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SARCOPLASMIC RETICULUM CA RELEASE IN INTACT VENTRICULAR MYOCYTES

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TABLE OF CONTENTS

1. ABSTRACT

Sarcoplasmic reticulum (SR) Ca release in intact ventricular myocytes is the major source of Ca which activates cardiac contraction (although Ca influx makes a non-negligible contribution in most species). The fundamental events of SR Ca release are known as Ca sparks. The twitch Ca transient is composed of ~10,000 Ca sparks occurring in a given cell, and they are synchronized by the action potential and Ca current. Many factors influence SR Ca release amplitude and kinetics, and the focus here is on understanding how these factors work in the intact cellular environment. The intracellular [Ca] ($[Ca]_i$) and intra-SR [Ca] ($[Ca]_{SR}$) are two of the most important dynamic modulators of SR Ca release. Indeed, while $[Ca]_i$ (and Ca current which initiates systolic SR Ca release) is widely acknowledged to be important, it is increasingly clear that $[Ca]_{SR}$ changes dynamically under physiological conditions and that this has very important regulatory effects on the SR Ca release process. While elevation of $[Ca]_{SR}$ obviously increases the driving force and amount of SR Ca available for release, it also increases the fractional release and can be responsible for spontaneous diastolic SR Ca release. These issues are discussed in both normal physiological and pathophysiological contexts.

2. INTRODUCTION

During cardiac excitation-contraction (E-C) coupling Ca enters the cell via L-type Ca channels as Ca current (I_{Ca}), while a smaller amount of Ca enters the cell via Na/Ca exchange (NCX) working in the outward NCX current ($I_{Na/Ca}$) mode (Figure 1 ; ref 1,2). Ca influx via I_{Ca} triggers Ca release from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR). While Ca entry via NCX can modulate or in extreme cases contribute to direct triggering of SR Ca release, this and other potential trigger mechanisms will be discussed below. The combination of Ca influx and SR Ca release raises free cytosolic [Ca] ($[Ca]_i$) which drives Ca binding to troponin C that switches on the contractile machinery. The rise in $[Ca]_i$ during a twitch is typically from ~100 nM to 1 μM. However, because $[Ca]_i$ is buffered (e.g. by 70 μM troponin C and 50

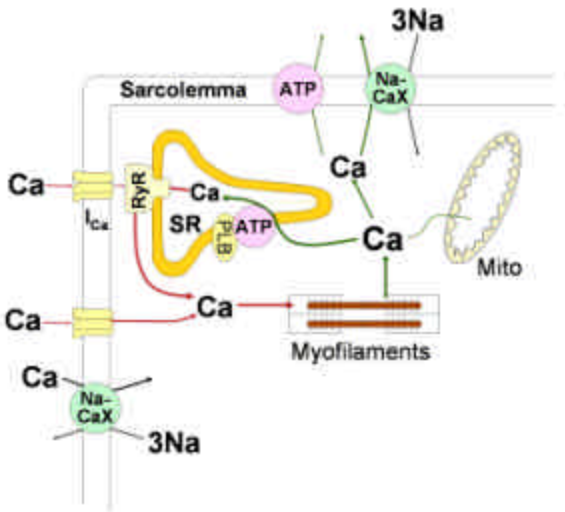

Figure 1. Ca movements in cardiac myocytes. Ca enters via Ca current (I_{Ca}) and a smaller amount via Na/Ca exchange (NaCaX), triggering SR Ca release via RyR at the cleft. Ca is transported out of the cytosol (allowing Ca dissociation from the myofilaments) via the SR Ca-ATPase (regulated by phospholamban, PLB), NaCaX, sarcolemmal Ca-ATPase and mitochondrial (mito) Ca uniport (ATP indicates the two ATPases).

μM SR Ca-ATPase) the total amount of Ca added to the cytosol to achieve this $[Ca]_i$ is about 50-100 μmol/l cytosol.

For relaxation to occur, $[Ca]_i$ must decline and this requires Ca transport out of the cytosol. Four transport systems can contribute to this $[Ca]_i$ decline: 1) the SR Ca-ATPase, 2) NCX in the Ca extrusion mode, 3) sarcolemmal Ca-ATPase and 4) mitochondrial Ca uniporter. The SR Ca-ATPase and NCX are the dominant mechanisms by far, but their relative contributions vary. In most mammalian ventricular myocytes (including rabbit, guinea-pig, ferret, dog, cat and human), the SR Ca-ATPase is responsible for about 60-75% of Ca extrusion with 25-40% attributable to NCX. In rat and mouse ventricular myocytes the SR Ca-ATPase is more dominant, taking up more than 90% of the Ca involved in E-C coupling. This balance can differ in different conditions. For example, at higher frequency and/or with sympathetic stimulation the SR becomes more dominant, while during heart failure the transsarcolemmal Ca fluxes are even more important (see section 6). In general this means that, unlike in skeletal muscle, Ca influx and extrusion plays an important quantitative role in E-C coupling in heart.

Much work has been done at the subcellular and molecular level as to how the key molecular players (I_{Ca}, RyR, SERCA2, phospholamban, NCX) function and are regulated. Indeed, this has provided a very rich array of important fundamental mechanistic information. However, it is crucial that the characteristics of these transporters be understood in the real cellular environment. While this inevitably entails sacrificing some degree of control over intracellular conditions, the advantages of understanding how the system works in the natural cellular environment make this a scientific imperative. Thus, the focus of this article will be to extend basic properties of Ca transport systems to the integrated cellular environment. In line with the focus of this volume, I will deal primarily with the SR Ca release in intact ventricular myocytes.

3. Ca SPARKS AND TWITCH Ca TRANSIENTS

The fundamental unit of SR Ca release in cardiac muscle is the Ca spark (3). Ca sparks are local SR Ca release events that likely represent the concerted opening of 6-20 individual RyR channels in a fairly stereotypical process (4-8). Once one RyR in a junctional cluster opens, it raises local $[Ca]_i$ causing an entire cluster to fire. Several RyRs may also be mechanically coupled (9), thereby assuring the local regenerative nature of Ca-induced Ca-release (CICR) within a junction (a cylinder ~100 nm in diameter and 15 nm high). However, in the space between individual junctions (300-2,000 nm) local $[Ca]_i$ declines, thus limiting propagation of SR Ca release from one junction to another under normal conditions. When cellular Ca loads are very high, the RyR sensitivity is increased and CICR can propagate as a Ca wave along the entire length of a cell at ~100 μm/s.

Ca sparks occur with a low stochastic probability in the resting myocyte ($P_o \sim 10^{-4}$), but are spatially and temporally synchronized during E-C coupling, when almost every junctional coupling region contributes to SR Ca release (10-13). When Ca influx via I_{Ca} raises local $[Ca]_i$ in the junctional cleft to 5-10 μM (within 2-5 ms of depolarization), this abruptly and dramatically increases RyR open probability, at all junctions in the cell. Because of spatial and temporal overlap during E-C coupling, Ca sparks cannot normally be well visualized, unless most I_{Ca} is blocked to diminish overlap (11,13) or high Ca buffer concentrations are used to trap released Ca more locally (14). Thus, the normal myocyte Ca transient is the summation of some 10,000 synchronized Ca sparks (1 per junction) and each Ca spark releases $2\text{-}3 \times 10^{-19}$ mol Ca, or 12-18,000 Ca ions.

Because Ca sparks and twitch Ca transients have a similar basis, we can expect that things that alter diastolic Ca sparks may have qualitatively similar effects on RyR during E-C coupling. Other chapters herein will address some of the details summarized above in more detail. Here the focus is extrapolation and integration up to the cellular level, while keeping characterizations at more fundamental levels in mind.

4. FACTORS AFFECTING SR Ca RELEASE

Numerous factors alter the frequency and amplitude of diastolic Ca sparks and the sensitivity of the RyR to activation by local intracellular Ca, which is generally considered to be the primary physiological activator of SR Ca release. How $[Ca]_i$ and several modulators of RyR gating may affect SR Ca release in intact myocytes is described below.

4.1. Intracellular Free [Ca]

Since Fabiato described CICR in permeabilized cardiac myocytes (15) it has become increasingly clear that

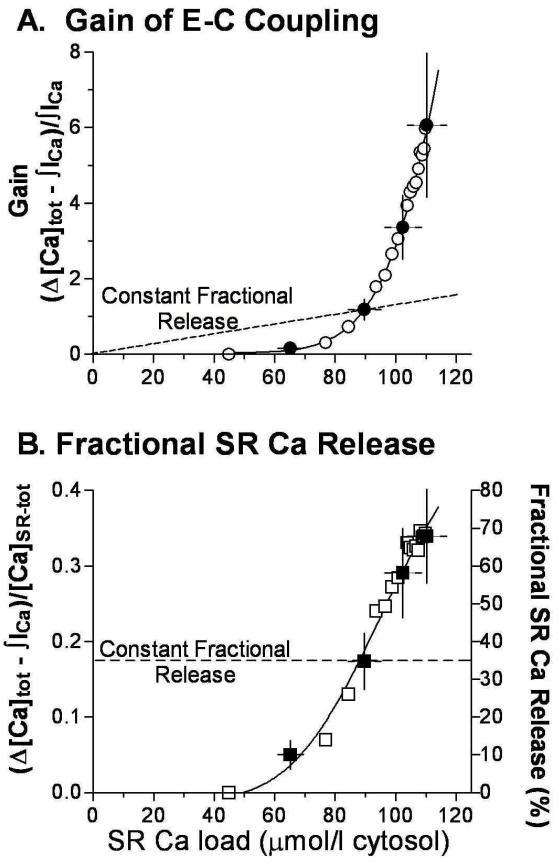

Figure 2. Fractional SR Ca release and gain of E-C coupling. Data from Shannon *et al*. (25) are regraphed, showing the SR Ca load-dependence of E-C coupling gain (A) or fractional release (B). Gain is defined as the total Ca transient amplitude ($\Delta[Ca]_i$ translated to total Ca $[Ca]_{tot}$) minus integrated I_{Ca}, divided by the integrated I_{Ca}. Fractional SR Ca release is the same $\Delta[Ca]_{tot}$ minus integrated I_{Ca}, but divided by the SR Ca load at the time of the twitch. Since some Ca can be taken up and released again, the right axis may reflect the total fractional release.

he amount of Ca entry can regulate SR Ca release. This is also completely consistent with the cytosolic [Ca]-dependence of RyR open probability measured in lipid bilayers studies (16). Indeed, when cellular SR Ca load in intact cells is held constant, altering I_{Ca} amplitude by changing extracellular [Ca] ($[Ca]_o$) or test membrane potential (E_m) produces gradation of SR Ca release (17,18). However, since individual junctions fire largely in a stereotypical all-or-none fashion, the gradation may come mostly from the fraction of junctions which contribute to a Ca spark. On the other hand, there is also some evidence that the amplitude of the local SR Ca release can also be modulated by I_{Ca} trigger size (19). Intact cell voltage clamp experiments have also suggested that opening of a single L-type Ca channel is sufficient to activate a Ca spark and that ~2 Ca ions have to bind to an RyR. Single channel bilayers experiments suggest that up to 4 Ca ions may be required and that the apparent K_d for Ca is in the range of several μM (20). This is consistent with models of diffusion around the inner mouth of a Ca channel, where $[Ca]_i$ within a radius of ~15 nm is expected to rise to 10-20 μM within 1 ms of L-type Ca channel opening (21,22). The above bilayer studies suggested that there is also very little temporal delay (<1 ms) between the rise in local [Ca] and RyR opening. This agrees with our measurements in intact cells where the peak of SR Ca release measured locally (as the effect on I_{Ca} inactivation) occurred within 2-5 ms of the rise of the action potential, which is similar to the time to peak I_{Ca}.

While it is important to understand these local events which are critical determinants of E-C coupling, it is also important to keep in mind that it is the global cellular Ca and binding of Ca to troponin C which produces the end effect of E-C coupling. The peak of the global Ca transient and Ca release flux sensed by global Ca indicators is slower and smaller than that in the junctional cleft. Indeed, global $[Ca]_i$ only reaches ~1 μM during a twitch. This has important implications with respect to the local control of CICR indicated above. That is, activation of SR Ca release may normally require a rapid rise in local $[Ca]_i$ to >10 μM. As Ca diffuses away from the junction $[Ca]_i$ decreases due to dilution into the cytosolic volume, strong buffering and Ca removal fluxes. This means that at the neighboring junctions (>0.3 μm in the transverse and ~2 μm in the longitudinal direction) $[Ca]_i$ will be well below the threshold to trigger that neighboring SR Ca release unit. This normally prevents propagated SR Ca release in ventricular myocytes, which could cause asynchrony in the ventricle and contribute to arrhythmogenesis. As we will see, increasing SR Ca content (and other factors) may shift this apparent threshold for local $[Ca]_i$ to trigger neighboring junctions.

4.2. Intra-SR Free [Ca]

Clearly, one expects the amount of SR Ca release to depend on the amount of SR Ca available for release. Indeed, the release rate should be related to the free intra-SR [Ca] ($[Ca]_{SR}$) in a relatively direct way. If this were the only effect, we would expect the "gain" of E-C coupling ($Ca\ release/I_{Ca}$) to rise roughly linearly as a function of $[Ca]_{SR}$ and the fraction of SR Ca released to be relatively constant as a function of $[Ca]_{SR}$. Cellular data clearly indicate that this simple expectation is incorrect. That is, at SR Ca content below about 40% of normal (~40 μmol/l cytosol) a normal I_{Ca} trigger causes almost no SR Ca release, but as SR Ca rises there is an increasingly steep relationship between either gain or fractional SR Ca release and SR Ca content or $[Ca]_{SR}$ (18,23-26). Figure 2 shows the dependence of gain and fractional release on SR Ca content. It is also clear that increasing intra-SR Ca content greatly increases the frequency (as well as amplitude) of resting Ca sparks, even when diastolic $[Ca]_i$ is unchanged (3,27-29).

This effect is completely consistent with isolated RyR measurements in bilayers, where higher luminal [Ca] greatly increases RyR2 open probability (30-35). Indeed, higher $[Ca]_{SR}$ greatly shifts the $[Ca]_i$-dependence of RyR gating. The same $[Ca]_i$ that caused half-maximal activation of RyR P_o at $[Ca]_{SR}$ = 5 mM produced no RyR opening

when $[Ca]_{SR}$ was low (20 μM). Some investigators claim that the luminal Ca effect is due to more Ca passing through the channel and acting at the cytoplasmic activating site (33,34). However, Györke & Györke (35) showed that this activating effect of $[Ca]_{SR}$ on RyR gating was due to Ca binding on the luminal side, because the $[Ca]_{SR}$ effect was independent of driving voltage direction. In any event, at the cellular level $[Ca]_{SR}$ has a profound role in regulating the RyR. At low $[Ca]_{SR}$ the SR can fail to release Ca, while at high $[Ca]_{SR}$ the luminal [Ca] may be the important trigger for what is normally called spontaneous SR Ca release associated with cellular Ca overload and arrhythmogenic delayed afterdepolarizations (DADs). Since SR Ca content affects both the amount of Ca available for release and the gating of the RyR, it is an extremely powerful modulator of SR Ca release. Thus, I will address determinants of SR Ca load separately below (section 5).

4.3. Turn -off of SR Ca release

Since CICR is inherently a positive feedback mechanism, it is important to consider what turns off release. Moreover, there is good evidence indicating that only about half of the SR Ca content is actually released during a twitch. In addition, SR Ca release must turn off to allow diastolic refilling of the heart. Three possibilities contributors are: 1) local $[Ca]_{SR}$ depletion, 2) RyR inactivation (or adaptation) and 3) stochastic attrition (36-38). Stochastic attrition means that as channels gate stochastically, there is a finite probability that all of the L-type Ca channels and RyRs in a junction close simultaneously, in which case local $[Ca]_i$ would fall very rapidly and interrupt regenerative release. This could work for a small number of Ca channels, but with more realistic numbers of channels, it is far too unlikely that all would close at once. However, if RyRs gate as a synchronized or coupled group (9) stochastic attrition could contribute to the shut off of SR Ca release. However, it is not yet clear whether most RyRs in a spark site really work that way.

The local depletion of SR Ca cannot completely explain the turn-off of release, because very long lasting Ca sparks can be seen (e.g. with ryanodine or caffeine) that do not decline with time (>200 ms; ref 3,27). Diffusion from other regions of the SR might limit local SR Ca depletion, but during the global Ca transient, the entire $[Ca]_{SR}$ declines. Since $[Ca]_{SR}$ strongly modulates RyR gating, the decline in local $[Ca]_{SR}$ may contribute dynamically to the turn-off of global SR Ca release during a twitch. As stated above though, this cannot explain fully why either Ca sparks or global SR Ca release turn off.

Cardiac RyRs can also undergo inactivation or adaptation (both of which may depend on $[Ca]_i$). Classically, inactivation is absorbing (as in Na channels) such that the RyR would be unavailable for re-activation until it recovers (37,39-41). Adaptation refers to a reduction in RyR open probability from a peak upon rapid activation, but from which point the RyR can still be reactivated by a higher $[Ca]_i$ (39,42). Whether the decline in RyR open probability observed is solely inactivation, adaptation or some combination is controversial, and few cellular studies have addressed this in any unequivocal manner. Nevertheless, there is clearly some refractoriness in cellular and local SR Ca release events (27,37,43,44).

In summary, RyR inactivation and partial luminal SR Ca depletion (by reducing RyR opening) probably both contribute to the turn-off of release during a twitch Ca transient. Coupled gating of RyRs (so many RyRs gate as one) may also mean that a variant of stochastic attrition might contribute as well.

4.4. Restitution

After a twitch or spontaneous Ca spark RyR availability recovers with two time constants, a fast one (100-300 ms; ref 44) and a very slow one (several seconds; ref 27,45). RyR inactivation may be important in minimizing inappropriate SR Ca release events between heartbeats. It may also play a central role in the force-frequency relationship that is recorded in myocytes, muscles and the intact heart. In particular, if the SR Ca content does not increase, then increased pacing frequency results in decreased contractile force which cannot be attributed to altered SR Ca availability, I_{Ca}, action potential or myofilament properties. Thus, there is a refractoriness in the E-C coupling mechanism that has physiological impact. Based on Fabiato's early work on CICR, this sort of refractoriness is expected and ought to be sensitive to the level of $[Ca]_i$, in a manner analogous to the E_m-dependence of Ca or Na channel recovery from inactivation. That is, lower $[Ca]_i$ ought to favor more rapid restitution of RyR function. This has not been well tested, but elevations in diastolic $[Ca]_i$ in pathophysiological conditions could also compromise RyR availability. On the other hand, when $[Ca]_{SR}$ and $[Ca]_i$ are both elevated, Ca sparks occur immediately after a twitch (27). The high $[Ca]_{SR}$ may hasten recovery of RyR even when $[Ca]_i$ is relatively high.

4.5. Mg, ATP and pH

Many factors can modulate the RyR open probability, but for practical reasons most bilayer experiments have been done with relatively non-physiological solutions. Intracellular free [Mg] is normally 0.5-1 mM in cardiac myocytes, and mM Mg inhibits RyR gating. When the cardiac RyR is activated by Ca (without ATP), Mg inhibits P_o half-maximally at 2.3 mM (46). However, at physiological [ATP] (5 mM) the inhibitory effect of free [Mg] is modest at 2 mM, and half-inhibition occurs at 5 mM free [Mg]. The precise $[Ca]_i$ *vs.* P_o relationship *in vivo* is not known, but ATP shifts activation to lower [Ca], while Mg shifts it to the higher [Ca]. Mejia-Alvarez *et al.* (47) also made great efforts to study cardiac RyR single channel current under solution conditions which approach physiological. A unitary current of ~0.30 pA can be inferred at 2 mM $[Ca]_{SR}$ and 0.15 pA at 1 mM $[Ca]_{SR}$ (150 mM [K], and 1 mM [Mg]).

Since [ATP] and [Mg] do not change rapidly during E-C coupling these levels may set the physiological Ca-sensitivity of the RyR. For example, Valdivia *et al.* (42) found that mM Mg inhibits steady state cardiac RyR open probability (P_o) at a given free [Ca], but also accelerates the decline in P_o induced by a rapid increase in

local [Ca]. During ischemia free intracellular [Mg] increases several-fold as [ATP] falls, presumably because ATP is a major Mg buffer (48). These changes would reduce RyR sensitivity to Ca. Ischemia is also accompanied by acidosis and cardiac RyR P_o is reduced by >50% upon reduction of pH from 7.3 to 6.5 (46,49,50). Thus, ischemia may greatly depress the responsiveness of the RyR to a given local activating Ca.

4.6. Na/Ca exchange

Ca entry via NCX has been implicated as a potential trigger of SR Ca release in two different ways. First, the rapid Na current associated with the action potential upstroke may raise local submembrane [Na] ([Na]sm), causing Ca entry via outward INa/Ca to trigger SR Ca release (51-53). This interpretation has, however, been challenged (54-56). Data also suggests that Na channels are excluded from the junctional cleft and from regions where NCX is localized (57), making this mechanism less likely. Second, outward INa/Ca is activated directly by depolarization, and can trigger SR Ca release and contraction, especially at very positive Em and when ICa,L is blocked (58,59). However, a given Ca influx via INa/Ca is much less effective and slower than ICa,L in triggering SR Ca release (60). Thus, when both ICa and INa/Ca triggers coexist, CICR is controlled almost entirely by ICa. Na/Ca exchanger molecules may also be excluded from the junctional cleft, making this less physically feasible (57). Thus, outward INa/Ca can trigger SR Ca release, but its physiological role may be modest. It might gradually trigger SR Ca release if there is no nearby Ca channel activated, or it may raise local submembrane [Ca]i to allow a given ICa trigger to be more efficacious in causing SR Ca release.

4.7. Dihydropyridine receptor, Bay K 8644 and FK-506

In skeletal muscle there is fairly clear evidence that the skeletal L-type Ca channel (DHPR) physically interacts with the skeletal RyR, especially in the region of the loop between the second and third domain of the DHPR (II-III loop; reviewed in ref. 1). In heart, the situation is less clear, despite highly homologous RyR and DHPR proteins. The emerging cardiac picture is of a much less robust DHPR-RyR interaction than in skeletal muscle. This is consistent with the lack of voltage-dependent Ca release (VDCR, see below), a less ordered physical array of DHPR over RyR in junctions (61) and the 4-10-fold excess of RyR over DHPR (62). This excess of RyR implies that at most 10-25% of RyR could possibly interact with a DHPR. Nevertheless, cardiac DHPRs are concentrated at sarcolemmal-SR junctions, albeit not as tetrads (61). The carboxy half of a cardiac DHPR II-III loop peptide (Ac-10C, KERKKLARTA) was found to activate skeletal RyR1 and enhance SR Ca release in skinned skeletal muscle (63,64). We found that Ac-10C can inhibit cardiac Ca spark frequency by 63% in voltage clamped ventricular myocytes, for the same SR Ca load and diastolic $[Ca]_i$ (65). A peptide from the carboxy region common to both cardiac and skeletal DHPR also inhibits ryanodine binding to cardiac RyR (66). Thus, the analogous cardiac DHPR and RyR domains may interact, but clarifying work is needed.

The dihydropyridine L-type Ca-channel agonist, Bay K 8644 has also provided data suggestive of molecular communication between cardiac DHPR and RyR. Bay K 8644 (100 nM) accelerates resting loss of SR Ca in ventricular myocytes in a manner that is completely independent of Ca influx, and which is competitively inhibited by DHP antagonists (67-71). Bay K 8644, even at 100 times higher concentration, had no direct effect on cardiac RyR channel gating in bilayer experiments. Another Ca channel agonist (FPL-64176) which does not compete at the same DHPR site did not alter Ca sparks, but enhanced I_{Ca} in a manner similar to Bay K 8644. Bay K 8644 also increased ryanodine binding in intact cells, but not after mechanical disruption. We proposed that Bay K 8644 binds to the DHPR and transmits a Ca-independent signal to the RyR, altering its resting open probability. This effect of Bay K 8644 via the DHPR differs from effects on I_{Ca} that occur more slowly and in a depolarization-dependent manner (71). We concluded that after binding to the DHPR the pathways diverge for the I_{Ca} gating effect and the intramolecular effect on the RyR, manifest as increased resting Ca sparks.

Ca sparks occur in the complete absence of extracellular Ca, even without Bay K 8644. This emphasizes that Ca sparks do not require Ca entry. Moreover, even at this microscopic level action potentials produce no SR Ca release or change in resting Ca sparks in the absence of $[Ca]_o$ (with or without Bay K 8644). Thus, while these Bay K 8644 effects may imply a weak intramolecular link between cardiac DHPR and RyR, they do not support E_m-dependent SR Ca release in heart (VDCR). With respect to E-C coupling, it is notable that Bay K 8644 *depresses* E-C coupling (lower Ca release for a given I_{Ca} and SR Ca load; 72,73). This effect can be explained by the long open times induced by Bay K 8644 modified Ca channels, without invoking the modulation above. That is, a given whole cell I_{Ca} in the presence of Bay K 8644 requires fewer channels (because openings are longer). Since only the first couple of ms of I_{Ca} are needed to trigger SR Ca release, much of the Ca influx will be wasted with respect to triggering SR Ca release (i.e. lower Ca release for a given I_{Ca}). In conclusion, there may only be a weak DHPR-RyR link in heart.

4.8. CaMKII, Protein Kinase A

Phosphorylation of the cardiac RyR by cAMP-dependent protein kinase (PKA) alters RyR gating in bilayers. Valdivia *et al.* (42) found that PKA decreased basal P_o at 100 nM [Ca], but greatly increased peak P_o (to nearly 1.0) during a rapid photolytic increase of [Ca]. PKA also accelerated the subsequent decline in P_o attributed to adaptation. In contrast, Marx *et al.* (74) found that PKA-dependent RyR phosphorylation at Ser-2809 enhanced steady state open probability of single RyRs in bilayers, and attributed this to displacement of FKBP-12.6 from the RyR. However, in more intact cellular systems, we could not detect any effect of PKA-dependent RyR phosphorylation on resting Ca sparks in the absence of phospholamban (with unchanged SR Ca load; 29). RyR phosphorylation might also alter the intrinsic responsiveness of SR Ca release to an I_{Ca} trigger signal, but results are mixed, showing increase, decrease and lack of change (19,75,76). Thus, whether PKA-dependent phosphorylation alters RyR

behavior during rest or E-C coupling remains controversial. This is particularly challenging to measure in intact cells, because increases in I_{Ca} and SR Ca uptake make isolation of intrinsic RyR effects difficult.

Ca-Calmodulin dependent protein kinase (CaMKII) also phosphorylates the cardiac RyR at Ser-2809 (77). In bilayer recordings with cardiac RyR, CaMKII was reported to either increase or decrease channel P_o (77-79). This discrepancy may be partly explained by dynamic changes of RyR gating, as discussed above for PKA (but similar data are not available for CaMKII). In voltage clamped myocytes, we found that inhibition of CaMKII prevented a [Ca]i-dependent increase in the fraction of SR Ca released for the same ICa and SR Ca content (80). Introduction of phosphatases (PP1 & PP2A) into myocytes also depresses E-C coupling gain (81). Thus, in the intact cardiac cell repeated Ca transients may activate CaMKII, phosphorylate RyR2 and enhance the efficacy of E-C coupling.

Calmodulin (CaM) has independent effects on the RyR, which complicates CaMKII effects. Fruen *et al.* (82) reported that 4 CaM molecules bind per skeletal muscle RyR (RyR1) tetramer at both low and high [Ca]. They found the same thing for the cardiac RyR2 at 200 µM [Ca], but when [Ca] was 100 nM RyR2 bound only 1 CaM per RyR tetramer (with lower CaM affinity at low [Ca]). Balshaw *et al.* (83) showed 4 CaM molecules bind per RyR1 tetramer at both low and high [Ca], and the same was true for the RyR2 in the absence of Ca. However, CaM binding increased to 7.5 CaM molecules per RyR2 tetramer at 100 µM $[Ca]_i$. Thus, there is agreement that high [Ca] increases the CaM:RyR2 stoichiometry.

CaM also regulates RyR gating. CaM inhibits Ca release from both cardiac and skeletal SR at $[Ca]_i$>100 nM (82-88). For skeletal RyR1 at low [Ca] (< 100 nM) CaM activates RyR1 opening, but at higher [Ca] (> 1 µM) CaM inhibits RyR1 channel opening. Using a mutant CaM which is unable to bind Ca, Rodney *et al.* (89) showed enhanced RyR1 Ca affinity and single-channel activity at high [Ca], consistent with Ca bound CaM inhibiting RyR1. For cardiac RyR2 CaM inhibits RyR2 opening at both low and high [Ca], and shifts the Ca-dependence of RyR2 activation to higher [Ca] (83). In contrast, Fruen et al. (82) found no effect of CaM on RyR2 flux or ryanodine binding at 100 nM [Ca] (in the presence or absence of Mg). In conclusion, under physiological conditions we might expect CaM bound to RyR2 to exhibit either no effect or an inhibitory effect on the Ca-dependent activation of the RyR.

FK-506-binding proteins (FKBPs) bind to and co-purify with the RyR (90-93), but the intrinsic peptidyl-prolyl isomerase activity is not essential for RyR effects (94). FKBP-12 (MW 12,000) binds tightly to skeletal RyR, while heart expresses both FKBP-12 and -12.6, and the latter associates with the cardiac RyR2, due to a 600-fold higher affinity (93). FK-506 and rapamycin cause dissociation of FKBP from the RyR and modify RyR gating in bilayer studies (95-99). FKBP removal from skeletal RyR causes the appearance of subconductance states. Kaftan et al. (98) found analogous results with the cardiac RyR, with increased overall Po with FKBP removal (despite the lower conductance states). Indeed, when exogenous recombinant FKBP was added to recombinant RyR in bilayers the normal channel gating properties with FKBP were restored. On the other hand, Barg et al. (99) found no functional effect of FKBP on cardiac RyR. FK-506 also inhibits cardiac RyR adaptation (100) and increases Ca-sensitivity of RyR gating. Complementary measurements in intact cells show that FK-506 increases resting Ca spark frequency and causes resting SR Ca content to decline (100,101). Twitch Ca transients are also higher in ventricular myocyte where FKBP is either removed by FK-506 or genetically ablated (100-102). This confirms that the overall enhanced Po of RyR in bilayers after FKBP removal extends functionally to resting Ca leak in intact ventricular myocytes and to E-C coupling effects as well.

A current working model is that FKBP physically stabilizes the coordinated gating of the 4 RyRs in one homotetramer so that openings go from the fully closed to the fully open state, but with reduced overall P_o for a given [Ca] (e.g. shifting the P_o *vs.* [Ca] relationship to higher [Ca]). Marx *et al.* (103) showed that FKBP may also be involved in physical coupling between RyR tetramers, allowing more than one tetramer to gate simultaneously.

Sorcin, a ubiquitous 22 kDa Ca binding protein ($K_{m(Ca)}$=1 µM) associates with both cardiac RyR and DHPR (104,105). Sorcin could reduce RyR open probability and ryanodine binding (IC_{50} =480-700 nM), but this inhibitory effect was relieved by PKA-dependent phosphorylation of sorcin (106). Thus, sorcin and FKBP might serve as endogenous inhibitors of SR Ca release, that can be relieved by PKA-dependent phosphorylation (107).

Eisner *et al.*(108) suggested that altered systolic RyR gating properties alone in intact cells exert only transitory effects on Ca transient amplitude, invoking a sort of autoregulation. That is, abrupt increases in RyR opening or fractional SR Ca release cause greater Ca extrusion via Na/Ca exchange at the first beat, thereby decreasing SR Ca available for the next beat. In the steady state this lower SR Ca content offsets the increased fractional SR Ca release such that Ca transients are almost unchanged. This may limit the overall impact of RyR modulation in the intact cellular environment. On the other hand, there must also be some limit for this autoregulation, such that if the RyR is too leaky (especially through diastole), the SR Ca content could be reduced to the point that SR Ca release fails (as discussed above with respect to the effect of [Ca]SR on fractional release). Thus, the process of SR Ca release must be interpreted in the complex cellular environment of the myocyte. SR Ca load serves as an important subcellular preload that could be considered analogous with end diastolic volume. As such, factors regulating SR Ca load deserve a bit more detailed discussion.

5. SR Ca CONTENT

5.1. Thermodynamics and Leak vs. Pump-mediated Backflux

In principle, the maximal SR Ca content in the intact cell would occur in the absence of leak, when the SR

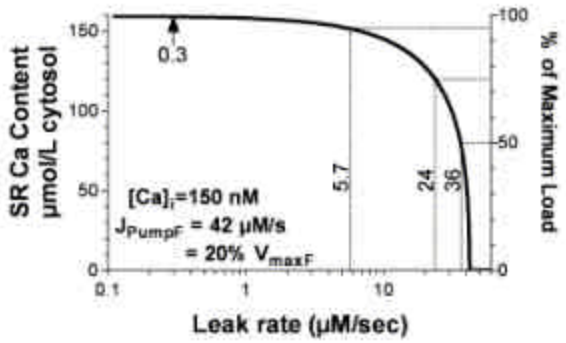

Figure 3. Dependence of SR Ca content on SR Ca leak rate. Curves are based on Eq 1 in the steady state ($J_{PumpF} - J_{PumpR} = J_{Leak}$), where $[Ca]_{SR}$ is a function of J_{Leak} and $[Ca]_i$: $[Ca]_{SR}=7000\{([Ca]_i^2 (V_{max}/J_{Leak} - 1) - (K_{mFor})^2)/ (V_{max}/J_{Leak} + 1))\}^{1/2}$ and SR Ca content, ($[Ca]_{SRtot} = [Ca]_{SR} + (B_{maxSR}/(1+\{K_{dSR}/[Ca]_{SR}\})$, where B_{maxSR} (3 mmol/l cytosol) is the intra-SR binding capacity and K_{dSR} (0.6 mM) is the dissociation constant. Thus $[Ca]_{SR}$ and $[Ca]_{SRtot}$ are functions of $[Ca]_i$ (150 nM), V_{max} (210 µmol/l cytosol/s), K_{mFor} (300 nM) and J_{Leak}.

Ca-ATPase reaches its thermodynamic limit. That is, for a stoichiometry of 2Ca ions transported per ATP consumed the free energy required from ATP is $\Delta G_{SR\text{-}CaP} = 2RT \bullet \ln([Ca]_{SR}/[Ca]_i)$. We measured a maximal SR [Ca] gradient of 7,000, consistent with $\Delta G_{SR\text{-}CaP}$ = 44 kJ/mol (109), which is 74% of the energy available from ATP (for ΔG_{ATP}= -59 kJ/mol, ref 110). Thus, for $[Ca]_i$ = 150 nM, $[Ca]_{SR}$ would be 1 mM, in agreement with estimates of $[Ca]_{SR}$ in the beating heart (111), and energetic efficiencies of other P-type ATPases.

The SR Ca-pump is reversible and Ca can move out of the SR and even make ATP in doing so (112,113). Of course net reverse Ca-pump flux is not expected physiologically, because $[Ca]_{SR}/[Ca]_i$ would have to exceed the ΔG which built the gradient in the first place. However, it is important to appreciate that at thermodynamic equilibrium (with leak of Ca via other pathways at zero) both forward and reverse flux through the SR Ca-pump occur, but are equal and opposite. This can be appreciated by the following equation (114) :

$$J_{Pump} = \frac{V_{mFor}([Ca]_i/K_{mFor})^2 - V_{mRev}([Ca]_{SR}/K_{mRev})^2}{1 + ([Ca]_i/K_{mFor})^2 + ([Ca]_{SR}/K_{mRev})^2} \quad (1)$$

where V_{mFor} and V_{mRev} are the forward and reverse maximum rates, and K_{mFor} and K_{mRev} are the forward and reverse dissociation constants. It can be seen that if $[Ca]_{SR}$ = 0, the last term in both numerator and denominator drop out and this reduces to the standard Hill equation ($V_{max}/(1+\{K_m/[Ca]_i\}^n)$). At equilibrium (with zero leak) the net SR Ca-ATPase flux (J_{Pump}) is 0. At this point the numerator in Eq 1 is zero so the two terms are equal. For the reasonable assumption (based on data) that V_{mFor} $=V_{mRev}$ this relationship implies that the limiting [Ca] gradient above, $[Ca]_{SR}/[Ca]_i = K_{mRev}/ K_{mFor}$ which is the well known Haldane relationship. It also means that the affinity of the Ca-pump for cytosolic Ca is 7000 times higher than that for intra-SR Ca (e.g. K_{mFor} = 300 nM, K_{mRev} = 2 mM). That is why Ca dissociates from the pump readily after it is transported into the SR. Thus, without any leak the SR Ca-pump should approach asymptotically a $[Ca]_{SR}/[Ca]_i$ gradient of 7000. However, leak of Ca from the SR (e.g. as Ca sparks) is not normally zero, so the net SR Ca flux (J_{SR}) is the sum of J_{Pump} (as in Eq 1) plus J_{Leak}. In this more realistic steady state scenario the forward pump rate (J_{PumpF}) must equal the sum of the pump backflux (J_{PumpR}) plus J_{Leak}.

Figure 3 shows how the SR Ca content is affected by J_{Leak} at $[Ca]_i$ of 150 nM (including consideration of intra-SR Ca buffering). When J_{Leak} is zero $[Ca]_{SR}$ is ~1 mM and the SR Ca content is 160 µmol/l cytosol. As J_{Leak} increases the SR Ca content declines. In intact ventricular myocytes from rat and rabbit we measured J_{Leak} to be ~0.3 µmol/l cytosol/s and found this to be consistent with resting Ca spark frequency (115). This would suggest that leak is not very important in limiting the SR Ca load. That was consistent with our follow up study in voltage clamped rabbit ventricular myocytes, where stimulation or inhibition of the SR Ca-ATPase failed to significantly change the maximal SR Ca load achieved at steady state (116). Of course achieving that $[Ca]_{SR}/[Ca]_i$ gradient took longer when the pump was partially inhibited. However, if leak were predominant the final load should have been significantly lower as well (Fig 3). At odds with these observations, blocking RyR-mediated leak with tetracaine can dramatically increase SR Ca content (117-119). This effect would not be expected if leak were very small and might imply a J_{Leak} closer to ~5-10 µmol/l cytosol/s (119). We have recently re-evaluated this issue with a novel experimental approach that allows us to measure SR Ca leak at different SR Ca loads (120). We find the same sort of steep SR Ca load-dependence of leak as we reported for either E-C coupling gain or fractional release (in Fig 2) and for Ca spark frequency (27). The result was that J_{Leak} varies over a broad range and might be closer to 5 µmol/l cytosol/s at relatively physiological SR Ca load and $[Ca]_i$. Considering that J_{Leak} increases steeply with $[Ca]_{SR}$ and that increasing J_{Leak} limits SR Ca load (Fig 3), it is easy to appreciate that this creates an intrinsic limit to SR Ca load. That is, if SR Ca is high, it increases leak, thereby limiting SR Ca content.

It is also worthwhile to consider the energetic consequences of J_{Leak}. If J_{Leak} were zero then the SR Ca content during diastole could be maintained with no net ATP consumption (since J_{PumpF} would equal J_{PumpR}). Of course, any intrinsic slippage or inefficiency in the pump mechanism would make some net energetic ATP cost to maintain $[Ca]_{SR}$. However, if J_{Leak} is 6 µmol/l cytosol/s this must be compensated by an equivalent net J_{PumpF}, requiring 3 µM ATP consumption per second. This constitutes an additional energetic demand for the heart. Figure 3 also shows that as J_{Leak} approaches 10-15% of the forward V_{max} of the pump the SR Ca load drops precipitously as a function of leak (because J_{PumpF} is ~15% of V_{max} at diastolic $[Ca]_i$). Thus if the physiological leak rate is very close to

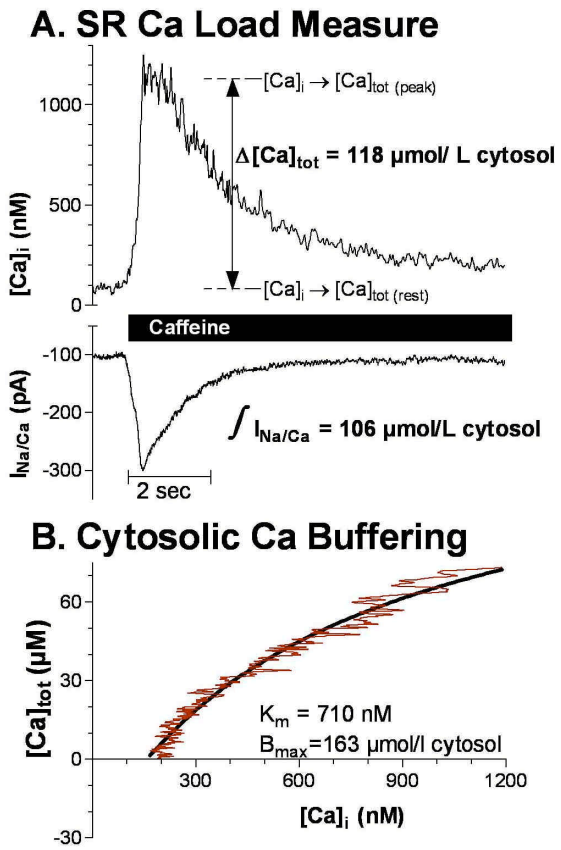

Figure 4. Measurement of SR Ca content and cytosolic Ca buffering during a caffeine-induced Ca transient in a rabbit ventricular myocyte under voltage clamp. **A**. Caffeine (10 mM) was applied rapidly and $[Ca]_i$ is converted to total cytosolic [Ca] ($[Ca]_{tot}$ in µmol/l cytosol) using previously measured Ca buffering ($272/\{1+(673\ nM/[Ca]_i)\}$ as in ref 1). This yields SR Ca content of 118 µmol/l cytosol. SR Ca content is also obtained by integrating $I_{Na/Ca}$ (multiplying by 6.44 pF/pL cytosol, and dividing by 96,490 C/mol and 0.93 to account for non-$I_{Na/Ca}$ mediated Ca transport). **B**. To measure Ca buffering $I_{Na/Ca}$ is integrated from the end backward as $[Ca]_{tot}$ and fit as a function of $[Ca]_i$ ($[Ca]_{tot} = \{B_{max}/(1+K_m/[Ca]_i)\}+B_{min}$, ref 120). Data recorded by K.S. Ginsburg and modified from ref. 1).

this steep region of the curve, small changes in SR Ca leak (as might occur in heart failure) could have a disproportionately large effect on SR Ca content.

During a normal cellular Ca transient $[Ca]_{SR}$ declines during SR Ca release and consequently reverse Ca-pump flux is decreased, while forward Ca-pump flux is stimulated by high $[Ca]_i$. Thus there is an increase in net SR Ca uptake by the pump. As $[Ca]_i$ declines and $[Ca]_{SR}$ rises the forward Ca-pump rate falls and reverse Ca-pump rate increases, until the net pump flux comes into balance with the SR Ca leak flux. At that point the SR Ca content has returned to the steady state level for the next beat. During the cardiac cycle the amount of Ca which entered via I_{Ca} will also have to be extruded by Na/Ca exchange. Of note in the non-steady state, either increased Ca extrusion by Na/Ca exchange or reduction of SR Ca-pump activity would result in less net Ca uptake by the SR and a lower steady state SR Ca content (and more extrusion of Ca from the cell). These perturbations may contribute to reduced SR Ca load and contractile dysfunction in heart failure (see Section 6).

An important functional consequence of this thermodynamic consideration is that SR Ca uptake (especially during late relaxation and diastole) will be sensitive to energetic limitations that may occur under pathophysiological conditions. For example, if [ATP] declines or [ADP] or $[PO_4]$ rise, the ΔG_{ATP} available to the Ca-pump will be reduced. While this may not alter V_{max} or initial rates of $[Ca]_i$ decline, it will reduce the [Ca] gradient that the SR Ca-pump can generate. This will have preferential effects on the latter phase of $[Ca]_i$ decline and on diastolic $[Ca]_i$ as the pump approaches a different thermodynamic equilibrium (at lower $[Ca]_{SR}$ and higher $[Ca]_i$). The lower SR Ca content would also disproportionately depress Ca transients by reducing both the amount of SR Ca available and also the fraction released.

5.2. Measurement of SR Ca content

SR Ca content has been measured in a variety of ways, including radiotracer ^{45}Ca fluxes, electron x-ray microprobe analysis, extracellular [Ca] depletions and rapid cooling contractures (see ref 1 for details and references). However, a convenient and widely used approach these days is the caffeine-induced Ca transient, as illustrated in Figure 4. This method has the advantage that it can be applied acutely to an isolated myocytes at any time (e.g. under different experimental conditions). Rapid application of 10-20 mM caffeine releases all SR Ca and prevents net reuptake because of open SR Ca release channels. Then quantitative measures of SR Ca load can be obtained from either the amplitude of the resultant contraction or Ca transient, or by integrating $I_{Na/Ca}$ (since most of the SR Ca is removed from the cell this way; see Fig 4A). Caffeine must be very rapidly applied in order to achieve a rapid and relatively uniform rise in $[Ca]_i$ throughout the cell. This is especially important when using either the contraction or Ca transient amplitude as an index of SR Ca content (less so when $I_{Na/Ca}$ is integrated).

If one measures caffeine-induced $\Delta[Ca]_i$ there are two important interpretational caveats. First, if there is significant Ca extrusion between the time that caffeine is applied and the peak $[Ca]_i$ is observed (e.g. due to NCX) the peak $[Ca]_i$ may be underestimated. Thus, caffeine-induced Ca transient are sometimes measured in conditions which inhibit NCX (e.g. 0Na, 0Ca solution; 116,121). Of course this can limit the ability to simultaneously integrate $I_{Na/Ca}$ (but see also ref 116). This issue makes it impractical to use caffeine-induced contractures or Ca transients in multicellular preparations, because $[Ca]_i$ may already decline in surface cells prior to SR Ca release in cells at the center of the preparation. Caffeine-induced contractures and $\Delta[Ca]_i$ can be useful from a comparative standpoint, but the second caveat is that to convert $\Delta[Ca]_i$ to SR Ca

content one must account for the buffering of Ca in the cytosol. This can be done using measured values of cytosolic Ca buffering (e.g. measured during $I_{Na/Ca}$ as in Fig 4; refs 1,122,123).

Integration of $I_{Na/Ca}$ during caffeine application can also provide a rather direct measure of the SR Ca content. This works because almost all of the Ca released by caffeine is extruded via $I_{Na/Ca}$. This same $I_{Na/Ca}$ data (where $[Ca]_i$ is also measured) can be used to measure cytosolic Ca buffering as illustrated in Fig 4B. This is like a simple reverse titration where integrated $I_{Na/Ca}$ counts the total number of Ca ions removed for a measured change in $[Ca]_i$. There are also three main caveats in using the $I_{Na/Ca}$ integral to measure SR Ca content. First, not all of the $[Ca]_i$ decline during a caffeine-induced Ca transient is due to $I_{Na/Ca}$, but can also include contributions from the sarcolemmal Ca-ATPase and mitochondrial Ca uniporter (1,121). While these are relatively minor, one can account for this by dividing the integrated $I_{Na/Ca}$ by the fraction of $[Ca]_i$ decline due to NCX (~0.75-0.93 depending on species). A second conversion required is to transform the surface Ca flux (usually in A/F) into "volume flux" (in µmol/l cytosol). We have measured the surface to volume ratio for ventricular myocytes in several species allowing this conversion (6.4-13 pF/pL cytosol; ref 124). A third potential issue is the possibility that NCX stoichiometry might not be 3Na:1Ca under all experimental conditions (125,126). It is less clear how to deal with this potential caveat and most investigators still assume that the NCX stoichiometry is fixed at 3Na:1Ca. Given the different assumptions and limitations between $\Delta[Ca]_i$ and integrated $I_{Na/Ca}$ methods, it is especially advantageous if one can measure SR Ca load in both complementary ways simultaneously, especially when the results are in good agreement, as in Fig 4. This also helps to reinforce the reasonable nature of the assumptions required. Maximal SR Ca content under relatively physiological conditions is about 90-130 µmol/L cytosol, or about twice the amount of Ca required to activate a twitch (116,127,128).

6. PATHOPHYSIOLOGICAL ALTERATIONS IN HEART FAILURE

Under pathophysiological conditions there may be a reduction in the amount of Ca released from the SR and this can be a significant contributing factor in contractile dysfunction (1). An important pathophysiological case in point (and major health problem) is heart failure, where contractile dysfunction has been shown to be caused in large part by altered myocyte Ca regulation (129-136). Most, but not all results have indicated that Ca current and myofilament Ca sensitivity are not significantly changed in heart failure (1). While alterations in these systems cannot be unequivocally excluded for participating in the heart failure phenotype, the focus here is on SR Ca release (and there is much experimental support for reduced SR Ca release in heart failure). There are three main mechanisms which have been implicated in the reduced SR Ca release in heart failure. Two of these are associated with reduced SR Ca content, which has been documented in human, canine and rabbit heart failure (74,134,136-138). The third is associated with unaltered SR Ca content, but reduced E-C coupling gain or fractional SR Ca release in rat or mouse heart failure (139-141).

6.1. Reduced SERCA2 and Enhanced Na/Ca Exchange

Many heart failure studies have demonstrated that the expression of SR Ca-ATPase is reduced, NCX is increased or both (130-135). Considering the fate of Ca during relaxation and $[Ca]_i$ decline, it is easy to understand how either reduced SR Ca-ATPase, or enhanced NCX (or both) will tend to reduce SR Ca content by shifting more Ca back out of the cell vs. the SR. On the other hand, if both of these changes occur, they can be offsetting with respect to the rate of $[Ca]_i$ decline during a twitch. That is, a large increase in NCX can compensate for a small reduction in SR Ca-ATPase function in terms of the rate of $[Ca]_i$ decline, but both changes would cause reduction in SR Ca load. The most compelling data for this mechanism is in heart failure in humans (ischemic and non-ischemic), rapid pacing-induced failure in the dog and non-ischemic pressure/volume overload in rabbit. In these cases, measurements of SR Ca-pump and NCX expression and function have been well documented and the reduction in SR Ca content has been measured via caffeine-induced Ca transients (134,136,137). It is of interest to note that either SERCA reduction alone or NCX increase alone can largely cause the reduced SR Ca content by shifting the competition between these systems away from the SR and toward extrusion via NCX during relaxation and $[Ca]_i$ decline (as demonstrated by experiments where SR Ca-ATPase is inhibited or NCX is overexpressed in normal cells, 142,143). Obviously, when both changes occur the results can be additive. The reduction in SR Ca content in heart failure reduces the amount of SR Ca available for release for a given I_{Ca} trigger, but this depressant effect is likely compounded by the inhibitory effect of low luminal $[Ca]_{SR}$ on the gating of the RyR during E-C coupling (as in Fig 2). Thus, reduced SR Ca content may be a crucial proximal cause of contractile dysfunction in heart failure.

6.2. Enhanced SR Ca Leak

Another mechanism that could reduce SR Ca content in heart failure is an enhanced diastolic leak of Ca from the SR (74,138). This would allow NCX another opportunity to compete with the SR Ca-pump for the Ca which had already been taken up by the SR during twitch $[Ca]_i$ decline. The strongest evidence for this possibility comes from studies by Marx *et al.* (74). They found that in heart failure RyRs are hyperphosphorylated by PKA (nearly 1 phosphate group per RyR monomer), and suggested that this was caused by a reduction in the amount of phosphatase associated specifically with the RyR. They showed also that this degree of RyR phosphorylation caused FKBP-12.6 to dissociate from the RyR and that this caused an increase in Ca-sensitivity of RyR channel opening in lipid bilayer recordings. This might increase the diastolic leak of Ca from the SR, which according to the curve in Fig 3 would reduce SR Ca content. This agrees with observations that Ca spark frequency is enhanced by FK-506 which displaces FKBP-12.6 from the cardiac RyR (100,101). Although we could not detect any effect on Ca spark frequency when we directly phosphorylated the RyR

by PKA in a cellular environment (29), this possibility remains intriguing and merits further study. Enhanced diastolic Ca leak could work synergistically with the changes in functional expression of the SR Ca-ATPase and NCX in reducing SR Ca load, and contribute to reduced SR Ca release during E-C coupling along the same lines just described.

6.3. Altered E-C coupling

In certain rat and mouse heart failure models E-C coupling is depressed without a reduction of I_{Ca}, SR Ca content or isolated RyR function. This includes spontaneous hypertensive heart failure (SHHF) rats, post-myocardial infarct rats and MLP knockout mice (139-141). In these cases a given I_{Ca} results in reduced SR Ca release (in some cases where SR Ca load was determined to be unchanged). It did not seem to be due to alteration in either the number or Ca-sensitivity of individual RyRs. This led these investigators to propose that there might be either a spatial change in the juxtaposition of Ca channels at the junction, or some other reduction in RyR Ca sensitivity that depends on the intact cellular environment. It is interesting that this mechanism has been most frequently reported in rats. While this might reflect a species-dependent difference in heart failure phenotype, this will require further study. This sort of intrinsic reduction in E-C coupling could also coexist with reduced SR Ca content as discussed above. Indeed, reduced SR Ca-ATPase expression and function has also been reported by several groups in rat models of hypertrophy and heart failure, especially at the transition from compensated to overt heart failure (130,144). Thus, while there are likely to be multiple contributing factors to heart failure, reduced SR Ca release probably plays a major role in the contractile dysfunction.

7. CONCLUSIONS

Release of Ca from the SR in the intact cardiac myocytes is influenced by numerous factors. We have learned much from studies in isolated systems (RyRs in bilayers and vesicles), but these results must be translated to the intact cellular environment. In some cases this can be approached with relatively direct cellular experiments, but this is not always the case. We do know that two of the most important physiological regulators of SR Ca release during diastole as well as systole are local $[Ca]_i$ and $[Ca]_{SR}$. Other factors may modulate this relationship (e.g. ATP, Mg, pH, calmodulin, FKBP-12.6, phosphorylation, redox state, nitric oxide), but Ca is probably the main dynamic regulator. I_{Ca} is the primary trigger for SR Ca release during E-C coupling. While Ca influx via other pathways (e.g. NCX) can make some contribution under certain circumstances, these effects are likely to be primarily modulatory. Intra-SR Ca is crucial in regulating SR Ca release in two ways. First, it determines the amount of SR Ca available for release. However, the effect of luminal $[Ca]_{SR}$ in regulating the gating of the RyR in the intact cell is also extremely important. This may increase the sensitivity of the RyR to cytosolic $[Ca]_i$. When SR Ca load is elevated, it may be the rise in $[Ca]_{SR}$ which triggers the so-called spontaneous SR Ca release during Ca overload that can be directly arrhythmogenic. Low SR Ca content may be a central cause of contractile dysfunction in heart failure, where it can be caused by reduced SR Ca-ATPase functional expression, increased NCX expression, increased diastolic SR Ca leak or even energetic limitations which affect ΔG_{ATP}. As a result, it is important to understand the functional cellular environment (and microenvironment) where the RyR exists in order to appreciate fully how it is functioning under any particular situation.

8. ACKNOWLEDGEMENTS

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