No Rest for the Weary: Diastolic Calcium Homeostasis in the Normal and Failing Myocardium

Following contraction of the heart, efficient relaxation (diastole) is essential for refilling the ventricles with blood. This review describes how ventricular relaxation is controlled by Ca$^{2+}$ homeostasis in cardiac muscle cells and how alterations in Ca$^{2+}$ cycling affect diastolic function in the normal and failing heart. These discussions illustrate that the diastolic phase is not simply a period of rest but rather involves highly regulated and dynamic Ca$^{2+}$ fluxes.

In cardiac myocytes, contraction and relaxation are triggered by a transient rise and decline in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}]_{i}$). The magnitude and kinetics of this Ca$^{2+}$ transient are tightly controlled by the activity of Ca$^{2+}$ handling proteins (FIGURE 1): Depolarization of the cell membrane during the travelling action potential elicits a small Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels localized in the t-tubules. Ca$^{2+}$ entering the cell binds to Ca$^{2+}$ release channels called ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR), triggering additional release of Ca$^{2+}$. This amplification process, known as Ca$^{2+}$-induced Ca$^{2+}$ release (24), occurs at functional units called dyads, where clusters of Ca$^{2+}$ channels and RyRs are in close proximity across a narrow (10–15 nm) cleft (11) (FIGURE 1). Ca$^{2+}$ release from a cluster of RyRs is called a Ca$^{2+}$ spark. Ca$^{2+}$ sparks can be elicited by Ca$^{2+}$-induced Ca$^{2+}$ release (“evoked sparks”) or can occur spontaneously in resting cells, without triggering by Ca$^{2+}$ channels (“spontaneous sparks”) (18). The spatiotemporal summation of thousands of evoked sparks elevates [Ca$^{2+}]_{i}$ from resting levels of ~100 nM to 600–1,000 nM at the peak of the Ca$^{2+}$ transient (12, 19). Since cardiac contractile proteins are activated by Ca$^{2+}$, cardiac contractile force is largely dependent on the amplitude and kinetics of Ca$^{2+}$ release. Impairments in the release process may contribute to reduced contractile force in systolic heart failure (12).

Following Ca$^{2+}$-induced Ca$^{2+}$ release at the dyad, relaxation occurs as Ca$^{2+}$ is recycled into the SR and extruded from the cell. SR Ca$^{2+}$ reuptake depends on the SR Ca$^{2+}$ ATPase (SERCA), whereas extrusion is accomplished predominantly via the Na$^{+}$-Ca$^{2+}$ exchanger (NCX) and to a much lesser extent the plasma membrane Ca$^{2+}$ ATPase (11). Tight coordination of Ca$^{2+}$ removal by these different systems ensures rapid relaxation of cardiomyocytes and thus efficient filling of the ventricles. From a clinical perspective, the importance of efficient relaxation has been highlighted in recent years by the observation that 50% of patients with heart failure have normal systolic function but exhibit myocardial dysfunction due to impaired diastole (20). Thus an improved understanding of the mechanisms controlling relaxation in normal and diseased hearts is essential. In the present review, we introduce the term “diastolic Ca$^{2+}$ homeostasis” and describe how the fine-tuning of this system promotes efficient relaxation in the healthy heart. We additionally discuss how disrupted Ca$^{2+}$ handling promotes diastolic dysfunction and arrhythmia during heart failure.

**Diastolic Ca$^{2+}$ Homeostasis**

There are two important interrelated aspects of Ca$^{2+}$ handling that determine diastolic function. The first is the rate of Ca$^{2+}$ decline following release, which regulates the speed of cardiomyocyte re-lengthening. The second aspect is the resting or end-diastolic [Ca$^{2+}]_{i}$, which sets the extent of cell relaxation or diastolic tension. The presence of resting cardiomyocyte tone has recently been elegantly demonstrated by showing that the resting length of cells increases when the interaction between the myofilament proteins actin and myosin is inhibited (32). Thus there is an “active,” Ca$^{2+}$-dependent component of cardiomyocyte stiffness, as well as a “passive” component that is due to the properties of the cytoskeleton (34). Active cardiomyocyte tension is regulated by Ca$^{2+}$ removal from the cytosol, which is, in turn, controlled by a complex interplay between SR Ca$^{2+}$ re-uptake and Ca$^{2+}$ extrusion from the cell. As will be discussed in the following sections, SR reuptake is regulated by SERCA and RyR acting as a functional system (FIGURE 1). Similarly, Ca$^{2+}$ extrusion from the cell is largely mediated by a close structural and functional relationship between NCX and the Na$^{+}$-K$^{+}$ ATPase (NKA). With active Ca$^{2+}$ transport by these two systems, the following discussion will illustrate...
that the diastolic phase is not simply a period of rest but rather involves dynamic regulation of $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_{\text{SR}}$.

Importantly, diastolic Ca$^{2+}$ homeostasis determines not only diastolic function, as reviewed here, but also systole. Resting $[\text{Ca}^{2+}]_i$ is an important determinant of contractility, since elevated resting Ca$^{2+}$ levels enhance contraction and force development (11, 74). Diastolic Ca$^{2+}$ fluxes also regulate contractility by altering SR Ca$^{2+}$ content and thus the magnitude of the Ca$^{2+}$ transient. Since the present review is focused on diastole, the reader is referred elsewhere for an overview of regulation of systolic function (11).

**The SERCA-RyR System**

Ca$^{2+}$ reuptake into the SR is dominant to Ca$^{2+}$ extrusion across the cell membrane, but there is a species-dependent variation in the relative contribution of these Ca$^{2+}$ removal systems. In humans, Ca$^{2+}$ removal is $\sim 70\%$ SR dependent, whereas in rats and mice $>90\%$ of Ca$^{2+}$ is cycled over the SR (11). SERCA function importantly influences not only the rate of Ca$^{2+}$ removal from the cytosol but...
also SR Ca\(^{2+}\) content. SERCA activity is controlled by the endogenous inhibitor phospholamban (PLB). Protein kinase A (PKA)-dependent phosphorylation of PLB at Ser16 relieves SERCA inhibition and is the key mechanism for increasing rate of relaxation and SR Ca\(^{2+}\) content during sympathetic stimulation (50). Ca\(^{2+}\)-/calmodulin-dependent protein kinase II (CaMKII) phosphorylation of PLB at Thr17 enhances SERCA function when diastolic [Ca\(^{2+}\)]\(_i\) is elevated since maximal pump rate is increased (58). Since Ca\(^{2+}\) is usually the limiting substrate for SERCA function, elevation of [Ca\(^{2+}\)]\(_i\) also directly enhances SERCA activity (30).

SR Ca\(^{2+}\) content links SERCA function to RyR activity, since RyRs are sensitive to SR luminal Ca\(^{2+}\) levels (45). With greater SERCA activity, one expects increased RyR open probability, resulting in increased diastolic Ca\(^{2+}\) “leak” from the SR (73). Increased RyR leak partially offsets Ca\(^{2+}\) reuptake via SERCA by increasing the loss of SR Ca\(^{2+}\) to the cytosol. Thus the net leak is the difference between RyR leak and SERCA-dependent Ca\(^{2+}\) reuptake. Net leak can only persist transiently as discussed below. The nature of SR Ca\(^{2+}\) leak, however, is still incompletely resolved, as recent studies show that spontaneous Ca\(^{2+}\) sparks cannot fully account for SR Ca\(^{2+}\) leak flux (68, 96). The term “silent leak” has been used to describe the non-spark fraction (~50%) of SR Ca\(^{2+}\) efflux during rest, and this may represent Ca\(^{2+}\) release from non-dyadic RyRs (68).

Altered balance between SERCA and RyR will primarily affect the rate of relaxation. For example, decreased SERCA activity slows relaxation and favors net loss of Ca\(^{2+}\) from the SR (37). In paced cells, this slowing of Ca\(^{2+}\) decline will result in increased diastolic [Ca\(^{2+}\)]\(_i\) if there is inadequate time for recovery to resting Ca\(^{2+}\) levels. However, the net effect of the SERCA-RyR system on diastolic [Ca\(^{2+}\)]\(_i\) is to a large extent determined by the activity of the sarcolemmal Ca\(^{2+}\) extrusion system, i.e., the interaction between NCX and NKA, as discussed below.

**The NCX-NKA System**

Ca\(^{2+}\) extrusion is predominantly mediated via NCX. Regulation of NCX activity is somewhat complex; although this protein does not appear to be a direct target for protein kinases such as PKA (93), NCX function is increased following PKA-dependent phosphorylation of its endogenous inhibitor, phospholemman (20). The NCX is electrogenic (1 Ca\(^{2+}\) is exchanged for 3 Na\(^{+}\)), and its function is therefore importantly influenced by the membrane potential: a more negative resting potential will promote Ca\(^{2+}\) extrusion, whereas more positive resting potential will inhibit Ca\(^{2+}\) extrusion. Due to the bi-directionality of NCX function, its activity is also regulated by local levels of Na\(^{+}\) and Ca\(^{2+}\). This implies that its precise localization is a critical functional determinant. Although this issue has been debated, several studies have suggested that a proportion of NCX molecules are localized in or very near the dyad (38, 56, 88). Importantly, the narrow dimensions of the dyadic cleft have been proposed to restrict the diffusion of ions, resulting in a local microdomain or so-called “fuzzy space” (FIGURE 1) (35). Dyadic [Na\(^{+}\)] is maintained by a balance between influx via neuronal-type Na\(^{+}\) channels (16, 38) and active Na\(^{+}\) removal by the Na\(^{+}\)-K\(^{+}\)-ATPase (NKA), specifically the \(\alpha_{1}\) NKA isoform (83, 84). Although NCX function is not directly dependent on ATP hydrolysis, nearby Na\(^{+}\) levels, which determine its activity, are actively maintained by NKA. Thus the NCX and NKA constitute a functional unit (FIGURE 1) that exports Ca\(^{2+}\) at the expense of ATP. Just as Ca\(^{2+}\) channels and RyRs are held in place at the dyad by junctophilin (86), the NCX-NKA system is also anchored to the SR by a protein called ankyrin-B (52). Thus structure is rigorously maintained to ensure precise control of ion concentrations in the microdomain.

The NCX-NKA system has somewhat complex effects on diastolic Ca\(^{2+}\) homeostasis (summarized in Table 1). The rate of NCX-mediated Ca\(^{2+}\) efflux regulates resting [Ca\(^{2+}\)]\(_i\) levels, as illustrated by results from modeling experiments shown in FIGURE 2A. The figure shows resting diastolic [Ca\(^{2+}\)]\(_i\), as a function of maximum NCX activity (\(V_{\text{max}}\)), which reflects the amount of NCX molecules in the membrane (set to 1 for a normal cardiomyocyte). Increasing

<table>
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<th>Parameter</th>
<th>Resting [Ca(^{2+})](_i)</th>
<th>Rate of [Ca(^{2+})](_i) Decline</th>
<th>SR Ca(^{2+}) Content</th>
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the amount of NCX reduces diastolic \([\text{Ca}^{2+}]_i\) (FIGURE 2B), which is consistent with the observation that rabbit cardiomyocytes overexpressing NCX exhibit lower resting \([\text{Ca}^{2+}]_i\) (63). However, no change in diastolic \([\text{Ca}^{2+}]_i\) is reported in mouse cardiomyocytes overexpressing NCX (1). This discrepancy may be explained by the fact that higher resting \(\text{Na}^+\) levels in mouse cells promote greater reverse-mode NCX function during overexpression (95). Modeling experiments predict that decreasing

**FIGURE 2.** Control of diastolic \([\text{Ca}^{2+}]_i\) by NCX

A: computational modeling was used to examine the influence of NCX activity on diastolic \([\text{Ca}^{2+}]_i\) in a resting cell by employing a standard algorithm for NCX current (37). NCX current was set to a fixed value calculated with membrane potential \(V_m\) = \(-80\) mV, \([\text{Na}^+]_i\) = 14 mM, and \([\text{Ca}^{2+}]_i\) = 100 nM. Assuming that diastolic \([\text{Ca}^{2+}]_i\) is solely determined by NCX activity, diastolic \([\text{Ca}^{2+}]_i\) was then calculated for a range of values for \(V_{\text{NCX}}^\text{max}\) (maximal NCX activity, proportional to protein expression levels), \(V_m\), and \([\text{Na}^+]_i\). Clearly, decreasing levels of NCX below normal rapidly leads to accumulation of \(\text{Ca}^{2+}\). Similarly, \(\text{Na}^+\) accumulation and/or decreasing membrane potential inhibit \(\text{Ca}^{2+}\) extrusion and \([\text{Ca}^{2+}]_i\) increases. B: in large, nonrodent species, higher NCX levels tend to decrease resting \(\text{Ca}^{2+}\) but also to reduce SR \(\text{Ca}^{2+}\) content as NCX “steals” \(\text{Ca}^{2+}\) away from SERCA. Interestingly, overall \(\text{Ca}^{2+}\) decline and relaxation are slowed during NCX upregulation (63, 69), since reduced diastolic \([\text{Ca}^{2+}]_i\) inhibits SERCA activity.
the amount of NCX would increase diastolic [Ca^{2+}]_i (FIGURE 2A). Indeed, bimodal pharmacological blockade of NCX has been shown to elevate diastolic [Ca^{2+}]_i in both pig and mouse myocytes (57). Interestingly, the resting Ca^{2+} level is unchanged in NCX knockout mice (28, 62), but this difference may be explained by the compensatory upregulation of SR Ca^{2+} cycling in these animals. FIGURE 2A shows that NCX-dependent regulation of diastolic [Ca^{2+}]_i is dependent not only on NCX expression levels (V_{max}) but also on the membrane potential and [Na^{+}]_i. Cytosolic Na^{+} accumulation markedly elevates cytosolic [Ca^{2+}]_i, in agreement with previous modeling predictions (29), as does membrane depolarization. Interestingly, FIGURE 2 shows that cytosolic Na^{+} levels and membrane potential have a more significant influence on resting Ca^{2+} levels than V_{max}.

Altering the rate of Ca^{2+} removal by NCX can affect the rate of decline of the Ca^{2+} transient, and thus cardiomyocyte relaxation. Since under most conditions the NCX functions predominantly in Ca^{2+} removal mode rather than Ca^{2+} entry mode, bidirectional NCX inhibition results in slowing of Ca^{2+} extrusion and relaxation (57). With reduced Ca^{2+} extrusion, more Ca^{2+} is available for re-sequestration by SERCA and SR Ca^{2+} content increases (57, 87). Overexpressing NCX in rabbit cells has the opposite effect, resulting in reduced SR Ca^{2+} stores (63, 69). Surprisingly, these cells exhibit a slower rather than a faster decline of the Ca^{2+} transient (63). The mechanism underlying this finding is unclear, but a likely explanation is that lower diastolic Ca^{2+} levels reduce SERCA activity either directly or via CaMKII-dependent phosphorylation of phospholamban (FIGURE 2B). Thus the NKA-NCX and SERCA-RyR systems are functionally linked. The consequences of this interaction for diastolic Ca^{2+} homeostasis are discussed in the following section.

**Interplay Between the SR Ca^{2+} Re-uptake and Ca^{2+} Extrusion Systems**

The SERCA-RyR and NCX-NKA systems effectively compete for the same pool of Ca^{2+}. Within the time provided by diastole, the outcome of the competition determines the rate of Ca^{2+} removal from the cytosol, SR Ca^{2+} content, and resting end-diastolic [Ca^{2+}]_i, which in turn control the rate and extent of relaxation and resting tension. In cells left unstimulated in experimental conditions, a steady state is reached where [Ca^{2+}]_i is stable. During a period of net SR Ca^{2+} leak, the NCX-NKA system will remove some of the Ca^{2+} released to the cytosol. Indeed, increasing leak with caffeine does not alter resting Ca^{2+} levels at steady state but rather results in loss of Ca^{2+} from the cell via NCX (89) (FIGURE 3A; Table 1). This loss of Ca^{2+} from the cell continues until the driving force for Ca^{2+} extrusion by NCX is reduced (NCX reaches its electrochemical equilibrium again) and/or the rate of RyR Ca^{2+} leak declines due to lowered SR content.

The interaction between the two Ca^{2+} removal systems becomes more complex when we move from our simplified example of a resting cell to a stimulated cell. Under in vivo conditions, the time span between Ca^{2+} release events is brief, meaning that Ca^{2+} removal across the SR as well as the sarcolemma is still ongoing at the time point of the next release (FIGURE 4). This issue is further complicated by the fact that the competition between NCX and SERCA depends on heart rate and that such effects are markedly species dependent. This phenomenon can be experimentally demonstrated by inserting a rest period of variable duration into the stimulation train. Rabbit and guinea pig myocytes exhibit post-rest decay of contractions, i.e., the first contraction after a period of rest is weaker than the beat preceding the rest period (8). This results from a net loss of Ca^{2+} from the cell as NCX competes with SERCA (8). Post-rest decay does not occur in rat and mouse cardiomyocytes, since SR Ca^{2+} uptake is highly favored over Ca^{2+} extrusion by NCX. Indeed, due to quite high resting [Na^{+}]_i in these cells, there is very little or no driving force for Ca^{2+} extrusion at diastolic membrane potential and [Ca^{2+}]_i (11). Higher pacing rates increase Na^{+} levels (21), as NKA activity becomes unable to keep up with Na^{+} influx, which gives greater dominance of SR Ca^{2+} removal (FIGURE 3B). Thus SR Ca^{2+} content increases (4), but so does RyR leak, and with reduced driving force for NCX-mediated Ca^{2+} extrusion and less time available for Ca^{2+} removal, diastolic [Ca^{2+}]_i increases (4, 54) (FIGURES 3B AND 4). This is reflected by a shortening of cell length at high frequencies (54). Increased diastolic [Ca^{2+}]_i also activates CaMKII, which enhances SERCA activity by PLB phosphorylation and thereby accelerates Ca^{2+} decline and relaxation at high heart rates (58) (FIGURES 3B AND 4).

The above discussion shows that the relationship between SR Ca^{2+} re-uptake and Ca^{2+} extrusion is quite dynamic and highly dependent on variations in [Na^{+}]_i and [Ca^{2+}]_i during the cardiac cycle. A well known example of how manipulation of local Na^{+} homeostasis affects global Ca^{2+} homeostasis is the use of cardio glycosides such as digitalis. These drugs inhibit NKA, resulting in accumulation of Na^{+} in the microdomain near NKA-NCX (FIGURE 3B). We have observed that in rodents these effects of cardio glycosides are mostly dependent on inhibition of the α1 isoform of NKA, which is localized in the t-tubules (81, 84). Similar to what is seen at high pacing rates (discussed above), Na^{+} accumulation limits Ca^{2+} extrusion by NCX resulting in elevated diastolic [Ca^{2+}]_i, and
stiffness (48, 71). There is also greater dominance of SERCA in cytosolic Ca\(^{2+}\) removal during NKA blockade, which increases SR Ca\(^{2+}\) load (71). This enhanced Ca\(^{2+}\) flux through SERCA results not only from inhibition of NCX but also from stimulation of SERCA activity by elevated \([Ca^{2+}]_i\) directly.
and/or via CaMKII (FIGURE 3B). Thus the rate of overall Ca\(^{2+}\) decline is increased during NKA blockade (71). These findings illustrate that local microdomains can importantly influence the competition between the SR and sarcolemmal Ca\(^{2+}\) handling systems.

Experimental SERCA inhibition also illustrates the interdependence of the SERCA-RyR and NCX-NKA systems (Table 1). As described above, reducing SERCA activity decreases the rate of Ca\(^{2+}\) decline and may elevate diastolic [Ca\(^{2+}\)]\(_i\) at high heart rates (37). With decreased competition from SERCA, Ca\(^{2+}\) removal by NCX results in net Ca\(^{2+}\) loss from the cell, leading to a reduction in SR Ca\(^{2+}\) content (FIGURE 5A). Indeed, we recently observed reduced SR Ca\(^{2+}\) content in myocytes isolated from mice with conditional knockout (KO) of the Serca2 gene 6 days after gene deletion (80). At this time point, a 53% reduction in SERCA protein expression occurred without altered expression of other Ca\(^{2+}\) handling proteins. Interestingly, Ca\(^{2+}\) leak rates were similar in KO cells and controls despite reduced SR content in KO myocytes (79). FIGURE 5A shows that this likely resulted from elevated diastolic [Ca\(^{2+}\)]\(_i\), directly triggering RyR opening and/or sensitizing RyRs to Ca\(^{2+}\) via CaMKII activation. At later time points following SERCA2 KO, further reduction in SERCA levels was associated with NCX upregulation. For example, at 4 wk after KO, a 67% reduction in SERCA activity was associated with a 2.5-fold increase in Ca\(^{2+}\) extrusion via NCX (3, 37, 41). Our mathematical modeling data (FIGURE 6) show that upregulated Ca\(^{2+}\) extrusion is essential for maintaining resting [Ca\(^{2+}\)]\(_i\) in these cells but that it also reduces SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient magnitude by “stealing” Ca\(^{2+}\) away from remaining SERCA molecules (37). However, somewhat surprisingly, NCX upregulation had very little influence on the time course of Ca\(^{2+}\) decay (FIGURE 6C). The explanation for this apparent discrepancy is illustrated in FIGURE 5. Although Ca\(^{2+}\) extrusion is enhanced by upregulated NCX, the resulting reduction in diastolic [Ca\(^{2+}\)]\(_i\) prevents any stimulation of SERCA due to direct effects of Ca\(^{2+}\) and/or CaMKII-dependent phosphorylation of PLB. Thus the overall rate of Ca\(^{2+}\) decline and relaxation remains relatively unchanged. Eventually, at 7 wk after KO when SERCA function is near zero, upregulated Ca\(^{2+}\) extrusion systems become essential for maintaining the rate of Ca\(^{2+}\) decline (41). In the following sections, we will see that insight into the balance of function of the Ca\(^{2+}\) extrusion systems is essential for understanding heart failure and arrhythmias.

**Diastolic Ca\(^{2+}\) Homeostasis in the Failing Myocardium**

**Impaired Ca\(^{2+}\) Removal**

In heart failure with primarily diastolic dysfunction, reduced myocardial compliance has been attributed to a stiffened extracellular matrix due to collagen deposition (3). Cardiomyocyte relaxation may additionally be impeded by altered isoform expression (55) and decreased phosphorylation (33) of the giant elastic protein titin. However, recent data suggest that there is also greater active, Ca\(^{2+}\)-dependent
stiffness in these cardiomyocytes. Selby and colleagues (72) observed that myocardial strips from biopsies of diastolic heart failure patients relaxed incompletely at high stimulation frequencies, suggesting that slow and/or incomplete Ca\(^{2+}\) removal contributes to elevated resting tension in this condition.

FIGURE 5. The relationship between NCX and SERCA fluxes illustrated in cardiomyocytes from the SERCA KO mouse

A: reduced SERCA activity decreases the rate of Ca\(^{2+}\) decline and elevates diastolic [Ca\(^{2+}\)]. With decreased competition from SERCA, Ca\(^{2+}\) removal by NCX results in net Ca\(^{2+}\) loss from the cell, leading to a reduction in SR content. However, elevated diastolic [Ca\(^{2+}\)] gives greater than expected Ca\(^{2+}\) leak due to direct triggering of RyR opening by Ca\(^{2+}\) and/or CaMKII-dependent RyR phosphorylation. Similar mechanisms stimulate SERCA, giving somewhat greater than expected rates of SR reuptake.

B: when SERCA reduction occurs simultaneously with NCX upregulation, elevation of diastolic [Ca\(^{2+}\)], is prevented, but so are the secondary effects of cytosolic Ca\(^{2+}\) accumulation on SERCA and RyR. Furthermore, with greater loss of Ca\(^{2+}\) from the cell via NCX, SR Ca\(^{2+}\) content is more markedly reduced.
Although the mechanisms underlying diastolic heart failure are only starting to be elucidated, systolic heart failure is much more extensively studied and, importantly, often associated with diastolic dysfunction including slowed relaxation and greater diastolic tone. Decreased SERCA activity has been widely reported in systolic heart failure, and this may result from either reduced SERCA expression or greater SERCA inhibition due to attenuated PLB phosphorylation by PKA (12, 67). However, an even more general finding in heart failure with different etiologies seems to be increased NCX-to-SERCA ratio (13, 53, 60).

Hasenfuss and colleagues were able to divide explanted hearts from patients with end-stage failure into three separate groups based on the diastolic function of cardiac muscle strips (26). All groups exhibited increased NCX-to-SERCA ratio, but increased NCX abundance was associated with less increase of diastolic stiffness at higher frequencies. This observation is in agreement with our observations from the SERCA KO mouse, described above (FIGURE 5), and implies that upregulation of the NCX-NKA system can functionally compensate for impairment of the SERCA-RyR system. Importantly, this compensative

![FIGURE 6. Experimental and mathematical modeling data from SERCA KO cardiomyocytes](image)

Ca²⁺ homeostasis in the SERCA2 KO mouse depends to a greater extent on sarcolemmal Ca²⁺ cycling, with upregulated pathways for Ca²⁺ entry and removal from the cell. The influence of such compensations on diastolic Ca²⁺ homeostasis was examined by using experimentally measured Ca²⁺ fluxes to parameterize a mathematical model of the mouse cardiomyocyte (37). Simulation of Ca²⁺ homeostasis in SERCA2 KO myocytes showed that diastolic [Ca²⁺] depends to a large degree on NCX, as does SR Ca²⁺ content. Specifically, NCX upregulation maintains diastolic [Ca²⁺] in the normal range at the expense of SR Ca²⁺ content and Ca²⁺ transient magnitude. However, rate of Ca²⁺ removal relies mainly on SERCA activity, for reasons described in FIGURE 3. Computational and experimental data adapted from Ref. 37 and with permission from Biophysical Journal.
capacity of the NCX-NKA system is dependent on maintenance of appropriate Na\(^+\) homeostasis. We observed that, in late stages following SERCA2 KO, Na\(^+\) accumulation had occurred and that this impaired Ca\(^{2+}\) extrusion despite increased expression of both NCX and plasma membrane Ca\(^{2+}\) ATPase (FIGURE 7) (36, 41). Experimental and modeling data showed that the Na\(^+\) gain in SERCA KO myocytes in part resulted from loss of NKA (36, 41), an observation consistent with other heart failure models (6, 22). Swift and colleagues observed that loss of the \(\alpha_2\) NKA isoform in failing myocytes impairs Ca\(^{2+}\) removal by NCX (81, 84), similar to mechanisms underlying the actions of ouabain (FIGURE 3B). Current work is aimed at determining whether loss of ankyrin B underlies these alterations by disrupting the anchoring of the NCX-NKA system (17).

Na\(^+\) handling may also be disrupted in heart failure by altered activity of other Na\(^+\) handling proteins. An increase in the slow-inactivating “late” Na\(^+\) current, probably due to a CaMKII-dependent mechanism, impairs diastolic function (77, 92). Conversely, inhibition of the late Na\(^+\) current by ranolazine has been shown to improve diastolic function in human failing myocardium (78). Also, the Na\(^+\)-H\(^+\) exchanger has been proposed to contribute to Na\(^+\) loading in failing cardiomyocytes (6, 36), suggesting that inhibitors such as cariporide may have therapeutic potential. However, the precise localization of the Na\(^+\)-H\(^+\) exchanger is unknown, making it difficult to predict the impact of changes in its function on local Na\(^+\) handling in microdomains. Indeed, microdomain alterations are likely quite complex in heart failure, and unraveling the functional consequences of such alterations presents a significant challenge.

**FIGURE 7. Importance of [Na\(^+\)]\(_i\) for rate of Ca\(^{2+}\) extrusion**

A: with progressive loss of SERCA between 4 and 7 wk following gene ablation, SERCA KO cardiomyocytes exhibit slowing of Ca\(^{2+}\) extrusion (measured as the rate of decay of Ca\(^{2+}\) transients stimulated in the present of caffeine). This slowing of extrusion results from inhibition of NCX function due to Na\(^+\) accumulation in the cytosol of KO cells (B) [increase in [Na\(^+\)]\(_i\) of ~5 mM in 7-wk KO (36)] despite progressive up-regulation of Ca\(^{2+}\) extrusion pathways (C). At the late stage of KO, rates of Ca\(^{2+}\) decay depend almost exclusively on Ca\(^{2+}\) extrusion, whereas at earlier stages with substantial SERCA activity remaining, SR Ca\(^{2+}\) reuptake governs rates of decline (FIGURE 4). Figure adapted from Ref. 41 with permission.
**Dysynchrony of Ca\(^{2+}\) Removal**

Altered synchrony of Ca\(^{2+}\) release has been quite thoroughly examined in heart failure, particularly in regard to t-tubule disorganization and/or loss (27, 42, 43, 75, 82). Such structural disorganization leads to the formation of orphaned RyRs, where Ca\(^{2+}\) release occurs only after diffusion from other dyads (FIGURE 8A). The resulting dysynchronous Ca\(^{2+}\) release produces an overall slowed rising phase of the Ca\(^{2+}\) transient. It could be expected that structural changes would also affect Ca\(^{2+}\) removal and slow the declining phase of the Ca\(^{2+}\) transient. For example, regions with delayed Ca\(^{2+}\) release at “orphaned” RyRs following detubulation also exhibit delayed Ca\(^{2+}\) decline (15). Altered levels and/or activity of proteins involved in diastolic Ca\(^{2+}\) homeostasis might further exacerbate such alterations. Preliminary observations from the SERCA KO mouse provide some insight into this phenomenon. As shown in the example line-scan images in FIGURE 8B, Ca\(^{2+}\) decline in the KO cells is considerably more dysynchronous than in controls. It is not yet clear whether this effect results from an overall loss of SERCA molecules or regional loss of SERCA in some regions before others.

**Altered Action Potential Configuration**

Changes in action potential configuration can also affect Ca\(^{2+}\) extrusion. Action potential prolongation is a hallmark of heart failure, and with greater time spent at depolarized potentials Ca\(^{2+}\) entry via NCX is enhanced and Ca\(^{2+}\) removal slowed. In large species, this is evidenced by a prolonged decay of the Ca\(^{2+}\) transient (66). This effect is likely partly due to the fact that action potential prolongation is also known to desynchronize and slow Ca\(^{2+}\) release (40, 66) and thus would be expected to delay Ca\(^{2+}\) re-uptake and extrusion. Importantly, altered action potential configuration has much more dramatic effects on Ca\(^{2+}\) transient kinetics in large species than in small species (40, 66). Indeed, with the very brief action potential in mice and rats, the cell is repolarized by the time the Ca\(^{2+}\) transient reaches its peak. The declining phase of the Ca\(^{2+}\) transient in these species is therefore determined by rates of Ca\(^{2+}\) re-uptake and extrusion and not action potential configuration. This fact should be considered when mouse heart failure models are evaluated (40, 54).

Importantly, alterations in resting membrane potential are also reported in heart failure. We have observed that failing rat myocytes are depolarized and that such depolarization increases SR content by inhibiting of Ca\(^{2+}\) extrusion via NCX (14) (see FIGURE 2A). Such alterations may offset reductions in SR Ca\(^{2+}\) content associated with decreased SERCA activity.

**Increased SR Ca\(^{2+}\) Leak**

In recent years, accumulating evidence has pointed to an important increase in SR Ca\(^{2+}\) leak in heart failure due to CaMKII-dependent phosphorylation of RyR (2, 90). As described above, a leakier RyR favors net Ca\(^{2+}\) loss from the cell and reduction in SR Ca\(^{2+}\) content (89) (FIGURE 3A). Indeed, a recent study showed that reductions in SR content in failing human myocardium could be reversed by CaMKII inhibition (76). It is worth considering that since increased diastolic \([Ca^{2+}]_i\), activates CaMKII, impaired RyR function in heart failure could initiate or maintain a vicious cycle of Ca\(^{2+}\) leak and CaMKII activation. Furthermore, the importance of considering SERCA and RyR as parts of a common system is illustrated by recent findings that restoration of SERCA activity in heart failure by gene transfer restores normal diastolic \([Ca^{2+}]_i\), decreases CaMKII activation, and reduces leak (46).

Greater Ca\(^{2+}\) leak in heart failure not only disrupts relaxation by elevating resting \([Ca^{2+}]_i\), but may potentially also result in delayed afterdepolarizations, spontaneous action potentials, and triggered arrhythmias (90, 91). The process underlying this chain of events begins with uncontrolled SR Ca\(^{2+}\) release in diastole. A local release, i.e., spontaneous Ca\(^{2+}\) spark, diffuses through the cytosol and triggers further release from neighboring release units. This may initiate a self-propagating process called a Ca\(^{2+}\) wave (FIGURE 9A) (18). Since some Ca\(^{2+}\) released by the wave will be removed by NCX in exchange for Na\(^{+}\), a transient inward current results. This current causes a delayed after-depolarization (FIGURE 9A) that may reach the threshold for triggering an action potential (70).

As seen from the description above, several “check points” must be passed for the uncontrolled Ca release to result in arrhythmias. First, local SR Ca\(^{2+}\) release must be of a certain magnitude to elicit a Ca\(^{2+}\) wave (FIGURE 9A) (18). Since some Ca\(^{2+}\) released by the wave will be removed by NCX in exchange for Na\(^{+}\), a transient inward current results. This current causes a delayed after-depolarization (FIGURE 9A) that may reach the threshold for triggering an action potential (70).

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FIGURE 8. Synchrony of Ca\textsuperscript{2+} homeostasis in normal and failing cardiomyocytes

A: t-tubules are lost (*) or disorganized (**) in heart failure, impairing the integrity of dyads and microdomains. This leads to orphaned RyRs and dysynchronous Ca\textsuperscript{2+} release but also likely slowed dysynchronous Ca\textsuperscript{2+} reuptake. Microdomains are additionally altered by loss of NKA, which locally elevates Na\textsuperscript{+} levels and impairs Ca\textsuperscript{2+} removal by NCX (**). Greater Ca\textsuperscript{2+} leak is widely reported in heart failure due to CaMKII-dependent RyR phosphorylation. B: synchrony of Ca\textsuperscript{2+} decline is additionally believed to be modulated by the activity of Ca\textsuperscript{2+} handling proteins. For example, Ca\textsuperscript{2+} transients exhibit non-uniform decay in SERCA KO cells.
release. This explains why less Ca\(^{2+}\) is needed in the SR for uncontrolled local release to occur and also why released Ca\(^{2+}\) is more likely to spread as a Ca\(^{2+}\) wave. Thus loss of control of diastolic Ca\(^{2+}\) can result in both contractile dysfunction and arrhythmias (9).

**Other Alterations in Ca\(^{2+}\) Fluxes in Failing Cardiomyocytes**

This review has so far focused on the SERCA-RyR and NCX-NKA systems, which are the dominant mechanisms for Ca\(^{2+}\) removal in cardiomyocytes.

![Diastolic Ca\(^{2+}\) release from the SR depends on the interplay between SERCA and RyR](image)

**FIGURE 9.** Diastolic Ca\(^{2+}\) release from the SR depends on the interplay between SERCA and RyR

A: spontaneous SR Ca\(^{2+}\) release can initiate a self-propagating process called a Ca\(^{2+}\) wave. This is visible in the example confocal line-scan image as a rise in Ca\(^{2+}\)-dependent fluorescence traveling across the cell. Removal of the released Ca\(^{2+}\) by NCX causes delayed after-depolarizations, which, if large enough, may trigger spontaneous action potentials. B: spontaneous Ca\(^{2+}\) occurs when SR Ca\(^{2+}\) exceeds a set threshold, which is mainly determined by the RyR. i) Healthy cardiomyocytes are quiescent in the diastolic phase since SR Ca\(^{2+}\) content is below the threshold for release. ii) If SR Ca\(^{2+}\) content is temporarily increased above the threshold level, diastolic release occurs even in healthy myocytes. iii) In heart failure, SR Ca\(^{2+}\) is reduced due to lower SERCA2 activity. This tends to reduce the likelihood for diastolic release, since the threshold is not reached. iv) However, heart failure often involves increased chronic PKA signaling and importantly increased CaMKII activity, which sensitizes the RyR and decreases the threshold for release.
However, other more minor contributors to global Ca\textsuperscript{2+} cycling may be of increased importance in failing myocytes. For example, the plasma membrane Ca\textsuperscript{2+} ATPase (FIGURE 1) is thought to contribute little to overall Ca\textsuperscript{2+} removal [\textapprox 1\% (7)]. However, new evidence suggests that the Ca\textsuperscript{2+} ATPase is important for controlling a local pool of Ca\textsuperscript{2+} near the cell membrane involved in Ca\textsuperscript{2+}-dependent hypertrophy signaling (94). Thus up-regulation of the Ca\textsuperscript{2+} ATPase in models such as the SERCA knockout mouse (3, 41) may protect against or delay hypertrophy and heart failure development. Similarly, reduced Ca\textsuperscript{2+} extrusion via the plasmalemmal Ca\textsuperscript{2+} ATPase has been linked to hypertrophy and diastolic dysfunction in mice expressing SERCA2b in the absence of phospholamban (44).

Although the relationship between mitochondrial function and cytosolic Ca\textsuperscript{2+} homeostasis is much debated, these organelles occupy \textasciitilde30\% of the cardiomyocyte volume (compared with 3\% occupied by the SR) (11) and may influence diastolic Ca\textsuperscript{2+} through a number of mechanisms (reviewed in Refs. 64, 65). In healthy cells, mitochondria have been estimated to contribute \textasciitilde1\% of total Ca\textsuperscript{2+} removal during the global Ca\textsuperscript{2+} transient (7). However, it has been suggested that mitochondria and SR may be functionally linked due to the presence of structural interactions between these organelles (5, 23, 25, 85). Of particular interest is the proximity of structural interactions between these organelles/SR may be functionally linked due to the presence of a special subpopulation of mitochondria to RyR (5, 23, 25, 85). Of particular interest is the proximity of structural interactions between these organelles/and Na\textsuperscript{+} ATPases such as SERCA and NKA may occur (47, 64).

In addition, conditions that deplete the cell of ATP favor mitochondrial production of reactive oxygen species (ROS). ROS-dependent modulation of RyR is receiving increasing attention and has been implicated in SR Ca\textsuperscript{2+} leak and arrhythmogenic Ca\textsuperscript{2+} release in heart failure (10). Thus mitochondrial metabolism could importantly affect diastolic Ca\textsuperscript{2+} homeostasis by altering the SERCA-RyR balance.

Conclusion

The above discussions have illustrated that the diastolic phase of the cardiac cycle is not simply a period of rest but involves highly dynamic Ca\textsuperscript{2+} fluxes. In summary, diastolic function is dependent on both the rate and extent of Ca\textsuperscript{2+} removal from the cytosol, which respectively regulate the speed and extent of cardiomyocyte relengthening. Decay rates and diastolic levels of [Ca\textsuperscript{2+}] are therefore of integral importance, and alterations in either of these parameters can have serious consequences in diseases such as heart failure. Determining the precise nature of diastolic Ca\textsuperscript{2+} homeostasis is not, however, as straightforward as one might think but requires detailed insight into the function of the two systems that regulate Ca\textsuperscript{2+} removal: the SERCA-RyR system and the NKA-NCX system. Future strategies aimed at improving diastolic function in heart failure should be carefully directed at manipulation of these systems.

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