Abnormal intrastore calcium signaling in chronic heart failure


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Diminished Ca release from the sarcoplasmic reticulum (SR) is an important contributor to the impaired contractility of the failing heart. Despite extensive effort, the underlying causes of abnormal SR Ca release in heart failure (HF) remain unknown. We used a combination of simultaneous imaging of cytosolic and SR intraluminal [Ca] in isolated cardiomyocytes and recordings from single-ryanodine receptor (RyR) channels reconstituted into lipid bilayers to investigate alterations in intracellular Ca handling in an experimental model of chronic HF. We found that diastolic free [Ca] inside the SR was dramatically reduced because of a Ca leak across the SR membrane, mediated by spontaneous local release events (Ca sparks), in HF myocytes. Additionally, the magnitudes of intrastore Ca depletion signals during global and focal Ca release events were blunted, and [Ca]r recovery was slowed after global but not focal Ca release in HF myocytes. At the single-RyR level, the sensitivity of RyRs to activation by luminal Ca was greatly enhanced, providing a molecular mechanism for the maintained potentiation of Ca sparks (and increased Ca leak) at reduced intra-SR [Ca] in HF. This work shows that the diminished SR Ca release characteristic of failing myocardium could be explained by increased sensitivity of RyRs to luminal Ca, leading to enhanced spark-mediated SR Ca leak and reduced intra-SR [Ca].

excitation–contraction coupling | ryanodine receptor | sarcoplasmic reticulum

In mammalian heart, the major source of Ca required for contractile activation is the sarcoplasmic reticulum (SR). Ca sequestered in this organelle can be rapidly released in response to a small entry of Ca from the extracellular milieu to the cytosol. This process, termed Ca-induced Ca release, involves specialized intracellular Ca release channels, the ryanodine receptors (RyRs), which are clustered in the SR membrane (1, 2). Ca levels inside the SR determine the functional role of this organelle in two major ways: firstly, they establish the overall size of the Ca pool available for release; secondly, SR luminal Ca regulates the functional state of the RyR Ca release channel. RyR open probability changes as a direct function of [Ca] at the luminal side of the channel (3, 4). The responsiveness of RyRs to luminal Ca seems to be mediated by the auxiliary proteins triadin, junctin, and calsequestrin (CASQ2), which are coupled to RyRs at the luminal surface of the SR (5). During the release process, the increase in cytosolic Ca is accompanied by a simultaneous, reciprocal decline in intra-SR Ca (6–8). This reduction in [Ca]sr leads to deactivation or closure of RyRs, contributing to Ca-induced Ca release termination (9, 10). At the same time, stimulatory effects of high luminal Ca on RyR channel open probability are responsible for the activation of a Ca leak pathway, which plays a role in setting the SR Ca content during the diastolic phase by leaking excess Ca from the SR (11, 12).

Heart failure (HF) occurs when there is a reduction in cardiac output that is inadequate to meet the metabolic demands of the body. The most common defect in HF is impaired contractility of the ventricles. Evidence suggests that the amount of Ca released from the SR into the cytosol is reduced, accounting for, or contributing to, the reduced contractile force generated by the failing heart. In a majority of studies, including those in human cardiomyocytes, reduced SR Ca release has been shown to be associated with a decrease in the SR Ca content (2, 13–17). Several explanations have been put forth for the diminished SR Ca stores in HF. In a number of reports, the reduction of SR Ca content has been attributed to depressed SR Ca-ATPase function and/or enhanced NCX activity during HF (12, 17, 18). These changes are expected to facilitate Ca removal from the cell at the expense of its uptake into the SR and result in under-filled SR Ca stores in HF. Another potential cause of reduced SR Ca content is enhanced diastolic leak of Ca via the RyRs (18–20). Although an enhanced RyR-mediated Ca leak could explain the reduced SR Ca content observed in HF, direct experimental support for this possibility in myocytes from failing heart is lacking. Thus, further studies are needed to determine the mechanisms of altered SR Ca handling in HF.

This report presents an investigation of subcellular and molecular features of HF in a canine model of chronic tachypacing-induced HF (21). To investigate the causes of aberrant SR Ca function in chronic HF, we performed measurements of myocyte cytosolic and SR luminal Ca, and conducted recordings from single-RyR channels. This work shows that the diminished SR Ca release that is characteristic of failing myocardium could be explained by increased sensitivity of RyRs to luminal Ca, leading to enhanced spark-mediated SR Ca leakage and reduced intra-SR [Ca].

Materials and Methods

Chronic, stable left ventricular dysfunction was induced by right ventricular apical tachypacing (modified Preval 8086 pacemaker, Medtronic, Minneapolis) in a canine model for 13–23 months. Myocytes were isolated by using standard techniques. Cytosolic and intra-SR [Ca] changes were monitored by using confocal microscopy, and whole-cell currents were recorded with the patch clamp technique. Single-channel recordings were carried out as described in ref. 3. Expression of the major SR Ca handling proteins was determined by immunoblot analysis. Detailed material and methods can be found in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

Results

I_{Ca}, Intracellular Ca Transients, and SR Ca Content in Intact Myocytes.

Cell capacitance in HF myocytes was dramatically increased (126 ± 21 pF, n = 8, in control, and 214 ± 39 pF, n = 9 in HF

This paper was submitted directly (Track II) to the PNAS office. Abbreviations: CASQ2, calsequestrin; HF, heart failure; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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myocytes), consistent with ventricular hypertrophy. Depolarization-induced ICa and spatially resolved intracellular Ca transients were measured in cardiomyocytes dialyzed with fluo-3. Fig. 1A shows representative traces of ICa and confocal line-scan images along with the spatial average of Ca transients recorded during a depolarizing step from −50 to 0 mV in myocytes from control and failing hearts. The amplitude of Ca transients was reduced and their duration prolonged in HF myocytes. Whereas the amplitude of current density (ICa) did not change, the time constant of ICa inactivation was slowed in HF myocytes, apparently because of diminished Ca-dependent inactivation of the L-type Ca channels (Table 1, which is published as supporting information on the PNAS web site).

Averaged Ca transient and ICa amplitudes are plotted as a function of voltage in Fig. 1B. The observed decreases in Ca transient amplitude in HF myocytes with respect to control were significant throughout the whole range of membrane potentials tested (from −40 to 60 mV). At the same time, ICa density was preserved, suggesting that the reduced SR Ca release is not simply due to a reduced Ca trigger in myocytes from failing hearts.

The SR Ca content was inferred from recordings of Ca transients and Na/Ca exchange currents recorded on application of caffeine. Based on these measurements, the SR Ca content was reduced to ∼65% of control in HF myocytes (Fig. 1C). Similar decreases in ICa-induced Ca transients and SR Ca content have been described previously in experimental models of hypertrophy and HF in the dog (22, 23). The primary focus of the present study was to determine the factors responsible for the abnormal Ca releasing and storing capacities of SR in chronic HF.

Ca Sparks and SR Ca Content. To further explore the effects of HF on the SR Ca release mechanism, we performed measurements of elementary Ca release events, Ca sparks, in saponin-permeabilized myocytes (Fig. 2A). The myocytes were bathed in a fluo-3-containing internal solution at a buffered [Ca] (≈50 nmol/liter Ca/0.1 mmol/liter EGTA). In control myocytes, spontaneous Ca sparks occurred with an average frequency of ∼3.0 ± 0.3 s⁻¹. In myocytes from failing hearts, spark frequency was increased to 6.6 ± 1.2 (Fig. 2B), whereas event amplitude was significantly decreased (Fig. 2C). Additionally, although spark width was not altered there was a small but significant increase in event duration in HF myocytes compared with controls. These HF-related changes in Ca sparks were confirmed in intact myocytes loaded with fluo-3 acetoxyethyl ester (Fig. 8 and Tables 2 and 3, which are published as supporting information on the PNAS web site).

Ca spark-mediated leak of Ca from the SR plays an important role in setting the SR Ca content (11). The observed increase in frequency of sparks would be expected to result in reduced sequestration of Ca in the SR in HF myocytes, unless the SR Ca uptake rate was also increased. The total SR Ca content in the same two groups of cells was estimated by application of caffeine (10 mmol/liter). As shown in Fig. 2D, the amplitude of caffeine-induced Ca transients was decreased to ∼75% of control in HF.
myocytes. These results suggest that Ca spark frequency (and the associated Ca leak) is increased, potentially accounting for the reduced SR Ca content in HF myocytes.

**Intra-SR [Ca].** To further understand the abnormal SR Ca handling in HF, we performed measurements of intra-SR [Ca] in permeabilized myocytes. [Ca]$_{SR}$ was monitored with the low affinity Ca indicator fluo-5N loaded into the SR. Fig. 3 shows representative two-dimensional (X–Y) images of fluo5N-loaded control and HF myocytes. Fluo-5N fluorescence intensity, corresponding to [Ca]$_{SR}$, was invariably lower throughout the image plane in HF myocytes compared with controls (Fig. 3A), indicating that not only focal plane-averaged (Fig. 3B and C) but also local diastolic [Ca]$_{SR}$ was reduced in HF myocytes. For simultaneous imaging of cytosolic Ca and SR Ca in fluo-5N-loaded myocytes, rhod-2 was introduced into the bathing solution. Myocytes incubated in a solution containing 50 nmol/liter Ca and 50 µmol/liter EGTA exhibited periodic spontaneous Ca waves (Fig. 4A and B). Additionally, Ca sparks similar to those recorded with fluo-3 could be readily observed, again more often in HF myocytes than in control myocytes (Fig. 4A). During Ca release, rhod-2 fluorescence transiently increases, whereas fluo-5N fluorescence transiently decreases, thus forming mirror images of Ca waves and sparks in the cytosolic and luminal compartments, respectively. In both control and HF cells, Ca waves arise with the same frequency of $\approx0.25$ s$^{-1}$ (Fig. 4A). At the same time, the amplitude of the cytosolically measured Ca waves was reduced in HF myocytes (Fig. 4B and Table 4, which is published as supporting information on the PNAS web site), consistent with the blunted depolarization- and caffeine-induced Ca transients in HF myocytes. Additionally, the velocity of Ca wave propagation was reduced in HF myocytes as indexed by the prolonged slope of the Ca wave front on the myocyte line-scan images (Fig. 4A). Similar HF-induced changes in properties of Ca waves were observed in intact myocytes loaded with fluo-3 acetoxyethyl ester (Fig. 8 and Table 5, which are published as supporting information on the PNAS web site).

Strikingly, the SR-entrapped fluo-5N revealed a dramatic reduction in the basal [Ca]$_{SR}$ in HF myocytes compared with controls (Fig. 4A–C and Table 6, which is published as supporting information on the PNAS web site). Additionally, the amplitudes of both the global and focal Ca depletion signals (i.e., during Ca waves and sparks, respectively) were diminished in HF cells compared with controls (Figs. 4A and B and 5A and B). The amplitude of the Ca wave depletion signal constituted $\approx45\%$ of the total reduction of fluo-5N fluorescence in caffeine, indicative of partial SR depletion during Ca wave-associated Ca release in both control and HF myocytes (10 mmol/liter, FCalc Fig. 4C). Recovery kinetics of [Ca]$_{SR}$ for Ca wave-associated Ca release were significantly slower in HF myocytes, consistent with a compromised ability of the SR to sequester and retain Ca in HF myocytes (Fig. 4C). However, [Ca]$_{SR}$ recovery after focal Ca release was similar in HF and control myocytes (Fig. 5B). The differential effects of HF on [Ca]$_{SR}$ recovery after large scale vs. spatially limited SR Ca release suggests different SR refilling mechanisms after global and localized Ca release events (24). Collectively, these results showed that SR luminal [Ca] is reduced in HF myocytes. They also demonstrated that generation of spontaneous Ca waves and sparks in HF myocytes occurs at abnormally low intra-SR Ca levels, thereby accounting for, or contributing to, the reduced sequestration of Ca in the SR in HF myocytes. The increased occurrence of spontaneous release events at reduced SR Ca content in HF myocytes is reminiscent of the effects of the RyR-sensitizing agent caffeine (11, 25) and consistent with potential changes in the functional activity of RyRs in failing hearts.

**Single-RyR Channel Activity.** To directly compare the sensitivities of RyR channels from normal and failing hearts to luminal Ca, we performed single-RyR channel recordings using the planar lipid bilayer technique. SR microsomes were isolated from normal and failing dog hearts by using differential centrifugations, vesicles were incorporated into planar lipid bilayers, and single-RyR channels were measured by using Cs$^+$ as the charge carrier in the presence of cytosolic Mg$^{2+}$ and ATP. Representative single-channel recordings at varying luminal Ca concentrations are shown in Fig. 6A, whereas Fig. 6B plots averaged RyR open probability ($P_o$) as a function of trans [Ca] for control and HF channels. As demonstrated previously in RyRs from normal dog hearts (3, 4), increasing trans [Ca] from the micromolar to the millimolar range resulted in increased channel activity ($EC_{50} \approx 0.6$ mmol/liter). In RyRs from failing hearts, the luminal Ca sensitivity was profoundly shifted toward lower concentrations compared with controls such that the channel was substantially activated at trans [Ca] as low as 20 µmol/liter ($EC_{50} \approx 5$ µmol/liter; Fig. 6). In HF myocytes, reductions in luminal Ca below 1 µmol/liter were required to abolish luminal Ca activation. These results showed that RyR sensitivity to luminal Ca is greatly enhanced in HF.

**Levels of SR Proteins.** We performed immunoblot analyses to determine the contents of the major SR Ca handling proteins cardiac RyR (RyR2), CASQ2, SR CaATPase (SERCA2a), phospholamban (PLB), triadin, and junctin in cell lysates from control and failing hearts (Fig. 7). We did not detect changes in the expression of CASQ2, triadin, junctin, SERCA2a, or PLB,
whereas RyR2 content was significantly reduced in homogenates from failing hearts. Therefore, altered stoichiometry between RyR and other proteins of the Ca release channel complex could contribute to the abnormal Ca handling in HF.

Discussion

We investigated alterations in intracellular Ca handling in chronic HF using a combination of simultaneous imaging of cytosolic and SR intraluminal [Ca] in isolated cardiomyocytes and recordings from single-RyR channels reconstituted into lipid bilayers. We found that diastolic [Ca] inside the SR was dramatically reduced because of increased Ca spark-mediated Ca leak across the SR membrane in HF myocytes. At the single-RyR level, the sensitivity of RyRs to activation by luminal Ca was greatly enhanced, providing a molecular mechanism for the maintained potentiation of Ca sparks (and increased Ca leak) despite a reduced intra-SR [Ca] in HF. These results show that defective intrastore Ca signaling is involved in the pathogenesis of HF.

Defective RyR Luminal Ca Regulation as a Cause of Abnormal Ca Handling in HF. [Ca] inside the SR is an important determinant of the functional activity of the RyR Ca release channels (5, 26). Normally, RyR luminal Ca regulation appears to play a stabilizing role by countering the intrinsic positive feedback of Ca-induced Ca release during the release process. Specifically, dissociation of Ca from the luminal sensing sites is involved in termination of SR Ca release, permitting cardiac relaxation (9, 10, 27). Additionally, RyRs sensitive to [Ca]SR provide a luminal Ca-dependent leak pathway and play a role in regulating SR Ca content in cardiac muscle (11, 12). Presumably, this mechanism operates to prevent SR Ca overload by releasing excess Ca from the store (11).

Our finding that, in HF, RyRs become hypersensitive to luminal Ca helps to reconcile some apparently contradictory results and improves our understanding of abnormal Ca handling in the failing heart. Previous studies in normal myocytes have shown that the frequency of Ca sparks and the incidence of Ca waves are increased when the SR Ca load is elevated, whereas reduced SR Ca content is accompanied by reduced Ca spark and Ca wave occurrence. Yet in HF, an increased frequency of sparks is associated with reduced SR Ca content (ref. 23 and this work). Our demonstration of enhanced RyR activity at a given luminal [Ca] provides a logical explanation for these seemingly discrepant results. Moreover, our finding may explain the paradoxically increased incidence of arrhythmogenic Ca waves and delayed after depolarizations in various models of HF, despite the reduced SR Ca content (28–30). Of note, we did observe premature ventricular depolarizations in some of the HF dogs, consistent with an arrhythmogenic response to impaired calcium regulation during HF.

Comparison with Previous HF Models. Whereas the observed reduction and prolongation of SR Ca release are hallmarks of failing myocardium (2, 14–16), the effects of HF on other EC parameters, including ICa and SR Ca load, vary widely between different species and models. Our finding that alterations of SR Ca release occurred in the absence of significant changes in ICa density but in parallel with a decrease in SR Ca content is consistent with results obtained previously in shorter term (3–6 weeks) canine models of tachypacing-induced HF (22, 31).

Similar to a recent report by Song et al. (23) in which a canine model of left ventricular hypertrophy due to chronic pressure...
overload was used, our experiments showed an increase in the frequency of sparks in HF myocytes. In the study by Song et al., the increase in the Ca spark rate was rationalized by proposing that local regions with elevated SR Ca content exist in HF myocytes that escape detection by the caffeine method used to measure total SR Ca content. However, our direct [Ca]SR measurements demonstrated that [Ca] was lowered throughout the entire SR network, and that the increased Ca spark rate was a result of enhanced sensitivity of RyRs to luminal Ca in myocytes from failing hearts. It is unknown whether calcium dysregulation and the underlying mechanisms differ between left ventricular hypertrophy and HF.

In previous studies measuring RyR function in lipid bilayers, no differences in channel P_o between preparations from control and failing canine hearts were reported (31). This result is in contrast to the altered RyR function that we observed. We suggest that the substantially longer duration of HF in our study may contribute to this difference. Alternatively, it is possible that the experimental conditions may underlie this discrepancy. We note that in our experiments, differences in channel behavior were observed only in the presence of cytosolic MgATP (unpublished results). This observation would be consistent with previous reports that the effects of luminal Ca on RyR activity rely on the presence of an allosteric activating ligand (ATP, caffeine, sulmazole) (3, 4).

Molecular Mechanisms of Abnormal RyR Function in HF. The specific molecular causes for altered RyR function in failing hearts are not known. Marks and coworkers (19) have suggested that RyR phosphorylation by PKA results in dissociation of FKBP12.6 from RyRs, rendering the channels hyperactive with associated Ca leak in HF. Other laboratories, however, have found no evidence for phosphorylation-induced detachment of FKBP12.6 from RyRs in normal and diseased hearts (31–34). Thus, further studies are needed to determine whether the abnormal RyR modulation by luminal Ca in HF is caused by an altered interaction with FKBP12.6.

RyR modulation by luminal Ca appears to involve auxiliary proteins such as CASQ2, triadin, and junctin that are removed by RyR purification (5) (Fig. 9, which is published as supporting information).
information on the PNAS web site); alterations in the expression or function of these regulatory proteins may contribute to our observations. Although CASQ2 levels do not change in various models of HF (ref. 2 and this work), CASQ2 glycosylation is dramatically altered in HF, indicative of a potential defect in junctural SR trafficking and Ca release complex assembly (35). There is little information regarding the expression, localization, and function of triadin and junctin in failing hearts. In the present study, the levels of these proteins did not change in HF. However, when considering the decrease in RyR abundance to approximately half of control values, the ratios of both triadin and junctin to RyRs could contribute to increased RyR function in HF, because both triadin and junctin appear to act on RyRs by increasing RyR activity (5, 36).

Finally, abnormal RyR function in HF could be due to acquired defects in the channel protein caused by the altered intracellular environment in HF. HF is accompanied by increased formation of metabolic by-products, including reactive oxygen and nitrogen species and aldehydes, with potentially deleterious effects on intracellular proteins (37). These compounds are capable of reacting with lysine, cysteine, tyrosine, and histidine residues, resulting in long-term or permanent modifications in the RyR structure. Because RyR is a long-lived protein (38), it is particularly vulnerable to such acquired chemical modifications. Of note, mutations of RyR have been reported to sensitize RyR to luminal Ca (39). It is conceivable that posttranslational modifications in the protein structure can lead to similar functional changes. Regardless of its specific biochemical cause, abnormal activation of RyR by luminal Ca is an important factor in the pathogenesis of HF. Determining the precise nature of RyR defects in HF will require an understanding of the molecular basis of RyR regulation by luminal Ca. The putative intracellular mechanisms revealed in the present study should be considered in conjunction with global phenomena such as desynchronization of myocardial contraction that may also contribute to impaired cardiac function in this model of HF (21).

Conclusions

Using a canine model of chronic HF, we showed that the diminished SR Ca release characteristic of failing myocardium is at least partially due to increased sensitivity of RyRs to luminal Ca, leading to enhanced spark-mediated SR Ca leak and reduced intra-SR [Ca]. The biochemical causes for the abnormal RyR luminal Ca-dependent gating behavior are not known. They could involve disrupted protein–protein interactions within the RyR complex, altered covalent modification of RyRs such as phosphorylation, or acquired defects caused by reactive intracellular metabolites. Sensitization of RyRs to luminal Ca in HF could reflect an adaptive transformation, allowing the SR to operate at reduced SR Ca loads (and reduced energy costs) in the ATP-starved failing myocardium. Alternatively, the RyR sensitization may play a pathophysiologic or maladaptive role in HF. More studies are needed to determine the nature of the altered intrastore control of Ca release in HF. This defect in RyR function may provide a novel therapeutic target to treat contractile dysfunction in HF.

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Purified RYR2

*trans*-\[Ca\], 0.1 \(\mu\)mol/L

\[Po = 0.109 \pm 0.035\]

*trans*-\[Ca\], 1 \(\mu\)mol/L

\[Po = 0.100 \pm 0.011\]

*trans*-\[Ca\], 500 \(\mu\)mol/L

\[Po = 0.092 \pm 0.008\]