Published in final edited form as:

J Mol Cell Cardiol. 2008 January ; 44(1): 31–43. doi:10.1016/j.yjmcc.2007.10.012.

Mechanisms and potential therapeutic targets for ventricular arrhythmias associated with impaired cardiac calcium cycling

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Although increasingly effective therapies have reduced overall cardiovascular mortality, the proportion of sudden cardiac death (SCD) caused by arrhythmias is actually on the rise [1]. It is well recognized that the vast majority of SCD occurs in patients with ventricular contractile dysfunction, usually associated with some extent of clinical heart failure [2,3]. Despite this close relationship, the mechanisms linking mechanical to electrophysiological dysfunction in the heart are poorly understood. Greater understanding of these mechanisms is critical to the development of new targets for predicting and ameliorating risk of SCD in patients.

Impaired intracellular calcium regulation is one of the most striking abnormalities associated with contractile dysfunction [4]. Could this also be a major factor underlying electrical instability in failing myocardium? Understandably, arrhythmia research initially focused on sarcolemmal ion channels, while the clinical relevance of sarcomeric calcium handling to arrhythmogenesis was unclear, or thought to occur only under non-physiological conditions. However, the importance of intracellular calcium in cardiac electrical instability over a broad range of arrhythmia mechanisms has gained significant interest. Cellular calcium handling has been implicated in arrhythmias associated with T-wave alternans,[5-8] heart failure (heart failure),[9-16] and mutations of calcium regulatory proteins under exercise or elevated catecholaminergic states [17-26]. The strong association between calcium regulation and arrhythmogenesis can be appreciated by recognizing the direct effect intracellular calcium has on several membrane ionic currents [27]. Complicating this relationship is the fact that membrane ionic currents can, both directly and indirectly (by their effect on the action potential), influence intracellular calcium. Despite the recognition of the importance of calcium mediated arrhythmogenesis, the underlying mechanisms are not well understood, partly due to the complex interplay between membrane currents and intracellular calcium and, importantly, the difficulty in measuring transmembrane potential and intracellular calcium from intact cardiac preparations. In this review, we highlight recent observations which have implicated intracellular calcium cycling as source of abnormal impulse formation (triggered activity) and abnormal impulse conduction (reentrant excitation), both of which contribute to cardiac arrhythmogenesis. These findings suggest that a variety of calcium handling proteins involved in excitation-contraction coupling may provide future targets for antiarrhythmic therapies. .

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Calcium Homeostasis in Normal Myocytes

Calcium homeostasis is the maintenance of stable intracellular calcium levels during systole and diastole, and is governed by numerous channels and exchangers that regulate the flow of calcium ions between the extracellular space, cytoplasm, and intracellular stores (Figure 1). During systole, myocyte contraction is initiated by a sharp rise in cytoplasmic calcium levels ($\sim 1 \mu\text{M}$). This sharp rise in calcium is caused by calcium entering the cell through the L-type channel immediately following depolarization, which triggers calcium release from the sarcoplasmic reticulum (SR), in a process referred to as calcium-induced calcium-release (CICR). CICR occurs at narrow gaps between L-type calcium channels in the sarcolemma and SR protrusions (i.e. junctional processes or feet) that are part of the SR calcium release 40 channel. Calcium entering through the L-type channel binds to the SR release channel and initiates calcium release from the SR, which contains a relatively large concentration of calcium ions. A single SR calcium release channel is a macromolecular complex comprised of four ryanodine receptor (RyR) proteins, stabilizing proteins such as FKBP12.6, kinases, phosphatases, and many other proteins including calsequestrin, triadin, junctin and junctophilin [28]. L-type calcium channels and SR calcium release channels are located along the surfaced membrane and T-tubules to ensure that upon electrical excitation calcium release and, thus, contraction is uniform and synchronous within an entire cell. In the absence of T-tubules or L-type calcium channel activation, calcium waves can occur that propagate by CICR but are not, as a result, temporally synchronized. The calcium spark, a fundamental SR calcium release event, is likely comprised of calcium release from a cluster of SR calcium release channels that are functionally related and closely coupled (i.e. FKBP12.6-mediated coupled gating) [29]. The amount of calcium released from the SR is not all or none, and has a critical dependency on several factors including, primarily, the amount of calcium entering through the L-type calcium channel,[30] the amount of calcium in the SR,[31-34] and recovery (inactivation and adaptation) of RyR [35].

Modulation of the SR calcium release channel is critical to survival, and consequently influenced by multiple regulatory mechanisms. For example, PKA-dependent phosphorylation of RyR has been shown to have varied phosphorylation sites[36,37] which effects open probability (P_o) [38]. Ca-Calmodulin dependent protein kinase (CaMKII) can also phosphorylate RyR [39]. The FK-506 binding protein, FKBP12.6, is also an important modulator of SR calcium release. One FKBP12.6 binds tightly to each RyR protein subunit of the tetramer to prevent sub-conductance states and aberrant activation during diastole. This stabilizes the RyR in the closed and open states. In addition, multiple RyR channels are functionally related and closely coupled (i.e. FKBP12.6-mediated coupled gating) such that synchronous activation and inactivation of multiple RyR channels occurs [29]. It has been suggested that beta-adrenergic stimulation plays an important role in modulating the effect of FKBP12.6;[37,40,41] however, this remains controversial [42]. Finally, the proteins calsequestrin, triadin, junctin on the luminal side of the SR, assemble with RyR and are known to play important regulatory roles [28].

To maintain homeostasis, the same amount of calcium released from the SR into the cytoplasm must be reclaimed from the cytoplasm during diastole. Cellular relaxation during diastole is accomplished by lowering cytoplasmic calcium to resting levels ($\sim 100 \text{ nM}$). Calcium is removed from the cell by the sodium calcium ($\text{Na}^+ - \text{Ca}^{++}$) exchanger operating in the forward mode, where 3 Na^+ ions enter the cell and are exchanged for 1 Ca^{++} ion. The net transfer of charge during forward mode operation results in an inward (i.e. depolarizing) current that can prolong action potential duration (APD). In addition to lowering cytoplasmic calcium levels, the $\text{Na}^+ - \text{Ca}^{++}$ exchanger can also increase intracellular calcium levels when operating in the reverse mode, where 1 calcium ion enters the cell in exchange for 3 Na^+ ions leaving. When operating in the reverse mode an outward current results which can shorten APD. While it is

possible that $\text{Na}^+\text{-Ca}^{++}$ exchanger operating in the reverse mode can induce calcium release from the SR, this is not believed to be its main role.

A second mechanism for restoring cytosolic calcium to diastolic levels is through reuptake of calcium by the SR via the SR Ca^{++} ATPase (SERCA2a), which (to some extent) competes with the $\text{Na}^+\text{-Ca}^{++}$ exchanger for cytoplasmic calcium. SR Ca^{++} ATPase transports calcium from the cytoplasmic side of the SR to the luminal side and requires ATP. Phospholamban (PLB) appears to be the main endogenous regulator of SR Ca^{++} ATPase which acts to reduce SR Ca^{++} ATPase activity unless phosphorylated [43]. PLB is present at high concentrations in ventricular myocytes, but overexpression can cause further inhibition of SR Ca^{++} ATPase. Likewise, reduced expression of PLB, can significantly enhance SR calcium re-uptake [44]. Unlike the $\text{Na}^+\text{-Ca}^{++}$ exchanger, calcium re-uptake via SR Ca^{++} ATPase is significantly influenced by phosphorylation. PKA dependent phosphorylation of PLB is the likely mechanism of accelerated relaxation associated with catecholamines [45]. CaMKII can also accelerate SR calcium re-uptake by phosphorylation of PLB and, possibly, by a mechanism that does not require PLB [46].

The regulation of intracellular calcium might be irrelevant to mechanisms of arrhythmias and SCD if it were not for the effect intracellular calcium has on membrane potential. There are several mechanisms by which intracellular calcium can influence membrane potential. As mentioned above, the $\text{Na}^+\text{-Ca}^{++}$ exchanger can depolarize (forward mode) or repolarize (reverse mode) membrane potential. Typically, if intracellular calcium increases on one beat, (e.g. a larger calcium transient amplitude) it is expected that APD duration would increase, and vice versa. This positive correlation between APD and calcium transient amplitude has been termed positive Ca- V_m coupling [47]. However, given that the mode of $\text{Na}^+\text{-Ca}^{++}$ exchanger operation depends on Ca^{++} and Na^{++} concentrations and membrane potential, can make the resultant change in APD often difficult to predict. For example, even though $\text{Na}^+\text{-Ca}^{++}$ exchanger expression is increased in heart failure does not necessarily imply that reverse mode and, thus membrane depolarization and APD are increased.

Intracellular calcium can also influence membrane potential by its effect on L-type calcium channel inactivation. Calcium released from the SR, which is initiated and occurs nearby L-type calcium channels, inactivates L-type calcium current. As a result, an increase in calcium release from the SR on one beat (i.e. larger calcium transient amplitude) is expected to reduced L-type calcium current and, thus, shortens APD. In addition, the slow delayed rectifier K^+ current (I_{Ks}) is enhanced by intracellular calcium such that a larger calcium transient amplitude would also shorten APD. Such negative coupling between calcium transient amplitude and APD has been termed negative Ca- V_m coupling. Finally, in addition to the currents already mentioned, the calcium activated transient outward chloride current (I_{to2}) and calcium activated nonselective current ($I_{ns(\text{Ca})}$) can also influence membrane potential, but their effects on APD are species-specific and expected to be minimal.

Arrhythmias Caused by Abnormal Impulse Initiation (Triggered Activity)

Triggered activity can be described as an ectopic (e.g. unstimulated) beat that requires at least one preceding (i.e. triggering) beat. The mechanism of triggered activity is often defined by the timing of the ectopic beat relative to the triggering beat(s). Ectopy that occurs before final repolarization of the triggering beat is caused by early afterdepolarization (EAD) activity, and ectopy that occurs after final repolarization is caused by delayed afterdepolarization (DAD) activity. Both EAD and DAD activity are manifest as small oscillations in membrane potential, which, when reaching threshold can trigger additional beats. EAD activity is associated with a prolongation of APD allowing recovery from time-dependent inactivation of the L-type calcium channel “window” current producing secondary depolarizations [48]. DAD activity is

initiated by a spontaneous (i.e. non-electrically driven) calcium release from internal stores (e.g. SR or myofilaments). In response to such a spontaneous calcium release (SCR) event, calcium-sensitive ion channels and exchangers can depolarize the cell membrane (see below). If EAD or DAD amplitude is large enough to initiate an action potential, triggered activity will result. An EAD that occurs later during final repolarization (late EAD), may have a mechanism that is more similar to a DAD than an early EAD. The remainder of this section focuses on triggered activity associated with SCR events.

Triggered Activity Associated with Normal RyR Function

Digitalis toxicity is classically associated with triggered activity caused by DADs. Shown in Figure 2 is an example of triggered activity caused by DADs as measured in a Purkinje fiber during digitalis administration [49]. Digitalis and other cardiac glycosides (e.g. ouabain) inhibit $\text{Na}^+\text{-K}^+$ ATPase which results in the accumulation of intracellular Na^+ and, consequentially, an enhancement of the $\text{Na}^+\text{-Ca}^{++}$ exchanger in the reverse mode (sodium removal, calcium entry) and an accumulation of intracellular calcium. In addition to enhanced inotropy, if sufficient calcium accumulates inside the cell, calcium overload and, thus, SCR activity can result. An SCR event is believed to activate I_{Ti} [50,51] comprised of I_{NaCa} , $I_{\text{ns(Ca)}}$, and/or I_{to2} [51,52]. This I_{Ti} current will depolarize the membrane, creating DAD activity. In this classic example and as shown under a variety of conditions, when intracellular calcium levels are abnormally high, calcium overload and, thus, SCR activity can occur even when RyR is otherwise normal [52-59]. For example, calcium overload can also occur when sympathetic activity is enhanced. Enhanced calcium entry through the L-type calcium channel has been shown to cause calcium overload and triggered activity under elevated β -adrenergic stimulation and exercise [60-63]. However, in normal myocardium, the marked increase in calcium entry during beta-adrenergic stimulation is typically balanced by a shortening of APD due to enhanced I_{Ks} (delayed rectifier). Therefore, the blockade of I_{Ks} along with beta-adrenergic stimulation, which mimics long QT type 1 (LQT1) syndrome, has served as a useful model to study calcium overload and calcium-mediated triggered activity [55,64-66]. Even though triggered activity has been studied extensively in single cells, direct evidence of DAD activity from intact tissue preparations and in vivo [60] has previously been limited, partly due to the difficulty measuring SCR and DAD activity.

It has long been questioned as to whether DADs that are suitable in amplitude to drive the membrane to threshold potential in isolated myocytes can do so in multi-cellular preparations or the intact heart. Electrical coupling between neighboring myocytes can induce a current load, which retards DAD amplitude. Using dual calcium voltage optical mapping techniques, we have shown in intact myocardial preparations that under calcium overload conditions (I_{Ks} blockade and β -stimulation) triggered activity is caused by multiple, simultaneous SCR events [67]. Shown in Figure 3 (Panel A) are membrane potential (V_m) and intracellular calcium (Ca) signals recorded from the canine left ventricular wedge preparation during I_{Ks} blockade and Isoproterenol administration. Immediately after halting rapid pacing (Panel A), an ectopic beat (asterisk) occurs near the endocardium (Panel B), followed by a delayed afterdepolarization (DAD) and a spontaneous calcium release (SCR). Furthermore, just preceding the ectopic beat, V_m and Ca levels at the endocardial site slowly increase (arrow) and have a similar initial time course and amplitude as the DAD and SCR following the ectopic beat. In contrast, there was no slow increase in V_m and Ca during baseline pacing (left) or at the epicardial site (right). We found that under conditions of calcium overload most episodes of triggered activity occurred closer to the endocardium (Panel C), where diastolic calcium levels were highest [67]. The underlying subcellular mechanisms that trigger calcium release from the SR under these conditions are unknown. Several mechanisms have been proposed; the most prominent being cytoplasmic calcium interaction with RyR [68,69] or SR (i.e. luminal) calcium interaction with RyR [30,32,70,71]. From these and other studies, it is fairly clear that

both cytoplasmic and SR calcium play an important role in triggering an SCR. The primary governing mechanism is, however, still unknown.

Using ratiometric imaging techniques, we were able to provide some of the first evidence of the spatial extent of SCR events in tissue. Shown in Figure 4 (Panel A) is a contour map of SCR amplitude in the absence of a triggered beat. Regions of largest calcium release are indicated by lighter shades of gray. Interestingly, we observed multiple simultaneous SCR events in a single recording. In the example shown, two significant release events occurred, one near the endocardium (site a) and a second in the midmyocardium (site c). Panel B shows calcium transients recorded from sites a, b, c and d. SCR amplitude and time to peak at site a (endocardium) were larger and earlier compared to b and c. No SCR event was observed at site d (epicardium). Another interesting finding was that each SCR event did not occur from a single cell but from a region of myocardium comprised of many cells, as indicated by the SCRs occurring over a relatively large area ($\sim 3 \times 3$ mm) [67]. This suggests that before an action potential is triggered, non-electrically driven calcium release occurs over many cells rather than just one. This is consistent with the notion that a SCRs and corresponding afterdepolarizations involving relatively large clusters of cells may be required to overcome electrotonic loading and initiate a propagated impulse [72]. Finally, it is these SCR events occurred in working myocytes rather than Purkinje fibers, indicating that DAD-mediated triggered activity can occur in a variety of cell types.

While calcium release from the SR is one source of SCR activity, another source can be from myofilaments. In an elegant series of studies from Dr. ter Keurs laboratory, it has demonstrated that nonuniform contraction resulting in local rapid relaxation, can cause calcium to be released from myofilaments [73,74]. This release of calcium can then cause release of calcium from nearby SR calcium release channels, similar to CICR. In this manner, calcium waves can propagate within the cell (triggered propagated contraction) in the absence of electrical excitation. It has been proposed that this mechanism of DAD activity may explain triggered arrhythmias associated with myocardial infarction and ischemic heart failure, where non-contracting tissue sits alongside contracting tissue at the ischemic border zone [73,75]. Moreover, these reports suggest an alternative mechanism of triggered activity that is not caused by dysregulation of SR calcium regulatory proteins or SR calcium overload. Several studies in Purkinje cell aggregates[76] and trabeculae[74] have demonstrated that calcium waves can even propagate from cell-to-cell in the absence of an action potential. Such calcium waves propagate at a much slower velocity (~ 5 mm/sec) compared to the action potential propagation (~ 500 mm/sec). Importantly, calcium released during such a wave can activate I_{Ca} , as described above, and cause DAD and triggered activity. This may be a mechanism by which calcium release can occur over a significantly large area (Figure 5) to overcome electrotonic forces that might otherwise suppress local oscillations of membrane potential (i.e. DAD).

Triggered Activity Associated with Abnormal RyR Function

Abnormal RyR function can also result in spontaneous release of calcium from the SR. For example, recent evidence suggests that some arrhythmias are caused by mutations in the human cardiac RyR (hRyR2), resulting in a “leaky” channel and, thus, spontaneous calcium releases from the SR [25,77]. Studies in isolated cells have shown that known mutations associated with catecholaminergic polymorphic ventricular tachycardia can enhance RyR open probability during diastole by enhancing sensitivity to SR luminal[78] or cytoplasmic[79] calcium levels and promote alterations in channel structural stability [80]. Similarly, mutations in the calsequestrin gene have been associated with CPVT[81] and impaired regulation of RyR [82,83]. Abnormal RyR function has been linked to glycolysis[84] and FKBP12.6 deficiencies. Normally, multiple RyR channels are functionally related and closely coupled (i.e. FKBP12.6-

mediated coupled gating) such that synchronous activation and inactivation of multiple RyR channels occurs [29]. Therefore, dissociation of the FKBP12.6 molecule will also cause RyR channels to gate independently (i.e. desynchronized release) and alter individual RyR channel gating such that the RyR leaks calcium during diastole. FKBP12.6-deficient mice have been shown to be prone to triggered arrhythmias [23]. Studies using Rapamycin, which is known to dissociate FKBP12.6 from RyR, have also demonstrated abnormal RyR function [29, 85-89]. Using optical mapping techniques to measure intracellular calcium and transmembrane potential simultaneously, we have shown recently that Rapamycin administered with isoproterenol can significantly increase the occurrence of SCR, DAD, and triggered activity in the canine ventricular wedge preparation [85]. The role of FKBP12.6-dissociation in triggering spontaneous calcium release and causing fatal arrhythmias is, however, not well understood and very controversial.

Triggered activity has also been associated with acquired RyR dysfunction associated with heart failure [76,90]. Pogwizd et al[10,91] have shown in humans and experimental models that arrhythmias associated with heart failure can be non-reentrant. Moreover, the cellular mechanisms responsible are primarily related to calcium mediated DADs [11,92]. Several studies have shown that the SR calcium release channel is leaky under heart failure conditions, [37,93,94] and FKBP12.6 seems to be down-regulated in heart failure and hypertrophy [93, 95-97]. In addition, Yano et al[13] have shown that inhibition of FKBP12.6 dissociation (via JTV519) can prevent abnormal calcium leak from the SR associated with heart failure. These studies suggest that leaky RyR may be a mechanism of arrhythmias in failing myocardium. However, the role of FKBP12.6 has been disputed[42,98] and there are a number of other possible mechanisms that may play an important role in the high incidence of non-reentrant rhythms in heart failure. For example, SR calcium reuptake is abnormally slow in heart failure, [4] which could lead to an accumulation of cytoplasmic calcium and, thus, calcium overload. In addition, I_{NaCa} , a main component of I_{ti} is greater in heart failure compared to normal hearts. Xiong et al[99] have shown in particular that I_{NaCa} is selectively up regulated near the endocardium in heart failure. As a result, larger DADs that are more capable of triggering beats are more likely in heart failure. Similarly, it is possible that ionic currents that normally stabilize resting membrane potential (i.e. I_{K1}) are weaker in heart failure compared to normal hearts [11,89]. As a result, I_{ti} would have a much greater effect on the amplitude and occurrence of DADs. Enhanced phosphorylation of RyR is another means by which calcium dysregulation is associated with heart failure. For example, it is possible that residual β -adrenergic responsiveness associated with heart failure will enhance SR calcium load despite the fact that SERCA2a is reduced in heart failure [11]. Finally, CaMKII has been shown to play an important role in phosphorylation of RyR and, hence, diastolic calcium leak [100]. The mechanisms of arrhythmogenesis in heart failure are complex and multifactorial; yet, intracellular calcium regulation seems to be at the core of the problem.

Arrhythmias Caused by Abnormal Impulse Conduction (Reentry)

The relationship between intracellular calcium regulation and reentrant arrhythmias is less straightforward as that for triggered activity. Our laboratory[101,102] and others[103-106] have shown that intracellular calcium handling plays an important mechanistic role in T-wave alternans, a subtle beat-to-beat alternation of the ECG T-wave, and a sensitive marker of susceptibility to SCD [107]. Visible T-wave alternans has been associated with ventricular arrhythmias in diverse experimental and clinical settings[108-110] raising speculation that cardiac alternans is a frequent precursor to cardiac arrhythmias [111,112]. We have recently described a novel mechanism linking beat-to-beat alternation of membrane voltage (Vm-ALT) (i.e. cellular repolarization) to reentrant arrhythmogenesis [113]. When Vm-ALT is first initiated, it occurs with identical phase in all cells of a particular region of ventricular myocardium (i.e. spatially concordant alternans). However, above a critical heart rate threshold

Vm-ALT switches phase in some cells but not others, such that some cells undergo a prolongation of APD while neighboring cells undergo APD shortening on the same beat (i.e. spatially discordant alternans) [113,114]. The transformation from spatially concordant to discordant alternans is directly responsible for the emergence of marked spatial dispersion of repolarization. Hence, spatially discordant alternans amplifies physiological heterogeneities of repolarization present at baseline into pathophysiological heterogeneities of sufficient magnitude to produce conduction block and reentrant excitation [113-115]. While spatially discordant Vm-ALT is important to the mechanism of arrhythmogenesis, equally important are the mechanisms that cause Vm-ALT to occur in the first place.

Intracellular Calcium Cycling as Mechanism of Repolarization Alternans

Although a variety of sarcolemmal currents can exhibit alternating-type activity including I_{to} , [116] I_{Ca} , [117] I_{Kr} , [118,118,119] few have been definitively shown to be mechanistically responsible for producing Vm-ALT. In contrast, there are convincing data for a primary role of intracellular calcium regulation (i.e. calcium cycling) in the mechanism of Vm-ALT [101, 104,105,105,106,120-124]. However, given the complex regulatory feedback between cytoplasmic calcium and sarcolemmal ion channels governing the action potential, deciphering the role of calcium cycling versus sarcolemmal currents in the mechanism of Vm-ALT is a challenging “chicken and egg” problem. However, the seminal observations of Chudin et al [103] that alternans of calcium transient amplitude (Ca-ALT) is similarly induced under current-clamp (where Vm-ALT occurs) and voltage-clamp (i.e. where Vm-ALT is prevented) conditions proved that Ca-ALT is not dependent on Vm-ALT, and strongly supported the notion that cellular alternans arises from calcium cycling.

Recently, we have demonstrated several independent lines of evidence supporting the primary role of calcium cycling in the mechanism of Vm-ALT [8,101,102]. Using dual calcium voltage optical mapping techniques we found that in the intact heart the development of Vm-ALT and Ca-ALT are very closely related [101]. Shown in Figure 5 (Panel A) are action potentials (Vm) and calcium transients (CaF) recorded simultaneously at increasing pacing rates. At baseline (150 bpm), no Vm-ALT or Ca-ALT was observed. As pacing rate increased (375 bpm), subtle Vm-ALT and Ca-ALT appeared simultaneously. An additional increase in pacing rate (461 bpm) resulted in pronounced Vm-ALT and Ca-ALT. Shown in Panel B is the averaged magnitude of APD-ALT and Ca-ALT as a function of pacing rate. In this representative example, the Ca-ALT and Vm-ALT threshold and the exponential increase in both Vm-ALT and Ca-ALT occurred in synchrony. The same is true during the development of spatially discordant Vm-ALT (data not shown). Interestingly, in the normal guinea pig heart, long APDs were always associated with large calcium transient amplitude and short APD with small calcium transient amplitude (i.e. concordant electrical and mechanical activity due to positive Ca-Vm coupling). Others have shown electrical mechanical discordance (short APD associated with large calcium transient) in ischemic hearts [125]. Nevertheless, our results demonstrate that in the normal heart the development of beat-to-beat Vm-ALT closely coincides with the development of Ca-ALT. In this same study, we also found that Vm-ALT could not be explained by restitution kinetics. The “restitution hypothesis” states that Vm-ALT occurs when the slope of the APD restitution curve is >1 , which has been taken as evidence that sarcolemmal rather than SR ion channels determine Vm-ALT [112,126-130]. We took advantage of a unique property of the guinea pig model of T-wave alternans, which includes an epicardial gradient of APD restitution, to test the hypothesis that myocytes with steepest restitution slope are most susceptible to Vm-ALT. To the contrary, we found that when compared to Vm-ALT resistant myocytes, Vm-ALT prone myocytes failed to exhibit steep restitution or prolonged APD [101]. Rather, we found that the onset of alternans (Ca-ALT and Vm-ALT) consistently occurs near the LV base, where calcium release is weakest (LV base) [131]. Finally, we have also found that Vm-ALT exhibits a hysteresis effect whereby alternans persists at heart rates below

those required to induce it. Importantly, we found that BAPTA-AM (intracellular calcium chelator) significantly reduced hysteresis, further supporting an important role of calcium cycling in the mechanism of alternans [102]. These data and that from other laboratories, strongly suggest that calcium cycling dictates cellular alternans. However, given the complex nature of calcium regulation in cardiac myocytes and depending on which of the multitude of disease models it occurs in (LQTS, ischemia, heart failure, etc), it is quite likely that the actual subcellular and molecular mechanisms for cardiac alternans vary.

To further investigate the mechanisms of Ca-ALT, we have examined the molecular profile of alternans prone and resistant cells [8]. Shown in Figure 6 is the protein expression of key calcium regulatory proteins in homogenates isolated from alternans susceptible (endocardium) and alternans resistant (epicardium) tissue. The alternans susceptible myocytes expresses significantly less SERCA2a and RyR compared to the alternans resistant myocytes, suggesting a molecular basis for cellular alternans, and specifically implicating calcium cycling proteins in this mechanism. Moreover, experiments performed in isolated myocytes subjected to pharmacological inhibition of either RyR or SERCA2a have demonstrated, at least conceptually, that dysfunction of either of these key calcium cycling proteins can cause Ca-ALT [8,132,133].

Alternans Associated with Impaired SR Calcium Release

Two important mechanisms have been proposed for Ca-ALT attributable to SR calcium release dysfunction. The debates between these mechanisms centers on whether Ca-ALT is associated with alternating SR calcium content or not [133]. Using metabolic inhibition in cat atrial and ventricular myocytes to inhibit RyR phosphorylation, Huser et al[133] reported Ca-ALT without beat to beat fluctuations in SR content, suggesting that refractory-like properties of RyR can produce alternating open probabilities of the channel irrespective of SR calcium load. Diaz, et al[132] used an innovative, albeit non-physiological, stimulation protocol to induce Ca-ALT without pharmacological inhibition of RyR (thereby avoiding non-selective drug effects). Ventricular myocytes were repetitively voltage-clamped below the activation voltage for I_{Ca} . The resulting weak CICR produced desynchronized RyR release, which dramatically steepened the relationship (feedback gain) between SR calcium content (i.e. luminal calcium) and the subsequent SR calcium release. These subcellular conditions highly favored the development of Ca-ALT dynamics, as a small transient increase in luminal SR calcium produced relatively large RyR release, leading to depletion of SR calcium, and a small calcium release on the subsequent beat, leading to restoration of SR calcium content, and a large calcium release, as the alternating pattern continues. Under these circumstances, Ca-ALT is associated with beat to beat alternation of SR calcium content. Shiferaw et al[134] have also shown using computer modeling that Ca-ALT is dependent on the steep, non-linear, relationship between calcium release and SR content. However, others have shown that Ca-ALT alternans does not depend solely on SR content. Picht et al[135] used direct continuous measurements of intra-SR free calcium to show that pacing induced Ca-ALT does not require SR calcium content alternans but, rather, may depend more on RyR recovery. Moreover, RyR dysfunction independent of SR calcium content may also be mechanism of Ca-ALT, such as that associated with local inhibition of glycolysis,[133,136] tetracaine or acidification,[137] and a leaky RyR [138].

Alternans Associated with Impaired SR Calcium Re-uptake

SR Ca^{++} ATPase maintains a calcium gradient between the SR matrix and the cytosol which is close to the theoretical limit based on the free energy available from ATP hydrolysis [139]. Therefore, one would predict that elevations in HR could easily overcome the capability of the SR Ca^{++} ATPase, leading to alternation in calcium reuptake as subpopulations of SR Ca^{++} ATPase fully respond on alternating beats only. When SR Ca^{++} ATPase is impaired, the amount

of released calcium can only be fully reclaimed on an alternating beat basis, giving rise to Ca-ALT. Shown in Figure 7 is an example of Ca-ALT and its dependence on the decay of the calcium transient in the canine left ventricular wedge preparation. Calcium transients measured near the epicardium (EPI) and endocardium (ENDO) during an abrupt decrease in pacing cycle length from 600 ms to 300 ms demonstrate a transitory increase in Ca-ALT near the endocardium and epicardium. At steady state (~4 seconds) Ca-ALT near the endocardium (85%) was much larger than that near the epicardium (15%). The reason why alternans occurred preferentially near the endocardium was likely due to the inability to restore calcium to resting diastolic levels upon rapid pacing, as evidenced by the slower decline of the calcium transient (i.e. larger τ) and greater accumulation of cytoplasmic calcium near the endocardium compared to the epicardium [124].

It is abundantly clear that heart failure is associated with significant blunting of SR calcium reuptake, caused by reduced SERCA2a expression [4,140-142]. Expression of the SR Ca^{++} ATPase regulatory protein PLB is also diminished [143-145]. heart failure causes decreased SR calcium content due to decreased SR Ca^{++} ATPase function and expression as well as increased NCX expression, both leading to less cytosolic calcium being taken up into the SR [94,145]. Upregulation of $\text{Na}^{+}\text{-Ca}^{++}$ exchanger is almost uniformly reported in human and animal models of heart failure [4,146]. In heart failure, increased $\text{Na}^{+}\text{-Ca}^{++}$ exchanger may be a compensatory response to decreased SERCA2a expression in order to extrude calcium and improve diastolic function [147]. In addition, faster stimulation rates result in decreased SR calcium uptake in heart failure vs. normal hearts, indicating a decreased ability for calcium cycling to keep up with increasing HR [148]. This, in addition to abnormalities of SR calcium release, may explain why alternans is increased in failing myocytes. Finally, it is possible that other changes associated with heart failure can secondarily impact calcium cycling and arrhythmogenesis. For example, a partial loss of T-tubules [149] and the disruption of their regular organization [150] is associated with heart failure. In either case, these abnormalities can cause local delays and desynchronized SR calcium release in ventricular cells, which has been associated with alternans [137].

Electrogenic Mechanisms of Vm-ALT—As with triggered activity, Ca-ALT would be irrelevant to arrhythmogenesis if it were not for the effect calcium has on transmembrane potential. As mentioned previously, there are several mechanisms by which calcium can influence membrane potential. The two most likely mechanisms are calcium inactivation of the L-type calcium channel (negative Ca-Vm coupling) and the enhancement of $\text{Na}^{+}\text{-Ca}^{++}$ exchanger (positive Ca-Vm coupling). I_{to} and I_{Ks} are Ca—dependent ion channels that can also modulate transmembrane potential in response to changing calcium, and these mechanisms are not mutually exclusive. Our experience indicates that alternating activity of $\text{Na}^{+}\text{-Ca}^{++}$ exchanger is the most likely electrogenic mechanism for Vm-ALT based on our dual calcium voltage optical mapping experiments, which have consistently revealed similar phase of Ca-ALT and Vm-ALT (i.e. positive Ca-Vm coupling) over a wide range of pacing rates. The opposite (i.e. negative Ca-Vm coupling) would be expected if I_{Ca} , I_{Ks} , or I_{to} , are the electrogenic mechanisms. Also, in atrial and ventricular myocytes L-type calcium current is constant under alternans conditions, [133] further implicating $\text{Na}^{+}\text{-Ca}^{++}$ exchanger. In disease, ion channel expression can be significantly altered such that the primary electrogenic mechanism is enhanced or switched to another ion channel. For example, since $\text{Na}^{+}\text{-Ca}^{++}$ exchanger is upregulated in heart failure, it is possible that there is also enhanced electrogenic “gain” or a change in the direction of coupling from positive to negative Ca-Vm coupling, providing an additional mechanism for enhanced susceptibility to Vm-ALT in heart failure. Interestingly, the balance of positive and negative Ca-Vm coupling could also influence the formation of spatially discordant alternans, an important substrate for reentrant excitation [151].

Summary and Clinical Implications

Understanding the mechanisms linking mechanical to electrical dysfunction in the heart will be critical to improving understanding of the complex sequence of events which trigger life-threatening ventricular arrhythmias in some patients, while not in others. Intracellular calcium dysregulation is likely to be an important link, through a broad range of arrhythmia mechanisms. As outlined in this review, abnormal calcium regulation can enhance RyR open probability, leading to spontaneous (i.e. non-electrically driven) calcium release and arrhythmias caused by abnormal impulse formation. Additionally, abnormal calcium regulation can cause calcium transient alternans, which is a key mechanism of repolarization alternans, a potent substrate for reentrant excitation. Therefore, our knowledge of calcium dysregulation at the cellular and molecular level is essential; however, relating such abnormalities to arrhythmias that occur in the intact heart is equally important.

Improving our basic understanding will lead to important developments in the identification and treatment of life threatening arrhythmias associated with mechanical dysfunction in heart disease. Abnormal intracellular calcium regulation at the level of the SR is a novel target with numerous points of attack. For example, Marks et al[138] and others[152] have suggested that restoring FKBP12.6 binding to RyR can reduce diastolic calcium leak from the SR. Similarly, calcium regulatory proteins on the luminal side of the SR (e.g. junctin and calsequestrin) as well as preventing SR calcium overload may also serve as therapeutic targets to prevent spontaneous calcium release. Arrhythmia risk may also be reduced by enhancing the abnormally slow rate of calcium cycling that has been previously associated with cardiac alternans and heart failure. For example, increasing the reuptake rate of calcium by the SR in heart failure may reduce alternans. This, however, may overload the SR and promote triggered activity. Obviously, the unintended effects of manipulating such novel targets need to be fully evaluated. Nevertheless, the number of potentially new targets is very promising. Moreover, unlike sarcolemmal calcium channel blockers, SR targets may have an added benefit that mechanical function will be improved or minimally impacted while reducing the risk of SCD associated with heart failure.

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Figure 1.

A simplified schematic of normal myocyte intracellular calcium cycling. Shown are the main proteins and their primary responsibility in the rapid increase of cytoplasm calcium (red) during systole and the subsequent decrease in cytoplasmic calcium levels (blue) during diastole.

During steady state conditions, intracellular calcium homeostasis is maintained by an equal amount of calcium entering and exiting the cytoplasm for each beat. Shown below for reference is the time course of transmembrane potential and cytoplasmic calcium levels during a single beat.

Figure 2.
Example of triggered activity and DADs in a Purkinje fiber during Digitalis administration. The arrow indicates the last paced beat, which is followed by two triggered (i.e. ectopic) beats and damped oscillations of membrane potential the represent DAD activity. Reproduced with permission.

Figure 3.

Triggered activity in the canine wedge preparation under conditions of enhanced calcium entry. Shown in Panel A are representative action potentials (V_m) and calcium transients (Ca) recorded during baseline pacing (600ms) from the endocardium (left) and rapid pacing (180ms) followed by an abrupt halt in pacing from the same endocardial site (middle) and an epicardial site (right). After cessation of rapid pacing, an ectopic beat is evident (asterisk). In contrast to the epicardium, a delayed-afterdepolarization (DAD) and a spontaneous calcium release (SCR) occurred at the endocardium that did not elicit an ectopic beat. The activation map of the ectopic beat (Panel B) shows its origin (asterisk) close to the endocardium yet away from the site of stimulation (stimulus symbol). Overall experiments (Panel C), triggered activity (TA) occurred more frequently at the endocardium compared to other regions ($p < 0.001$). Reproduced with permission.

Figure 4.

Multiple simultaneous SCR events in the canine wedge preparation under conditions of enhanced calcium entry. In Panel A, a representative transmurular map of SCR amplitude (SCR_{amp}) reveals 2 “hot spots” of high SCR amplitude near the endocardium (a) and midmyocardial region (c). Panel B shows the ECG and calcium transients recorded at a, b, c and d. SCR event at site a had an earlier occurrence and larger amplitude compared to b and c. Note that no SCR was observed at d (epicardium). Reproduced with permission.

Figure 5.

ECG, Vm, and CaF recordings at 3 different pacing rates (A). The number below each Vm and CaF recording represents APD (ms) and normalized CaF amplitude (%), respectively. The development of T-wave alternans was closely paralleled by APD-ALT and CaF-ALT. At 150 bpm (left), all signals were stable over time, and no alternans was observed. At 375 bpm (middle), subtle beat-to-beat alternans was observed, which was further increased at a pacing rate of 461 bpm. B, Representative experiment showing that the magnitude of APD-ALT and CaF-ALT increased as a function of pacing rate. Reproduced with permission.

Figure 6.

Molecular profile of alternans susceptible VS. resistant myocytes. Endocardial myocyte (left) undergoes significant Vm-ALT and Ca-ALT (black & orange traces) while under identical conditions the epicardial myocyte (right) does not. Western immunoblots show decreased RyR and SERCA2a in alternans susceptible myocytes. Reproduced with permission.

Figure 7.

ECG and calcium transients near the epicardium (EPI) and endocardium (ENDO) recorded during an abrupt increase in pacing cycle length from 600 to 300 msec (arrow). Near ENDO and EPI, calcium transient alternans was persistent following 4 seconds of rapid pacing. At ENDO, where the decay of the calcium transient was slower (174 ms) compared to EPI (93 ms), the magnitude of calcium transient alternans was greater. At ENDO, the calcium transient amplitude for beat a was 68% larger than that for beat b. In contrast, at EPI the degree of calcium transient alternans was less obvious (9%). Reproduced with permission.