[Frontiers in Bioscience 7, d1454-1463, June 1, 2002]

REGULATION OF SARCOPLASMIC RETICULUM CALCIUM RELEASE BY LUMINAL CALCIUM IN CARDIAC MUSCLE

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1. ABSTRACT

The amount of Ca^{2+} released from the sarcoplasmic reticulum (SR) is a principal determinant of cardiac contractility. Normally, the SR Ca^{2+} stores are mobilized through the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR). In this process, Ca^{2+} enters the cell through plasmalemmal voltage-dependent Ca^{2+} channels to activate the Ca^{2+} release channels in the SR membrane. Consequently, the control of Ca^{2+} release by cytosolic Ca^{2+} has traditionally been the main focus of cardiac excitation-contraction (EC) coupling research. Evidence obtained recently suggests that SR Ca release is controlled not only by cytosolic Ca^{2+}, but also by Ca^{2+} in the lumen of the SR. The presence of a luminal Ca^{2+} sensor regulating release of SR luminal Ca^{2+} potentially has profound implications for our understanding of EC coupling and intracellular Ca^{2+} cycling. Here we review evidence, obtained using *in situ* and *in vitro* approaches, in support of such a luminal Ca^{2+} sensor in cardiac muscle. We also discuss the role of control of Ca^{2+} release channels by luminal Ca^{2+} in termination and stabilization of CICR, as well as in shaping the response of cardiac myocytes to various inotropic influences and diseased states such as Ca^{2+} overload and heart failure.

2. INTRODUCTION

In cardiac muscle, most of the Ca^{2+} required for contractile activation is derived from a specialized intracellular Ca^{2+} release and storage organelle, the SR. During electrical activation, the Ca^{2+} that enters the cell through plasmalemmal voltage dependent Ca^{2+} channels binds to and activates the Ca^{2+} release channels, also known as ryanodine receptors (RyRs), clustered in release units in the membrane of the SR. When the Ca^{2+} release channels open, a much larger amount of Ca^{2+} is released from the SR, resulting in activation of contractile proteins. This mechanism is known as CICR (for reviews see refs. 1-4). Intuitively, CICR, at least in individual RyR clusters, should be self-regenerating and continue until completion because of the positive feedback of released Ca^{2+} on further release. However, relaxation of cardiac muscle requires a robust termination of Ca^{2+} release, so that resting cytosolic Ca^{2+} can be restored by Ca^{2+} transporters present in both the SR membrane and plasmalemma. Intense research has been brought to bear on the mechanisms that terminate and stabilize CICR. Much attention has been focused on the role of cytosolic Ca^{2+} in regulating Ca^{2+} release. In particular, it has been suggested that binding of Ca^{2+} to inhibition sites on the RyR causes channel activity to decrease through processes referred to as Ca^{2+}-dependent inactivation or adaptation accounting for the early termination of Ca^{2+} release. However, the role of these mechanisms that involve changes in cytosolic $[Ca^{2+}]$ remains controversial (See ref. 5 for a review).

The key to resolving the paradoxes of CICR may reside on the luminal side of the SR. Growing evidence suggests that the SR Ca^{2+} release process is regulated not only by cytosolic Ca^{2+} but also by Ca^{2+} inside the lumen of the SR. The regulatory mechanism appears to be much more sophisticated than simply its influence upon the concentration gradient between the SR and the cytosol. An emerging view is that the size and the functional state of the SR Ca^{2+} store is controlled by Ca^{2+} sensing sites on the luminal side of the Ca^{2+} release channel. By linking the loading status of the SR to the activity of RyRs, the luminal Ca^{2+} sensor stabilizes CICR and influences the way in which the cell responds to pharmacological agents that affect SR Ca^{2+} cycling. Furthermore, alterations in this mechanism may contribute to certain pathological conditions such as Ca^{2+}-dependent arrythmias and heart failure. In this article, we summarize experimental evidence for luminal Ca^{2+} regulation of Ca^{2+} release. We also review potential molecular mechanisms and discuss functional implications of this control mechanism in normal and diseased heart.

3. Ca^{2+} IN THE SR

The amount of Ca^{2+} stored in the SR is determined by the balance between Ca^{2+} uptake by the SR Ca^{2+} pump (sarco/endoplasmic reticulum Ca^{2+}-ATPase, SERCA), binding of Ca^{2+} to luminal buffers such as calsequestrin (CSQ), and Ca^{2+} leak from the SR via the RyRs. For the purpose of this review, it is important to distinguish between the free and total SR $[Ca^{2+}]$ ($[Ca^{2+}]_{SR}$). During release, Ca^{2+} bound to luminal buffers is expected to dissociate from these binding sites, thus contributing to the Ca^{2+} released. Therefore, the amount of bound Ca^{2+} may be an important determinant of the functional size of the pool. However, it is the free $[Ca^{2+}]_{SR}$ that determines the concentration gradient and electrochemical driving force for Ca^{2+} across the SR membrane. Similarly, it is the free Ca^{2+} that is likely to govern various Ca^{2+}-dependent processes in the lumen of the SR, including modulation of the functional activity of the Ca^{2+} release channels. In the sections below we briefly summarize the data available regarding estimates of the total and free $[Ca^{2+}]$ in the SR and the properties and role of the main luminal Ca^{2+} buffer, CSQ.

3.1. Total $[Ca^{2+}]_{SR}$ and calsequestrin

The total SR Ca^{2+} content in intact cells is commonly estimated by measuring the amount of Ca^{2+} released from the SR to the cytoplasm upon addition of caffeine. The amount of released Ca^{2+} is inferred by using fluorescent Ca^{2+} dyes or by integrating the Na^{+}/Ca^{2+} exchange current (e.g. refs. 6-9). Most studies performed under normal cellular conditions have estimated the resting total SR Ca^{2+} content to be in the range of 50-260 μmol/liter of cytosol in myocytes from various mammalian species. (e.g. 4). Assuming the SR comprises 3.5% of cell volume (4), the total SR Ca^{2+} content is 1.4-7.4 mmol/liter of SR volume. As discussed below, a substantial part of this Ca^{2+} may be bound to low affinity intraluminal buffers. The concentration of Ca^{2+} binding sites within the SR has been estimated to be 3 or 14 mM in intact ventricular myocytes (10) and isolated cardiac SR microsomes (11), respectively, with a K_D of 0.63 mM (11). The difference in luminal Ca^{2+} buffering between intact cells and SR vesicles is likely to reflect SR fragmentation caused by tissue homogenization (12). The Ca^{2+} binding properties of the SR in cardiac myocytes match reasonably well those of CSQ, supporting the notion that CSQ is a major site for storing Ca^{2+}. Cardiac CSQ binds ~40 Ca^{2+} ions/mole with an apparent affinity of 0.5 mM (13). Based on the reported yield of isolated cardiac CSQ (14; 40 mg/kg wet wt), the concentration of CSQ Ca^{2+} binding sites in cardiac SR can be estimated to be in the range of 3.2-6.4 mM (4). Thus, assuming a total concentration of Ca^{2+} in the SR of 4 mM and a concentration of SR Ca^{2+} binding sites of 4-5 mM with a K_D of 0.65 mM, the free $[Ca^{2+}]_{SR}$ would be about 0.6 - 1.0 mM and the fraction of intra SR Ca^{2+} bound to buffers would be approximately 50-75%. During a twitch, up to 60% of the total Ca^{2+} is released from the SR (7). Therefore, depending on the true amount of bound luminal Ca^{2+}, a substantial fraction (up to 50%) of Ca^{2+} that is released during a twitch may be released from CSQ. While the equilibrium binding properties of Ca^{2+} to CSQ are relatively well characterized, very little is known about the kinetics of binding. Apparently, no experimental studies of the association and dissociation rate constants have been reported in the literature. The paucity of kinetic data for Ca^{2+} binding to CSQ makes it difficult to assess the relative roles of binding and diffusion in establishing the concentration profile of free Ca^{2+} within the SR. Also the role of minor luminal Ca^{2+} binding proteins (sarcalumenin, histidine rich Ca^{2+}-binding protein, and calreticulin, ref. 4) in cardiac SR Ca^{2+} homeostasis remains to be determined.

10-20 fold over-expression of CSQ leads to development of severe heart failure in mice and reduction of the amplitude of Ca^{2+} transients in cells isolated from the failing hearts (15-17). At steady state, increasing Ca^{2+} binding site concentration would be expected to increase the amount of releasable Ca^{2+}. This is expected because the overexpressed CSQ provides a larger store of SR Ca^{2+}. However, in a beating cardiac cell, increased Ca^{2+} store size may slow the dynamic recovery of $[Ca^{2+}]_{SR}$ because of the longer times required to refill the store. This is a potential explanation for why EC coupling is depressed in mice overexpressing CSQ.

Recently it has been demonstrated that a missense mutation in a highly conserved region of CSQ is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in humans (PVT, ref. 18). The mutation converts a negatively charged aspartic acid into a positively charged histidine, in a highly negatively charged domain of calseqestrin, and is likely to compromise the ability of CSQ to bind Ca^{2+}. The specific mechanism whereby this defect induces PVT remains to be elucidated.

3.2. Free $[Ca^{2+}]_{SR}$

Few experimental studies have been performed to measure free $[Ca^{2+}]_{SR}$ in cardiac muscle. One obstacle has been the lack of low affinity fluorescent Ca^{2+} indicators that would be suitable for measuring Ca^{2+} in the millimolar range in which Ca^{2+} appears to be present in this compartment. Another difficulty is introducing the probe into the SR. Shannon and Bers (11) measured $[Ca^{2+}]_{SR}$ in rat isolated cardiac microsomes with the Ca-Mg indicator furaptra entrapped in the vesicles by homogenization of cardiac tissue in the presence of the indicator. They estimated that the resting free $[Ca^{2+}]_{SR}$ reaches 0.7 mM for cytosolic $[Ca^{2+}]$ of 100 nM. This value might underestimate the true intra-vesicular $[Ca^{2+}]$ because the fluorescence signal saturated also near 0.7 mM Ca^{2+}. On the other hand, the content of Ca^{2+} in the vesicles was likely to have been higher than normal because of the presence, in the experimental solutions, of ruthenium red to block the RyRs. Inhibition of Ca^{2+} leak via RyRs by this drug is known to lead to a substantial increase of the SR Ca^{2+} content (9). Chen et al. (19) used a low affinity Ca^{2+}-sensitive NMR probe (TF-BAPTA) to measured free $[Ca^{2+}]$ inside the SR in intact, perfused working hearts and reported a diastolic value of ~1.5 mM. Raising cytosolic free Ca^{2+} by exposing the heart to elevated extracellular KCl, resulted in an increase in $[Ca^{2+}]_{SR}$ to about 5 mM. During systole $[Ca^{2+}]_{SR}$ decreased only moderately (by ~30%), consistent with the notion that the Ca^{2+} is heavily buffered in the SR. It is unclear how such measurements of $[Ca^{2+}]$, averaged throughout the entire SR luminal compartment, reflect the local $[Ca^{2+}]$ changes near Ca^{2+} release sites. A mathematical model of Ca^{2+} release from the junctional SR (jSR) predicts that local release events (i.e. Ca^{2+} sparks) might be associated with significant depletion of local Ca^{2+} in the jSR elements (20). Clearly, more experimental and modeling studies are needed to define the changes in $[Ca^{2+}]_{SR}$ during both global and local Ca^{2+} release from the SR.

4. EXPERIMENTAL EVIDENCE FOR MODULATION OF THE Ca^{2+} RELEASE MECHANISM BY LUMINAL Ca^{2+}

A number of studies have explored the dependence of SR Ca^{2+} release on SR Ca^{2+} content in intact and permeabilized cardiac cells. In addition, the effects of luminal Ca^{2+} have been studied in RyR channels reconstituted into lipid bilayers. Most of these studies found that luminal Ca^{2+} influences the functional activity of the Ca^{2+} release channels. However, the interpretation of the results has been complicated by the existence of at least two potential mechanisms: 1) extra-SR effects involving the cytosolic Ca^{2+} activation sites of the RyR; and 2) intra-SR effects mediated by distinct luminal Ca^{2+} sensing sites on the RyR or associated proteins. It is likely that these two mechanisms co-exist in cardiac myocytes. Here we will summarize evidence, obtained using *in situ* and *in vitro* approaches, in favor and against a luminal Ca^{2+} sensor regulating RyR activity.

4.1. Cell studies

In 1992, Fabiato (21) provided the first experimental evidence for regulation of Ca^{2+} release by Ca^{2+} stored inside the SR of cardiac muscle. He showed that mechanically skinned cardiac myocytes exhibit spontaneous Ca^{2+} release, which required a SR Ca^{2+} overload. High cytosolic Ca^{2+} did not inactivate this release. This mechanism existed in addition to the time- and Ca^{2+}-dependent Ca^{2+} release that is first activated and then inactivated by an increase of Ca^{2+} at the cytosolic side of the SR. Fabiato (21) proposed that this second type of release is initiated by binding of Ca^{2+} to regulatory sites in the lumen of the SR.

In intact cardiac myocytes, a number of investigators have explored the effects of changes of SR Ca^{2+} content on Ca^{2+} release from the SR (for a review see 4). Most studies found that that SR Ca^{2+} release increases steeply with the increase in the SR Ca^{2+} content. For example, Shannon et al. (22) used conditioning pulses to progressively increase SR Ca^{2+} load. This study employed caffeine applications to empty the SR for assessment of its Ca^{2+} content. They found a steep nonlinear relationship between the fraction of Ca^{2+} released and SR Ca^{2+} content. The highly non-linear relationship between Ca^{2+} release and SR Ca^{2+} content, revealed in these studies, indicates that the effects of load may not be simply due to the increased amount of Ca^{2+} available for release. Rather, they likely involve alterations of RyR gating. It is important to note that, while consistent with regulation of the release mechanism by luminal Ca^{2+}, these effects can also be readily explained by extra-SR effects through cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Indeed, $[Ca^{2+}]_i$ near the release sites would be expected to be higher for any RyR opening at increased SR Ca^{2+} loads. This higher $[Ca^{2+}]_i$ would tend to activate more neighboring RyRs via CICR, thereby accounting for or contributing to disproportionately large SR Ca^{2+} release.

At the local Ca^{2+} release level, several studies have demonstrated that the rate of occurrence of elementary Ca^{2+} release events (i. e. sparks) depends positively on SR Ca^{2+} content (23-25). In these studies, the SR Ca^{2+} content was increased by elevating the $[Ca^{2+}]$ in the extracellular solution or by altering the rate of electrical stimulation of the cells. Subsequently, Song et al. (26) found no significant change in the frequency of sparks when the SR Ca^{2+} content was reduced by thapsigargin (a selective inhibitor of the SR Ca^{2+} pump), while correcting the spark statistics for changes in detectability of events. Because the detectability of small sparks against background noise is reduced in confocal microscopy, the data had to be corrected to avoid overestimating the reduction of spark frequency. These authors attributed the changes in spark frequency observed in previous studies to two factors. The first was altered detectability of sparks with different amplitudes against the background noise. The second was increases in cytosolic Ca^{2+} that usually accompany alterations in SR Ca^{2+} load upon elevating extracellular [Ca^{2+}]. Lukyanenko and co-workers (27) then re-examined the effects of alterations of SR Ca^{2+} content on Ca^{2+} sparks in permeabilized myocytes at constant (i.e. buffered) cytosolic [Ca^{2+}] and with corrections made to account for missed events. Enhancing the SR Ca^{2+} content by selectively stimulating the efficiency of the SR Ca^{2+} pump (using an anti-phospholamban antibody) increased the frequency of Ca^{2+} release events. Furthermore, in myocytes exposed to elevated cytosolic Ca^{2+} to increase the initial SR Ca^{2+} content, partial depletion of the SR by thapsigargin reduced the frequency of sparks (corrected for missed events). Thus, it is possible that the Song et al's (26) experiments were performed at relatively low SR Ca^{2+} loads, at which luminal Ca^{2+} was outside the range in which it could effectively influence release site activity. All together, the relationship between spark frequency and SR Ca^{2+} content seems to support active luminal regulation of release at luminal sites.

In addition to monitoring fractional Ca^{2+} release and spark frequency at different SR Ca^{2+} loads, another useful strategy for demonstrating the role of luminal Ca^{2+} in controlling Ca^{2+} release has been the use of RyR inhibitors. It has been shown that certain RyR inhibitors, such as tetracaine, only transiently suppress spontaneous (i.e. Ca^{2+} sparks and waves) and experimentally evoked Ca^{2+} release (27-31). The restoration of release in the continuous presence of the drugs was associated with an increase in the SR Ca^{2+} content caused by reduced leak of Ca^{2+} through the RyRs. One possibility is that the recovery of release in the presence of tetracaine is simply due to increased amount of releasable Ca^{2+} in the SR (29,30). The decreased number of open Ca^{2+} release channels may be offset by an enhanced Ca^{2+} flux through each channel that was not inhibited by the drug. Such a simple compensation of blockage could occur only if inhibition by the drug is partial, and at least some of the release sites remain available for liberation of Ca^{2+}. Further, at the local release level, such a mechanism would be expected to manifest itself only by an increase in the magnitude of sparks without any increase in their frequency. In contrast to these expectations, we found that the effects of tetracaine were transient not only with respect to the amplitude of release events, but also with respect to their frequency (27,28). In addition, the recovery of release was observed even with tetracaine concentrations that caused an initial complete inhibition of release (31). Such observations indicate that the recovery of release from inhibition is not simply due to an increase in the amount of releasable Ca^{2+}. Instead the release mechanism itself becomes altered by luminal Ca^{2+} in a way that makes it less sensitive to inhibition by tetracaine. These results cannot be explained easily by effects of Ca^{2+} on the cytosolic side of the SR and provide strong evidence in support of existence of distinct intraluminal Ca^{2+} sensing sites that regulate the behavior of the RyR allosterically.

4.2. Studies in RyRs reconstituted in bilayers

A number of investigators have shown that increasing Ca^{2+} on the luminal side of the RyR leads to an increase in RyR channel open probability (24,32-36). These effects occurred in the range of 0.1 – 10 mM Ca^{2+} (apparent K_D ~ 2 mM, Hill coefficient ~ 2; i.e. ref. 34) and were manifested predominantly by increased open times (35) or an increased frequency of events (34). While in some studies the presence of cytosolic Ca^{2+}, alone, was sufficient (albeit at high concentrations) to mediate the effects of luminal Ca^{2+} (35), other studies found that the activation by luminal Ca^{2+} required, in addition to cytosolic Ca^{2+}, the presence of allosteric modulators of RyR activity such as sulmazole, caffeine, or ATP on the cytosolic side of the channel (24,32-34). Similar to experiments with cardiac myocytes, two mechanisms of luminal Ca^{2+} regulation have been proposed. One suggestion is that Ca^{2+} flowing through the open RyR channel activates the channel by interacting with its cytosolic Ca^{2+} activation sites ('feed-through" regulation). The other suggestion is that luminal Ca^{2+} acts at distinct sites on the luminal side of the Ca^{2+} release channel complex (true luminal regulation). Locating the site of action of luminal Ca^{2+} is confounded by the fact that, with millimolar luminal Ca^{2+}, high Ca^{2+} flux from the luminal to the cytosolic side of the channel makes it difficult to exclude the possibility that luminal Ca^{2+} has some access to cytosolic sites.

In support for the "feed-through" regulation hypothesis, Xu and Meissner (35) found, in canine RyRs purified by sucrose gradient, that the effects of luminal Ca^{2+} are much larger at negative holding potentials than at positive holding potentials. Negative holding potentials favor luminal-to cytosolic Ca^{2+} fluxes. They were able to correlate the effects of luminal Ca^{2+} on RyR open probability (P_o) with the magnitude of luminal-to cytosolic fluxes. In the presence of caffeine and nanomolar cytosolic Ca^{2+}, estimated luminal-to-cytosolic fluxes of 0.25 pA increased channel P_o. At high cytosolic [Ca^{2+}], estimated luminal Ca^{2+} fluxes of 8 pA caused a decline in channel activity. The authors proposed that Ca^{2+} passing through the channel could both activate and inactivate the channel at cytosolic sites. These studies provide strong evidence for the ability of Ca^{2+} passing through the pore to influence channel activity at cytosolic regulatory sites. However, they do not necessarily rule out the possibility that luminal Ca^{2+} can also modulate channel activity at distinct luminal sites.
The existence of luminal sites was addressed directly (34) by performing measurements at high positive membrane potentials and at high cytosolic Ca^{2+} conditions, in which the electrochemical gradient does not support luminal-to-cytosolic Ca^{2+} fluxes. Luminal Ca^{2+} was found to potentiate native canine cardiac RyRs, regardless of whether Ca^{2+} flowed from the luminal to cytosolic side or from the cytosolic to luminal side of the channel. These results support the notion that luminal flux is not required for the effects of luminal Ca^{2+}. Recently Ching and co-workers (36) also reported convincing evidence of luminally-located Ca^{2+} regulatory sites in native sheep cardiac RyR using a tryptic digestion approach. After the RyRs were exposed to luminal trypsin, they lost their ability to respond to luminal Ca^{2+}. Apparently the luminal Ca^{2+} activation sites were

damaged by trypsin digestion. Thus, the single channel data accumulated to date show that, in some instances, luminal Ca^{2+} can have access to the cytosolic Ca^{2+} regulatory sites. At the same time, they also provide evidence for the existence of distinct regulatory sites on the luminal side of the channel. Taken together, the studies described above seem to support the existence of a true luminal Ca^{2+} sensor that controls the function of the RyR channel.

5. MOLECULAR STRUCTURE OF THE LUMINAL Ca^{2+} SENSOR

Very little is known about the structure of the luminal Ca^{2+} binding sites. Two obvious possibilities are that Ca^{2+} binds directly to the luminal aspect of the ryanodine receptor protein, or that it binds to an auxiliary protein with luminal location. The luminal loops connecting the putative transmembrane spanning domains M1-M2 and M3-M4 of the RyR possesses many negatively charged residues (37-39). Calcium ions could bind to these regions, altering the channel conformation to increase its open probability. Consistent with this possibility, the effects of luminal Ca^{2+} have been described in RyRs purified with CHAPS solubilization (32). It should be noted, however, that CHAPS solubilization does not necessarily lead to dissociation of all the proteins from the RyR (40). In addition, the results of experiments with purified RyRs could have been influenced by potential effects of luminal Ca^{2+} at the cytosolic activation sites, making it difficult to discriminate true luminal from feed-through effects of Ca^{2+}. Therefore the possibility that luminal Ca^{2+} exerts its modulatory influences indirectly, via interaction of the RyR with Ca^{2+}-sensitive luminal proteins, cannot be discarded.

Cardiac RyRs localized in the jSR appear to complex with a number of luminal proteins including CSQ, triadin and junctin (40). As discussed above, CSQ may bind a large portion of Ca^{2+} in the SR and provide a reserve for release. In addition, biochemical evidence obtained predominantly in skeletal muscle suggests that CSQ may actively participate in SR Ca^{2+} release by modulating the RyR (41,42). The actions of CSQ on the RyR could be direct or require the presence of intermediate linker proteins such as triadin or junctin. Consistent with the former possibility, the addition of CSQ to the luminal side of the skeletal RyR has been reported to enhance P_o of the channel in a Ca^{2+}-dependent manner (43,44). Junctin and triadin are structurally related integral membrane proteins that co-localize with the RyR and CSQ at the jSR membrane in cardiac and skeletal muscle (40,45-46). It appears that junctin and triadin interact directly in the jSR membrane and form a complex that anchors CSQ to the ryanodine receptor (40). Therefore, these proteins could mediate the proposed effects of CSQ on the RyR. The role of CSQ as a luminal Ca^{2+} sensor for the RyR was questioned by Ching and co-workers (36). These workers demonstrated that trypsin does not cleave CSQ, although exposure to the enzyme does abolish the luminal sensitivity of the RyR. This implies that the observed changes in luminal regulation of RyR were not caused by damage to CSQ, unless trypsin damaged certain structures involved in the interaction of the RyR and CSQ on the channel protein, itself, or on intermediate linker proteins. An alternative possibility is that the luminal Ca^{2+} sensor is formed by either junctin or triadin instead of CSQ. Their luminal domains are also rich in negatively charged residues that could form the Ca^{2+}-binding regions. Furthermore, these proteins do have putative sites for cleavage that could account for the loss of luminal Ca^{2+} sensitivity upon trypsin digestion.

6. FUNCTIONAL IMPLICATIONS FOR NORMAL PHYSIOLOGY AND DISEASE

The presence of a luminal Ca^{2+} sensor regulating release of luminal Ca^{2+} potentially has profound implications for our understanding of cardiac EC coupling and intracellular Ca^{2+} cycling in cardiac muscle. Here we will review the possible role for such a sensor in termination and long-term stabilization of CICR. We will also examine its potential role in such pathological conditions as regenerative Ca^{2+} waves and impaired Ca^{2+} release in heart failure

6.1. Termination of CICR

As a system in which Ca^{2+} is both the trigger and the output signal, CICR should be inherently unstable and self-regenerating. Yet, Ca^{2+} release is tightly graded with the magnitude of the Ca^{2+} trigger and robustly terminates. Despite intense effort, the mechanisms involved in control of CICR remain poorly understood. Lowering Ca^{2+} in the lumen of the SR would decrease channel activity, thereby providing a potential negative control mechanism to counter the positive feedback of CICR. Several recent studies are consistent with this scenario. For example, depletion of $[Ca^{2+}]_{SR}$ resulted in a disproportionately large decrease in the amount of Ca^{2+} released from the SR (7). This supports the notion that SR depletion may somehow contribute to the termination signal for Ca^{2+} release. Evidence for functional depletion of SR Ca^{2+} stores was also presented in recent studies of refractoriness of Ca^{2+} release. Following release of Ca^{2+}, time must pass before CICR can be activated again (23,47). This refractory behavior has been commonly attributed to inactivation by cytosolic Ca^{2+}. Recently, DelPrincipe and co-workers (48) showed that the SR Ca^{2+} release mechanism exhibits a much more prominent refractoriness following its activation on a global scale than following local activation of just a few release sites by photolysis of caged Ca^{2+}. They attributed this discrepancy to functional depletion of SR Ca^{2+}, which leaves the Ca^{2+} release channels unresponsive to Ca^{2+} trigger until the SR Ca^{2+} store is re-charged with Ca^{2+}. A direct experimental confirmation of the role of luminal Ca^{2+} in termination of CICR may come from studies that use low affinity Ca^{2+} buffers (ADA, citrate, or maleate) loaded into the SR. According to preliminary data obtained in our laboratory, clamping the level of Ca^{2+} in the SR by these exogenous buffers leads to dramatic increases in the amplitude and time-to-peak of sparks, as well as the duration of local Ca^{2+} release fluxes underlying Ca^{2+} sparks (49). These findings imply that the level of Ca^{2+} in the SR controls termination of Ca^{2+} release. At the whole-cell level, Ca^{2+} release loses its ability to respond to Ca^{2+}

stimuli in a graded fashion, in the presence of exogenous buffers in the SR (50). These data suggest that regulation of RyR openings by local intra-SR [Ca^{2+}] might be responsible for termination of Ca^{2+} sparks, and that robust termination of sparks is required for graded behavior of macroscopic Ca^{2+} release. Recently a mathematical model of Ca^{2+} sparks also has been suggested, in which RyR gating depends on luminal Ca^{2+} and the coupling between RyRs, in addition to the well-established activation by local Ca^{2+} in the dyadic cleft (51).

6.2. Dynamic control of SR Ca^{2+} content and release

In addition to providing an immediate "break" for regenerative CICR, the luminal Ca^{2+} sensor appears to continuously regulate the functional activity of the SR Ca^{2+} stores by linking SR Ca^{2+} content to the activity of the RyRs. Evidence in support of such a dynamic control mechanism has come from imaging spontaneous and electrically evoked Ca^{2+} sparks following pharmacological disturbances of the RyR channels or the Ca^{2+} pump in the SR membrane (27,28). In cardiac myocytes, stochastic openings of RyRs, manifested as spontaneous Ca^{2+} sparks, mediate a substantial leak (1,23-27) that plays a significant role in setting the SR Ca^{2+} content (9,27-29). The frequency of sparks increased at elevated loads and decreased at reduced loads (in the presence of a SERCA activating antibody or thapsigargin, respectively, ref. 27). Modulation of RyR channels by their inhibitors (tetracaine, Mg^{2+}) or agonists (caffeine) produced only transient changes (suppression or potentiation, respectively) in the frequency of sparks. These effects were accompanied by either an increase or a decrease, respectively, of the SR Ca^{2+} content. These results were attributed to a luminal Ca^{2+} sensor regulating the functional state of the SR Ca^{2+} release channel (27). For example, when the RyR channel blocker tetracaine is applied, the leak of Ca^{2+} through the channels (i.e. appearing as Ca^{2+} sparks) is decreased, resulting in accumulation of Ca^{2+} in the SR. The luminal Ca^{2+} sensor detects this elevation and increases the open channel probability of the RyR. This, in turn, leads to the recovery of sparking activity and counterbalances the inhibition by tetracaine. The sequence of events is the opposite when the RyR agonist caffeine is applied. Potentiation of RyRs leads to enhanced Ca^{2+} leak, causing the SR Ca^{2+} content to decline. The decreased $[Ca^{2+}]_{SR}$ leads to reduced RyR activity, thereby counterbalancing the primary potentiation of Ca^{2+} sparks by caffeine. Thus, by linking the open probability of the RyR to the loading state of the SR, the luminal Ca^{2+} sensor endows the myocytes with an ability to auto-regulate the functional size of their SR Ca^{2+} pool. In general, these results predict that because of the feedback regulation of the SR Ca^{2+} stores by luminal Ca^{2+} any maintained and selective modulation of RyRs would have only temporary effects on Ca^{2+} release.

Clearly, such a dynamic control mechanism, although possessing a certain time lag (due to the dynamics of changing SR load), should be advantageous for stabilizing Ca^{2+} cycling when either Ca^{2+} release or uptake is altered. The significance of luminal Ca^{2+} regulation can also be considered in the context of periodic Ca^{2+} cycling in cardiac cells. The periodic beating of the heart requires that Ca^{2+}, once released, is rapidly re-sequestered in the SR. To move Ca^{2+} rapidly, the SR Ca^{2+} pump may have to maintain high levels of cycling through the whole range of [Ca^{2+}] to which the pump is exposed in the cytosol. This Ca^{2+} transport mechanism, which is designed for rapid Ca^{2+} uptake, might be too coarse for precise adjustments of the SR Ca^{2+} content. Such fine adjustments might be necessary to avoid SR Ca^{2+} overload, which could result in loss of stability of CICR. The luminal Ca^{2+}-dependent leak may serve to fine tune the SR Ca^{2+} content by releasing excess Ca^{2+}. Thus RyRs, in addition to their role as a major Ca^{2+} release pathway, may also operate as "safety valves" to maintain stable Ca^{2+} load and release.

To fully understand Ca^{2+} cycling in beating myocytes, the intrinsic feedback regulation of SR function by luminal Ca^{2+} has to be considered together with other control processes, such as reciprocal interactions between different Ca^{2+} transport mechanisms in the plasmalemma (e.g. L-type Ca^{2+} channels, Na^{+}-Ca^{2+} exchanger) and the SR (e.g. RyRs, SERCA) (for a review, see ref. 52). All these mechanisms must act in synergy to maintain Ca^{2+} homeostasis. A special role of the luminal Ca^{2+} sensor maybe in providing a basic set point for adjusting the level of [Ca^{2+}] inside the SR.

6.3. Maintained regulation of Ca^{2+} release by cADPR

Given such dynamic regulation of release, however, why do certain substances such as cADP-ribose (cADPR), which are thought to interact specifically with RyRs, have maintained modulatory effects on release? This paradox can be resolved if such substances do not act directly upon the RyRs, but, in fact, influence the release channel indirectly through luminal modulation.

cADPR appears to present just such an example in which the release is enhanced solely by increasing the uptake, without any direct effects on the RyRs. In recent years, cADPR has emerged as a potential endogenous regulator of SR Ca^{2+} release (53-55). For example, it has been demonstrated that cADPR applied to the cytosol increases cell-averaged and local Ca^{2+} transients and contractions (56-58,64). The compound was initially viewed as a specific agonist of RyR channels, acting directly by increasing the open probability of the RyR (59). However, subsequent studies have indicated no effect of cADPR upon RyRs, or have detected influences that should be abolished in the presence of physiological concentrations of ATP (60,61). The conflicting data have led some investigators to rule out cADPR altogether as modulator of SR Ca^{2+} release (62).

This body of apparently contradictory results was reconciled by the recent finding that cADPR acts by enhancing SR Ca^{2+} uptake (63). Potentiation of Ca^{2+} release by cADPR appears to be mediated by increased SR Ca^{2+} load due to persistent enhancement of uptake, with subsequent luminal Ca^{2+}-dependent activation of RyRs. The evidence for this mechanism includes the following observations in response to cADPR application to permeabilized cardiac myocytes: increased frequency of local Ca^{2+} release events (i.e. sparks), increased SR Ca^{2+}

load, and reduced influence of cADPR in the presence of the SERCA2a inhibitor thapsigargin. At the same time, cADPR has negligible impact upon the activity of single RyRs in lipid bilayers, but significantly increased Ca^{2+} uptake by cardiac SR microsomes (63). The exact biochemical mechanism for the effect of cADPR upon SERCA activity is to date unknown, but could involve direct potentiation of the SERCA pump, or relief of the inhibition of the SERCA by dephosphorylated phospholamban. This mechanism of indirect modulation of RyR activity via the luminal sensor could therefore serve as a paradigm for other effectors of Ca^{2+} release that demonstrate maintained effects.

6.4. Ca^{2+} waves and arrhythmias

An excess of Ca^{2+} in the SR Ca^{2+} stores (Ca^{2+} overload) is a common feature in a variety of cell injuries (65). Ca^{2+} overload is known to promote the generation of spontaneous Ca^{2+} waves in cardiac myocytes (66-69). Regenerative Ca^{2+} waves are believed to be the underlying cause of both early and delayed afterdepolarizations (EAD and DAD), the basis of triggered arrhythmias in the heart (4,70,71). Considering the fact that increased $[Ca^{2+}]_{SR}$ enhances RyR P_o, how does the presence of the luminal Ca^{2+} sensor affect the generation of Ca^{2+} waves in Ca^{2+}-overloaded myocytes? Two specific scenarios have been discussed in this regard (21,23,69,67,72). According to the first mechanism, Ca^{2+} transported from the moving wavefront into the SR could trigger release by activation of RyRs from within the SR. The wave propagates as Ca^{2+} released from the SR is taken up into adjacent SR elements, raising local luminal Ca^{2+} above threshold for activation of the release mechanism at luminal sites. According to the second mechanism, elevated luminal Ca^{2+} sensitizes the Ca^{2+} release channels to cytosolic Ca^{2+}, enhancing the ability of cytosolic Ca^{2+} to activate adjacent release sites to cytosolic Ca^{2+} via CICR. From most experimental evidence, the second mechanism is more likely. For example, Engel and co-workers (72) investigated the temperature dependence of Ca^{2+} wave properties in isolated rat cardiomyocytes using digital Ca^{2+} imaging. They observed waves at 37ºC, 27ºC, and 17ºC. The velocities decreased by a factor of 1.8 over this range. At the same time, the half-maximal decay rates, which characterize local Ca^{2+} removal by the Ca^{2+} ATPase, increased by a factor of 3.5. The higher temperature sensitivity of Ca^{2+} removal compared with that of Ca^{2+} wave propagation is inconsistent with the hypothesis that Ca^{2+} wave propagation relies on Ca^{2+} ATPase-dependent uptake of Ca^{2+} from the spreading wave front into the SR. Also, using fluo-3 confocal microscopy, Cheng and co-workers (23) demonstrated that the local Ca^{2+} rise during spontaneous Ca^{2+} waves and electrically evoked Ca^{2+} transients has the same rapid time course. This is consistent with both phenomena having the same underlying mechanism, namely CICR. Lukyanenko and co-workers (69) used a pharmacological approach to discriminate between the cytosolic and luminal Ca^{2+} activation hypotheses. We examined the transition of focal caffeine-induced localized Ca^{2+} release into propagating Ca^{2+} waves under various experimental conditions. The conditions included increased SR Ca^{2+} loading, inhibition of SR Ca^{2+} uptake by thapsigargin, and sensitization of RyRs by caffeine. We were able to induce self-sustaining Ca^{2+} waves when the SR Ca^{2+} load was increased by exposing the cells to elevated extracellular Ca^{2+}. Inhibition of SR Ca^{2+} uptake by thapsigargin in cells preloaded with above normal levels of SR Ca^{2+} did not prevent local Ca^{2+} elevations from triggering propagating waves, but, instead, led to increased wave velocity. These results imply that Ca^{2+} wave propagation does not require translocation of Ca^{2+} from the spreading wave front into the SR. We were also able to induce propagating Ca^{2+} waves in cells with normal levels of SR Ca^{2+} load when the Ca^{2+} release mechanism was sensitized to cytosolic Ca^{2+} by low doses of caffeine (0.5 mM). This concentration of caffeine increases the P_o of RyR in lipid bilayers to about the same extent as does millimolar luminal Ca^{2+}. Therefore, it is clear that potentiation of RyR activity by elevated luminal Ca^{2+} could indeed contribute to higher incidence of Ca^{2+} waves in Ca^{2+}-overloaded myocytes.

How is this destabilizing influence of the luminal Ca^{2+} sensor reconciled with its potential stabilizing role discussed above? It appears that the dynamic control mechanism discussed above can operate effectively only within a certain range of SR Ca^{2+} load. When the increase in SR Ca^{2+} content falls outside this normal, "correctable" range as a result of cardiac cell injury, enhanced RyR channel activity, mediated by elevated luminal Ca^{2+}, tends to exacerbate the problem of instability, resulting in even more regenerative Ca^{2+} waves.

Recently it has been shown that a group of mutations in the cardiac RyR is associated with certain forms of ventricular arrhythmias causing sudden death (e.g. catecholaminergic polymorphic ventricular tachycardia, PVT, refs. 73-75). A similar condition has been linked to a mutation that disrupts Ca^{2+} binding in CSQ (18). The specific mechanisms thereby these genetic defects cause arrhythmias is not known. As discussed above, CSQ influences $[Ca^{2+}]_{SR}$ by binding luminal Ca^{2+} and may also modulate RyR open probability more directly as a putative luminal Ca^{2+} sensor. Given this and considering the central role regulation by luminal Ca^{2+} appears to play in controlling CICR and SR Ca^{2+} cycling, it is possible that altered RyR responsiveness to luminal Ca^{2+} is involved in the pathogenesis of these diseases.

6.5. Heart failure

Alterations in RyRs have been suggested to be a cause of reduced SR Ca^{2+} release and of diminished contractile response in failing hearts (76-80), although some other studies have found no alterations in expression or intrinsic gating behavior of RyRs in heart failure (81,82). As discussed above, one of the manifestations of SR Ca^{2+} release regulation by luminal Ca^{2+} is that any maintained and selective modulation of RyRs would be expected to have only transient effects on SR Ca^{2+} release (see also ref. 83). Therefore, alterations in RyR number or functional activity would not be expected to result in sustained changes in SR Ca^{2+} release, unless the dependence of the RyR on luminal Ca^{2+} were also altered or the aberrations of Ca^{2+} handling were to exceed the ability of the luminal

Ca^{2+}-mediated feedback mechanism to compensate the primary defects in RyRs (27). In accord with our preliminary data (84), luminal Ca^{2+} regulation of RyRs is indeed compromised in a dog model of heart failure. We can speculate that this reduced sensitivity of RyRs to luminal Ca^{2+} is an adaptive response to minimize the energy costs of Ca^{2+} cycling in failing hearts. Because luminal Ca^{2+}-dependent cycling may be important for stabilizing CICR, this adaptation may come at the price of reduced stability of CICR in heart failure. Thus, altered modulation of RyRs by luminal Ca^{2+} could potentially account for or contribute to fatal cardiac arrhythmias in failing hearts.

7. CONCLUSION

To summarize, luminal Ca^{2+} regulation of SR Ca^{2+} release has emerged as an important component of cardiac EC coupling and, therefore, should be included in any comprehensive description of the control of Ca^{2+} handling in cardiac muscle. Its consideration is also important to understand the impact of various inotropic influences and pathological conditions, such as heart failure upon Ca^{2+} cycling. Several key unknowns await determination for a complete understanding of this regulatory pathway. These include the molecular determinants of the luminal Ca^{2+} sensor and the precise levels of free $[Ca^{2+}]$ inside the SR to which the sensor is exposed to during Ca^{2+} cycling in heart cells.

8. ACKNOWLEDGMENTS

We thank Drs. R. Nathan and A. Zahradnikova for critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (HL 52620 and HL 03739 to S.G.) and the American Heart Association, Texas Affiliate (V.L.).

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Key Words: Excitation-Contraction Coupling, Ryanodine Receptor, Sarcoplasmic Reticulum, Review

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