI was enzymatically more active in the presence of increased Ca^{2+} (Lucero and Kaminer 1999), again showing modulation of enzyme activity by ER Ca^{2+} levels.

ERp72, a PDI-Like Protein

ERp72, an ER luminal protein with thioredoxin-like motifs, is homologous to the rat protein CaBP2 (calcium-binding protein 2) (Van et al. 1993). Its primary function appears to be as a molecular chaperone (Nigam et al. 1994) where it isomerizes disulfide bonds (Rupp et al. 1994). Though it does bind Ca^{2+} , the chaperone activity of ERp72 is unaffected by Ca^{2+} concentrations (Rupp et al. 1994) and

overexpression of ERp72 does not increase ER Ca^{2+} stores, suggesting its protein folding activity is more important than its Ca^{2+} binding (Lievremont et al. 1997).

The ER, as the principal intracellular Ca^{2+} store in nonstriated muscle cells, has evolved numerous proteins with the capability to buffer Ca^{2+} with variable capacities and affinities. Most Ca^{2+} -buffering proteins also serve as key modulators of protein folding, both upstream, as in calreticulin, which acts on unfolded proteins to ensure their correct folding, and downstream, as in BiP/GRP78, a key player in the unfolded protein response. In addition, most Ca^{2+} buffers are capable of dynamic responses to variations in ER Ca^{2+} levels, particularly with respect to conformation. It is therefore clear that ER Ca^{2+} buffering capacity is inextricably linked to other cellular processes that are the responsibility of the ER.

SARCOPLASMIC RETICULUM Ca^{2+} STORES

The sarcoplasmic reticulum (SR) is an organelle, closely related to the ER, that is found in cardiac, skeletal, and smooth muscle (Michalak and Opas 2009). Its major function is in regulation of Ca^{2+} fluxes responsible for muscular contraction by serving as the principal intracellular Ca^{2+} store within muscle cells; total SR Ca^{2+} levels, similarly to the ER, are in the millimolar range (Michalak and Opas 2009).

The most abundant Ca^{2+} -binding protein within the SR is calsequestrin, a high capacity Ca^{2+} binding protein. There are two isoforms of calsequestrin, skeletal muscle (calsequestrin-1, or Casq1) and cardiac muscle (calsequestrin-2, or Casq2) (Murphy et al. 2009). Interestingly, although fast-twitch muscle contains almost exclusively calsequestrin-1 isoform, slow-twitch muscle fibers also contain some calsequestrin-2 (Murphy et al. 2009). Calsequestrins across numerous species show high (>75%) homology (Beard et al. 2004); moreover, the two isoforms show high homology to each other (Wei et al. 2009b). Binding of Ca^{2+} to calsequestrin is based on extensive stretches of acidic amino acids within the carboxy-terminal region and

is high capacity, with calculated values of mol of Ca^{2+} bound per mol of cardiac calsequestrin ranging from 18 (Slupsky et al. 1987) to 60 (Park et al. 2004). Skeletal muscle calsequestrin binds more Ca^{2+} than its cardiac muscle counterpart: up to 80 mol of Ca^{2+} per mol of protein for skeletal muscle calsequestrin compared to 60 mol per mol of protein for cardiac muscle calsequestrin at saturating concentrations of Ca^{2+} (Park et al. 2004); (Wei et al. 2009b). Ca^{2+} binding to both isoforms of calsequestrin significantly affect the conformation of the protein, making it much more compact and playing a role in oligomerization (Ikemoto et al. 1972; Mitchell et al. 1988; He et al. 1993).

The crystal structure of calsequestrin revealed the presence of three almost identical domains, each of which shows high homology to *Escherichia coli* thioredoxin motifs, suggesting that calsequestrin may be yet another Ca^{2+} -binding protein with the ability to fold other proteins (Wang et al. 1998). In vivo, skeletal muscle calsequestrin consists of long, ribbon-like polymers that were described via electron microscopy (though not yet identified as calsequestrin) as early as 1970 (Franzini-Armstrong 1970). In the presence of Ca^{2+} , calsequestrin forms two different types of dimers, front-to-front and back-to-back. Both types are stabilized by salt bridges and Ca^{2+} ions binding into the negatively charged pocket between two copies of the protein (Wang et al. 1998). Back-to-back dimerization of skeletal calsequestrin occurs first; at a lower Ca^{2+} level (10 μM) whereas at a higher Ca^{2+} concentration (1 mM), calsequestrin forms front-to-front dimers followed by formation of linear polymers (Wang et al. 1998). Electron tomography shows that calsequestrin polymers are physically connected to the junctional region of the SR membrane in muscle cells (Franzini-Armstrong 1970; Wagenknecht et al. 2002). Polymerization behavior differs between the two calsequestrin isoforms: in vitro in the presence of 1 mM Ca^{2+} , calsequestrin-1 is mostly polymerized whereas calsequestrin-2 is primarily monomeric or dimeric (Park et al. 2003; Wei et al. 2009b). Calsequestrin-2's polymerization may be inhibited by its longer carboxy-terminal tail interfering with

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the Ca^{2+} -binding pocket (Park et al. 2004; Wei et al. 2009b). These differences in polymerization are thought to be related to the varying Ca^{2+} capacities of the two isoforms, though it is unclear if polymerization affects Ca^{2+} capacity or vice versa (Wei et al. 2009b).

Calsequestrin is also known to interact with the RyR. These interactions are thought to be mediated by the transmembrane proteins junctin and triadin, though recent results suggest that only junctin may be crucial for the regulation of RyR proteins by calsequestrin in skeletal muscle (Wei et al. 2009a). Interactions between calsequestrin and the RyR depend on the luminal Ca^{2+} concentration (Gyorke et al. 2004). In cardiac systems, calsequestrin-2 interacts with triadin to inhibit RyR2 channel opening, possibly regulated by SR luminal Ca^{2+} (Terentyev et al. 2007). Interactions between calsequestrin and RyR provide a range of sensitivity over the range of physiological Ca^{2+} concentration (Qin et al. 2008). The skeletal muscle isoform of calsequestrin also interacts with RyR proteins, though in a different and more complex fashion than does the cardiac isoform. Calsequestrin-1 is known to undergo reversible phosphorylation at low Ca^{2+} concentrations corresponding to depleted SR Ca^{2+} stores. Phosphorylated calsequestrin strongly interacts with junctin and hence inhibits the RyR1 channel (Beard et al. 2008). Junctin, but not triadin, is required for skeletal calsequestrin to exert regulatory control over RyR1 channels (Wei et al. 2009a). At resting SR Ca^{2+} levels, skeletal calsequestrin inhibits RyR1; by contrast, cardiac calsequestrin activates both RyR1 and RyR2 (Wei et al. 2009b).

Mice deficient in the *Casq2* gene have only 11% lower SR Ca^{2+} storage in cardiac muscle cells (Knollmann et al. 2006). The hearts of these mice display increased SR Ca^{2+} leak, especially after catecholaminergic stimulation, causing ventricular arrhythmias (Knollmann et al. 2006). Similarly, mutated forms of human cardiac calsequestrin cause certain forms of CPVT (catecholamine-induced polymorphic ventricular tachycardia), a disease associated with polymorphic ventricular tachycardias in response to adrenergic stimulation and exercise, often

culminating in sudden death (Terentyev et al. 2006). The exact mechanisms linking mutated cardiac muscle calsequestrin to impaired Ca^{2+} handling phenotypes vary from mutation to mutation. One human mutant of calsequestrin, $\text{G}^{112}+5\text{X}$, is a frame-shift mutation leading to premature termination; this protein is incapable of binding Ca^{2+} (di Barletta et al. 2006) or polymerizing (Terentyev et al. 2008). A different mutant of cardiac calsequestrin associated with CPVT, R^{33}Q , shows normal Ca^{2+} binding, but has lost the capability to inhibit RyR on emptying of SR Ca^{2+} stores, both at rest and after stimulation (Terentyev et al. 2006; Terentyev et al. 2008). The R^{33}Q mutation also impairs front-to-front dimerization, but not to such an extent as to abrogate polymerization (Valle et al. 2008); by contrast, the L^{167}H mutation completely eliminates polymer formation (Valle et al. 2008). The D^{307}H mutation almost entirely eliminates the Ca^{2+} sensitivity of calsequestrin's conformation and also impairs its binding to junctin, preventing interactions with the RyR channel (Houle et al. 2004; Viatchenko-Karpinski et al. 2004; Kalyanasundaram et al. 2009). From these results, it is clear that calsequestrin's importance is not limited to its buffering of Ca^{2+} but also its interaction with, and regulation of, other constituents of the Ca^{2+} release pathway. This conclusion is underscored by the fact that a 25% reduction in calsequestrin levels does not affect SR Ca^{2+} levels, but does increase Ca^{2+} leak via RyR channels, pointing to a regulatory function of calsequestrin on RyR (Chopra et al. 2007).

A mouse model deficient in skeletal muscle calsequestrin (*Casq1*) has also been generated (Paolini et al. 2007). The absence of calsequestrin-1 is not lethal and surprisingly has only a slight effect on the contraction of skeletal muscles (Paolini et al. 2007). Absence of calsequestrin correlates to reduced Ca^{2+} release from the SR (because of impaired Ca^{2+} accumulation near RyRs; partially compensated for by increased expression of release channels) and reduced Ca^{2+} reuptake by the SR (because of impaired Ca^{2+} buffering) (Paolini et al. 2007). *Casq1* null mice have only 20% of the SR Ca^{2+} stores of wild-type mice, suggesting that the

role of skeletal calsequestrin in fast-twitch muscles may be primarily its Ca^{2+} buffering, required to keep free luminal Ca^{2+} concentrations low, thus reducing SR Ca^{2+} leaking (Murphy et al. 2009). The increased susceptibility of *Casq1* null mice to heat stroke (Protasi et al. 2009) is likely a consequence of increased SR Ca^{2+} leakage in fast-twitch muscle fibers (Murphy et al. 2009). In mouse models of Duchenne muscular dystrophy, the most affected muscle fibers are associated with decreased levels of calsequestrin and, consequently, impaired Ca^{2+} handling (Pertille et al. 2009).

Experiments with calsequestrin overexpression (gain-of-function) have solidified its role as the key Ca^{2+} buffer within the lumen of the SR. Mouse models specifically overexpressing cardiac muscle calsequestrin in the heart showed cardiac hypertrophy and increased quantities of Ca^{2+} stored within the SR, both supportive of a role for calsequestrin as a Ca^{2+} buffer (Jones et al. 1998; Sato et al. 1998; Schmidt et al. 2000). Overexpression of calsequestrin increases SR Ca^{2+} stores and impairs restoration of free Ca^{2+} levels within the SR lumen (Terentyev et al. 2003; Terentyev et al. 2008), indicating that calsequestrin does buffer Ca^{2+} . Interestingly, the effects of overexpressing calsequestrin were similar to those of introducing a chemical Ca^{2+} buffer into the SR lumen (Terentyev et al. 2002; Terentyev et al. 2003), indicating that calsequestrin's functions in vivo include both Ca^{2+} buffering and regulation of the RyR proteins.

Calsequestrin, the primary Ca^{2+} buffer within the SR, is a fascinating protein to consider in terms of Ca^{2+} binding. Its conformation, oligomerization state, and interactions with other proteins all vary with physiological concentrations of Ca^{2+} . Although it is unquestionably a Ca^{2+} buffer, equally important is its regulation of SR Ca^{2+} release via communications with the RyR. As calsequestrin overexpression can be mimicked via introduction of a nonprotein Ca^{2+} buffer, it seems evident that although some copies of calsequestrin are involved in interactions with the RyR, the vast majority of calsequestrin is simply concerned with Ca^{2+} buffering in vivo.

Minor SR Ca^{2+} Buffering Proteins

The minor SR Ca^{2+} buffering protein HRC (histidine-rich Ca^{2+} binding protein) is a 165-kDa protein first identified and characterized in 1989 (Hofmann et al. 1989; Hofmann et al. 1991). HRC binds Ca^{2+} with high capacity and low affinity and, like calsequestrin, exists as a multimer within the SR lumen (Suk et al. 1999). However, in contrast to calsequestrin, which exists as higher order oligomers in the presence of increasing levels of Ca^{2+} , HRC dissociates from pentamers to dimers and trimers when Ca^{2+} levels are elevated (Suk et al. 1999). Moreover, unlike calsequestrin, HRC is less tightly folded and more sensitive to trypsin digestion in the presence of Ca^{2+} (Suk et al. 1999). HRC is known to be directly involved in Ca^{2+} binding, as shown through studies that correlated overexpression of HRC with impaired SR Ca^{2+} uptake (Gregory et al. 2006) and increased total SR Ca^{2+} stores (Kim et al. 2003). Early evidence showed that HRC, via its glutamate-enriched carboxy-terminal region, interacts with triadin in a Ca^{2+} -dependent manner to anchor HRC to the junctional membrane (Sacchetto et al. 1999; Lee et al. 2001; Sacchetto et al. 2001); this led to the hypothesis that HRC may also be responsible for regulating RyR activity through its interactions with triadin. HRC also interacts with SERCA in cardiac muscle (Arvanitis et al. 2007), leading to the intriguing hypothesis that HRC may link Ca^{2+} release (via triadin) and Ca^{2+} uptake (via SERCA) in the SR lumen (Pritchard and Kranias 2009). HRC is implicated in cardiovascular disease: overexpression (gain-of-function) of HRC provides protection against heart damage induced by ischemia/reperfusion (Zhou et al. 2007) whereas a S⁹⁶A mutation in HRC has been identified in human patients to correlate with decreased survival in idiopathic dilated cardiomyopathy (Arvanitis et al. 2008). Mice deficient (loss-of-function) in HRC are more liable to develop cardiac hypertrophy when treated with isoproterenol (Jaehnig et al. 2006).

Junctate, a 33-kDa protein localized to the SR membranes, is an alternative splice product of the same gene that produces junctin (Treves

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Figure 1. Organellar Ca^{2+} buffering and intracellular Ca^{2+} dynamics. Ca^{2+} is stored within several different organelles including the endoplasmic reticulum (ER), ERGIC, the Golgi apparatus, mitochondria, and peroxisomes. A typical Ca^{2+} release pathway is shown at *top*: an extracellular ligand binds to its receptor, leading to production of IP_3 , which binds to the IP_3R on the ER membrane, stimulating release of Ca^{2+} from the ER lumen. Note that the Golgi apparatus also contains IP_3R molecules and thus may contribute to Ca^{2+} release from stores; Golgi Ca^{2+} uptake occurs via SPCA pumps. As shown at *left*, Ca^{2+} released from the ER has several different fates, including regulation of gene transcription within the nucleus; uptake by mitochondria, which are typically closely apposed to the ER network and where Ca^{2+} can affect metabolism; or extrusion from the cell via $\text{Na}^+/\text{Ca}^{2+}$ exchanger plasma membrane proteins. Peroxisomes are known to maintain Ca^{2+} at higher levels than those of the cytoplasm, but how this gradient is developed and maintained is not yet known. Ca^{2+} levels are elevated in the ER, in the SR, in the ERGIC, and in the Golgi apparatus, with most Ca^{2+} bound to buffering proteins, as shown within these organelles. (See facing page for legend.)

et al. 2000). It is a high capacity, moderate affinity Ca^{2+} -binding protein (Treves et al. 2000); consequently, overexpressing juncate in skeletal muscle increases total SR Ca^{2+} stores (Divet et al. 2007). In addition to its luminal domain binding Ca^{2+} directly, in the ER, juncate interacts with IP_3R proteins and TRPC3 (transient receptor potential channel) channels to regulate Ca^{2+} homeostasis, both in response to agonists and to store depletion (Treves et al. 2004). In the SR of mouse cardiomyocytes, juncate interacts with SERCA2a, further linking it with Ca^{2+} handling (Kwon and Kim do 2009). Overexpression of juncate in the mouse heart leads to impaired Ca^{2+} transients, culminating in cardiac hypertrophy and arrhythmias (Hong et al. 2008).

GOLGI Ca^{2+} STORES

Although the ER and SR are accepted as the major organellar Ca^{2+} buffers, several other subcellular organelles, including the Golgi apparatus, mitochondria, peroxisomes, and endosomes/lysosomes, are known to maintain Ca^{2+} at significantly higher levels than those of the cytoplasm.

The Golgi apparatus was identified as containing Ca^{2+} in the millimolar range during the 1990s (Chandra et al. 1994; Pezzati et al. 1997). Golgi Ca^{2+} stores can be released in response to InsP_3 -triggered pathways and were shown to be developed by proteins residing within the Golgi, instead of existing solely because of vesicular trafficking from the Ca^{2+} -rich ER (Pinton et al. 1998). Ca^{2+} is bound within the Golgi by three proteins, Cab45, P54/NEFA, and CALNUC (nucleobindin), of which

the Ca^{2+} buffering capabilities of CALNUC are the most important and have been the most extensively investigated. Cab45 is a 45-kDa soluble protein with six Ca^{2+} -binding EF-hands; interestingly, it was the first soluble protein shown to be retained in the Golgi lumen (Scherer et al. 1996), though later results show Cab45 or its variants are also found in the cytoplasm (Lam et al. 2007) and secreted by pancreatic cancer cells (Gronborg et al. 2006). P54/NEFA (DNA binding/EF hand/acidic amino acid rich region protein) contains two EF-hands, but is thought to function in a regulatory role rather than as a Ca^{2+} buffer (Morel-Huau et al. 2002). Another Golgi Ca^{2+} -binding protein, CALNUC, shows some homology to calreticulin and is tightly associated with the inner membrane of the Golgi apparatus (Lin et al. 1998). CALNUC contains two EF-hand motifs, one of which has high affinity for Ca^{2+} , making the protein a low capacity, high-affinity Ca^{2+} buffer (Lin et al. 1999). CALNUC is extremely abundant in the Golgi lumen (approximately 0.4% of total Golgi protein), suggesting that it may be the principal Golgi Ca^{2+} store (Lin et al. 1999). The apparent affinity of CALNUC for Ca^{2+} has been reported as 6–7 μM (Lin et al. 1999; Kanuru et al. 2009). CALNUC is tightly folded in the presence of Ca^{2+} and that loss of Ca^{2+} exposes a large hydrophobic surface (de Alba and Tjandra 2004). They further postulate that this surface serves to modulate interactions with other proteins and that it may serve as both a Ca^{2+} buffer and a Ca^{2+} sensor (de Alba and Tjandra 2004). CALNUC has also been shown to be secreted (Lavoie et al. 2002) and may play a role in bone development (Petersson et al. 2004).

Figure 1. (Continued) The store-operated Ca^{2+} entry (SOC) is also represented. In response to depleted ER Ca^{2+} stores, an ER luminal Ca^{2+} sensor, STIM1, oligomerizes and migrates to subplasmalemmal punctae where it communicates with Orai1. Orai1 functions as a plasma membrane Ca^{2+} channel that allows for Ca^{2+} entry from the extracellular milieu into the cytoplasm, where it is taken up by SERCA into the ER lumen. BiP/GRP78 binding protein/glucose-regulated protein of 78-kDa; CRT, calreticulin; ERGIC, endoplasmic reticulum/Golgi intermediate complex; GRP94, glucose-regulated protein of 94-kDa; IP_3 , inositol trisphosphate; IP_3R , inositol trisphosphate receptor; NCX, $\text{Na}^+/\text{Ca}^{2+}$ exchanger; P54/NEFA, DNA binding/EF hand/acidic amino acid rich region protein; PDI, protein disulfide isomerase; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase; SPCA, secretory pathway Ca^{2+} -transport ATPase; STIM1, stromal interacting molecule 1.

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The importance of Golgi Ca^{2+} signaling is shown by the effects of its dysfunction. Hailey-Hailey disease, characterized by skin blistering and lesions, is caused by mutations in the gene encoding SPCA1, the secretory pathway Ca^{2+} ATPase responsible for maintenance of Golgi Ca^{2+} gradients (Hu et al. 2000; Sudbrak et al. 2000). Furthermore, reduced SPCA1 expression in a mouse model caused impaired neural polarity (Sepulveda et al. 2009).

ENDOPLASMIC RETICULUM GOLGI INTERMEDIATE COMPLEX

There is growing evidence that the ERGIC (endoplasmic reticulum Golgi intermediate complex) plays a role in Ca^{2+} storage, as would be expected for a compartment located between two Ca^{2+} -enriched organelles. The ERGIC contains SERCA and significant quantities of GRP94, usually thought of as an ER resident protein, allowing ERGIC to take up Ca^{2+} and buffer it (Ying et al. 2002). Calreticulin has also been detected within the ERGIC and may contribute to its Ca^{2+} buffering (Zuber et al. 2000).

ERGIC and the Golgi apparatus, as the two organelles following the ER in the secretory pathway, also contain Ca^{2+} at significantly higher levels than those found in the cytoplasm. It is therefore intriguing that both compartments have been shown to take up and buffer Ca^{2+} independently of trafficking from the ER. The proteins responsible for Ca^{2+} buffering within ERGIC and the Golgi apparatus are, as with many of the previously discussed Ca^{2+} buffers, multifunctional and responsible for communicating information about Ca^{2+} levels to other proteins.

MITOCHONDRIA

Mitochondria, the cellular organelles responsible primarily for energy generation, are also intracellular Ca^{2+} stores. Within the matrix of mitochondria, Ca^{2+} is stored not bound to Ca^{2+} buffering proteins but instead precipitated out as an insoluble salt, CaPO_4 . The handling of Ca^{2+} by mitochondria is too complex to be

covered in great detail here, but one aspect worth highlighting is how mitochondria communicate with the ER with respect to Ca^{2+} fluxes. Release of Ca^{2+} from the ER results in elevated cytoplasmic Ca^{2+} levels, after which mitochondria take up Ca^{2+} resulting in elevated matrix Ca^{2+} levels (Rizzuto et al. 1992). ER and mitochondria are often tightly apposed in regions known as mitochondria-associated membranes (MAM); the interactions between these two organelles are regulated by cytoplasmic Ca^{2+} levels (Wang et al. 2000). Interestingly, resting cytoplasmic Ca^{2+} levels promote dissociation of the two organelles whereas higher Ca^{2+} levels enhance their association, suggesting that mitochondria may act as buffers to soak up Ca^{2+} released from the ER lumen (Wang et al. 2000). The Ca^{2+} cross-talk between the ER and mitochondria is known to be implicated in cell death and, consequently, may be a target in cancer therapy (Rizzuto et al. 2009).

PEROXISOMES

Recent results indicate that peroxisomes are capable of taking up and storing Ca^{2+} within their lumens at concentrations much higher than those found in the cytoplasm (Raychaudhury et al. 2006; Lasorsa et al. 2008). However, it is not yet known how Ca^{2+} is stored within these organelles and whether there exist specific peroxisomal Ca^{2+} buffering proteins.

ENDOLYSOSOMAL COMPARTMENT

Recent results show that nicotinic acid adenine dinucleotide phosphate (NAADP) acts as a second messenger to mobilize intracellular Ca^{2+} stores through actions on two-pore channels (TPCs) in endosomal membranes (Calcraft et al. 2009). Endosomes and lysosomes may thus be considered to be Ca^{2+} storage organelles; the importance of lysosomal Ca^{2+} is shown by Niemann-Pick type C1 disease, a neurodegenerative lysosomal storage disease caused by perturbations in lysosomal Ca^{2+} handling (Lloyd-Evans et al. 2008). However, the mechanism of lysosomal Ca^{2+} buffering has not been described.

CONCLUSION

Eukaryotic cells use Ca^{2+} as an intracellular signal to regulate a wealth of processes; it is thus unsurprising to see that these cells have evolved equally varied proteins to buffer Ca^{2+} within the cell. The importance of these proteins is shown by consequences of their loss or mutation, which frequently result in cardiac and/or muscular phenotypes, emphasizing the importance of Ca^{2+} in muscular contraction. One characteristic uniting organellar Ca^{2+} buffers, which display such variety in their Ca^{2+} binding and their responses to Ca^{2+} , is their multifunctionality. Instead of being limited to serving as a passive sponge for Ca^{2+} within intracellular organelles, Ca^{2+} -buffering proteins are responsible for a variety of processes, including protein folding, regulation of apoptosis, and regulating Ca^{2+} release pathways. It thus may be limiting to name these proteins simply Ca^{2+} buffers, as this reduces their functions to just one, whereas they typically have other roles that may trump Ca^{2+} buffering in importance. Furthermore, the versatility of Ca^{2+} as a messenger suggests that the presence of proteins that respond to Ca^{2+} within so many organelles correlates to a network of Ca^{2+} signaling linking subcellular organelles, as shown in Figure 1.

ACKNOWLEDGMENTS

Work in our laboratory is supported by grants from the Canadian Institutes of Health Research Heart (MOP-53050, MOP-15415, MOP-15291) and Stroke Foundation of Alberta, Alberta Innovates–Health Sciences. D. Prins is supported by the Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarship–Master’s Award and a Studentship Award from the Alberta Innovates–Health Sciences.

ABBREVIATIONS USED

ER, endoplasmic reticulum; IP_3 , inositol-1,4,5-trisphosphate; IP_3R , inositol-1,4,5-trisphosphate receptor; PDI, protein disulphide isomerase, RyR, ryanodine receptor; SERCA,

sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SOCE, store-operated Ca^{2+} entry; SR, sarcoplasmic reticulum; STIM1, stromal-interacting molecule-1.

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