Perspectives on: Local calcium signaling

Subcellular Ca\textsuperscript{2+} signaling in the heart: the role of ryanodine receptor sensitivity

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Local Ca\textsuperscript{2+} signaling in the heart enables the triggering and regulation of the time-dependent changes of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), to be flexibly modulated with a high degree of stability and safety. The elementary physiological and pathophysiological components of subcellular Ca\textsuperscript{2+} signaling are regulated through a process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR), not by store overload–induced Ca\textsuperscript{2+} release (SOICR). The central element of CICR regulation is the sensitivity of the SR Ca\textsuperscript{2+} release channel, the RYR type 2 (RYR2), to [Ca\textsuperscript{2+}].

Here, in terms of past discoveries and future work, how RYR2 sensitivity to [Ca\textsuperscript{2+}], is influenced by key factors, including SR luminal Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{SR}), RYR2 phosphorylation, mutations in RYR2 and its interacting proteins, as well as cellular stretch. Finally, we explore the pathophysiological consequences of dysfunctional tuning of these regulatory factors in the context of dystrophic cardiomyopathy.

The spatial organization of the cardiac ventricular myocyte enables precise regulation of the cardiac [Ca\textsuperscript{2+}], transient (Bers, 2001; Cheng and Lederer, 2008). The details of this regulation at the subcellular level, however, remain both controversial and exciting. There is consensus that the “global” or cell-wide [Ca\textsuperscript{2+}] signal in ventricular myocytes is central to cardiac function; it underlies contraction and contributes to the regulation of electrical activity. Despite the certainty and clarity of these contributions, the subcellular details of Ca\textsuperscript{2+} signaling remain unsettled. How do subcellular organelles contribute to Ca\textsuperscript{2+} sparks? Although it is clear that sparks originate almost exclusively at the junctional SR (jSR), many aspects of Ca\textsuperscript{2+} spark signaling are still being investigated. For example, do mitochondria serve as dynamic Ca\textsuperscript{2+} stores? What “triggers” spontaneous Ca\textsuperscript{2+} sparks versus synchronized sparks? How does a propagating Ca\textsuperscript{2+} wave arise? What sustains the propagating wave of elevated [Ca\textsuperscript{2+}]? Does the cardiac cytoskeleton play both organizational and dynamic roles in the regulation of Ca\textsuperscript{2+} sparks? How does SR Ca\textsuperscript{2+} instability arise, and how does it lead to the generation of cardiac arrhythmias? How can mathematical modeling contribute to such investigations? How does Ca\textsuperscript{2+} signaling dysfunction influence or underlie disease progression? We present here some organizing principles that should help to resolve several issues and lay the foundation for future studies.

Spatial organization of the heart cell and the control of Ca\textsuperscript{2+} sparks

Subcellular anatomy: key to Ca\textsuperscript{2+} signaling stability. The SR includes two primary components, the jSR and the longitudinal SR (also called the free SR or the “network” SR; see Fig. 1) (Brochet et al., 2005). In addition, the SR is connected to the ER and the nuclear Ca\textsuperscript{2+} store (Wu and Bers, 2006), thus making the entire Ca\textsuperscript{2+} storage network fully interconnected. The primary Ca\textsuperscript{2+} release sites are located at the jSR, an extremely small pancake-shaped sub-volume in the system (each jSR contains \(\sim 1\) attoliter, \(\sim 1 \times 10^{-18}\) l, a size \(\sim 4,000\) times smaller than a Ca\textsuperscript{2+} spark). On one face, the jSR contains a paracrystalline cluster of 10–300 SR Ca\textsuperscript{2+} release channels, the RYR2s that span 15 nm (the “subspace”) to the surface membrane (i.e., the transverse tubule [TT] or surface sarcolemmal [SL] membranes) (Frazinzi-Armstrong et al., 1999; Baddeley et al., 2009). The other face of the jSR points toward the M line and is tens of nanometers away from the Z-disk end of the mitochondria and is connected through the thin nanoscopic tubules that constitute the longitudinal SR. The L-type Ca\textsuperscript{2+} channels (or dihydropyridine receptors) are transmembrane proteins that span the SL and TT membranes facing the subspace and thus appose the jSR. The lumen of the jSR also contains the Ca\textsuperscript{2+}-binding protein calsequestrin type 2 (CASQ2) and other regulatory proteins, such as junctin, triadin, junctophilin, and a small amount of...
other proteins including the ER Ca\(^{2+}\)-binding and chaperone protein calreticulin (Bers, 1991; Györke et al., 2007). The brief opening of either an L-type Ca\(^{2+}\) channel or an RYR2 under diastolic conditions leads to a local elevation of Ca\(^{2+}\) in the subspace ([Ca\(^{2+}\)\(_{\text{subspace}}\)] from the normal [Ca\(^{2+}\)] of 100 nM to \(~10\) µM (Cannell and Soeller, 1997; Soeller and Cannell, 1997; Sobie et al., 2002). This elevation of [Ca\(^{2+}\)\(_{\text{subspace}}\)] is sufficient to activate the RYR2 cluster to produce a Ca\(^{2+}\) spark, a process termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release, or CICR (Fabriato, 1983; Cheng et al., 1993; Cannell et al., 1994a,b; Sobie et al., 2002; Cheng and Lederer, 2008; Györke and Terentiev, 2008; Liu et al., 2010). The spatial organization of the jSR enables reliable activation of Ca\(^{2+}\) sparks by action potentials, but the relative insensitivity of RYR2s to Ca\(^{2+}\) protects the cell from instability and enables Ca\(^{2+}\) sparks during diastole to remain isolated from neighboring jSR spark sites. Thus, Ca\(^{2+}\) sparks do not normally activate other Ca\(^{2+}\) sparks (Cheng et al., 1993). High gain in the signaling pathway is thus created by the spatial organization of RYR2s in a cluster, and stability is maintained by the relative insensitivity of the RYR2s to [Ca\(^{2+}\)], (Cheng and Lederer, 2008).

Ca\(^{2+}\) sparks, Ca\(^{2+}\) blinks, and SR Ca\(^{2+}\) leak. The “life cycle” of a Ca\(^{2+}\) spark provides clues to its regulation. Should [Ca\(^{2+}\)\(_{\text{subspace}}\)] increase to \(~10\) µM, there is good probability that a Ca\(^{2+}\) spark will arise through CICR as noted above. Although Ca\(^{2+}\) spark termination is seen robustly in experiments, our understanding of how this occurs was more challenging. Mathematical modeling of spark behavior suggests that if a significant fraction of the local jSR Ca\(^{2+}\) is depleted during the Ca\(^{2+}\) spark (between 50 and 90%), a robust termination of the spark will be seen (Sobie et al., 2002). Importantly, this occurs without the need for “inactivation” of the RYR2s, unlike in other models (Stern et al., 1999). In support of this model, there is no experimental evidence to date that suggests rapid “fateful” inactivation of RYR2s (Liu et al., 2010). The [Ca\(^{2+}\)\(_{\text{SR}}\)] depletion hypothesis regarding Ca\(^{2+}\) spark termination has been supported by the observation of Ca\(^{2+}\) “blinks” (Brochet et al., 2005). A Ca\(^{2+}\) blink is the [Ca\(^{2+}\)\(_{\text{SR}}\)] depletion signal that occurs when a Ca\(^{2+}\) spark takes place. It is the reciprocal signal of a Ca\(^{2+}\) spark; specifically, [Ca\(^{2+}\)\(_{\text{SR}}\)] depletion is seen whenever sparks occur because the Ca\(^{2+}\) flux that underlies the Ca\(^{2+}\) spark comes from the jSR lumen. SR Ca\(^{2+}\) leak is the term of art now used to describe the non-synchronized loss of Ca\(^{2+}\) from the SR, which may occur as Ca\(^{2+}\) sparks or as very small release events that may be invisible when viewed with a confocal microscope (Sobie et al., 2006). How Ca\(^{2+}\) leak may occur, and how this leak may influence Ca\(^{2+}\) instability and cardiac arrhythmogenesis, is actively under examination (Wehrens et al., 2003; Lehnart et al., 2006, 2008; Sobie et al., 2006).

**Triggering Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves.** A Ca\(^{2+}\) spark is the local Ca\(^{2+}\) signal produced by an ensemble of RYR2s at a single jSR (Cheng and Lederer, 2008). Diastolic sparks can occur when a single RYR2 opens probabilistically.
and increases $[\text{Ca}^{2+}]_{\text{subspace}}$ to a high level (i.e., $\sim 10 \mu M$), activating the other RYR2s facing the same subspace and in the same jSR. In a similar manner, the opening of an L-type $\text{Ca}^{2+}$ channel at near-diastolic potentials will also increase $[\text{Ca}^{2+}]_{\text{subspace}}$ and activate a $\text{Ca}^{2+}$ spark (Cannell et al., 1995; Cheng et al., 1996; Santana et al., 1996). More recently (Jiang et al., 2004), an alternative hypothesis has been put forward suggesting that sparks are triggered by “store overload–induced $\text{Ca}^{2+}$ release,” or SOICR. Although we have not identified experimental evidence that supports the SOICR hypothesis and distinguishes it from CICR, there is strong evidence suggesting that $[\text{Ca}^{2+}]_{\text{SR}}$ regulates RYR2 $[\text{Ca}^{2+}]$, sensitivity. $[\text{Ca}^{2+}]_{\text{SR}}$ is certainly an integral feature of CICR because it influences the sensitivity of the RYR2 to be opened by $[\text{Ca}^{2+}]$. (Györke and Györke, 1998). The influence exerted by $[\text{Ca}^{2+}]_{\text{SR}}$ represents one factor, along with several others such as phosphorylation of the RYR2s, nitrosylation, oxidation, and channel mutations (Wehrens et al., 2003, 2004, 2005; Lehnart et al., 2004; Viatuchenko-Karpinski et al., 2004; Kubalova et al., 2005; Terentyev et al., 2003, 2005, 2006; di Barletta et al., 2006; Guo et al., 2006; Györke and Terentyev, 2008), which all can increase the voltage-independent probabilistic openings of RYR2s (Table I and Fig. 1 C). The distinction between the SOICR hypothesis and the CICR hypothesis is important to make. Both support $[\text{Ca}^{2+}]_{\text{SR}}$ influencing $\text{Ca}^{2+}$ sparks. SOICR, however, suggests that SR $\text{Ca}^{2+}$ overload triggers depolarization-independent $\text{Ca}^{2+}$ sparks (Jiang et al., 2004, 2007, 2008; Jones et al., 2008), underlies $\text{Ca}^{2+}$ waves, and is somehow distinctive from the probabilistic opening of RYR2. Just as the opening of L-type $\text{Ca}^{2+}$ channels apposing the jSR “triggers” $\text{Ca}^{2+}$ sparks by CICR, so does the probabilistic opening of RYR2s in a jSR cluster. Importantly, when a spark initiates a propagating $\text{Ca}^{2+}$ wave or contributes to the conduction of a wave, it is CICR that links the elevation of local $[\text{Ca}^{2+}]$, during the spark produced by one jSR to the triggering of a $\text{Ca}^{2+}$ spark in the next jSR. For $\text{Ca}^{2+}$ waves to arise, it has been hypothesized that an increase in RYR2 sensitivity to $[\text{Ca}^{2+}]$, occurs due to the underlying pathologies, which include SR $\text{Ca}^{2+}$ overload and catecholaminergic polymorphic ventricular tachycardia (CPVT) mutations of RYR2 and CASQ2 (Marks et al., 2002; Eldar et al., 2003; di Barletta et al., 2006; Györke, 2009). By this means, the $\text{Ca}^{2+}$ released by a spark diffuses and elevates the $[\text{Ca}^{2+}]$, in the subspace of a neighboring RYR2 cluster and triggers a spark at that site. This chain reaction can continue to produce the arrhythmogenic $\text{Ca}^{2+}$ wave that activates the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger and produces the arrhythmogenic transient inward current, $I_{\text{Tl}}$ (Györke et al., 2007). An increase in the sensitivity of the RYR2s to $[\text{Ca}^{2+}]$, is sufficient to enable this sequence. Although elevated $[\text{Ca}^{2+}]$, and $[\text{Ca}^{2+}]_{\text{SR}}$ are the simplest ways to enable this sequence, changes in RYR2 sensitivity by mutations to its primary sequence or alterations in modulatory proteins (e.g., CASQ2, calstabin2, and others) may also be sufficient. That increases in $[\text{Ca}^{2+}]_{\text{SR}}$ may increase RYR2 opening rate is a part of CICR that has been well articulated in the literature as noted above. It would thus seem that the CICR hypothesis can fully account for the triggering of $\text{Ca}^{2+}$ sparks and $\text{Ca}^{2+}$ waves in health and disease.

**Mitochondrial influences on $\text{Ca}^{2+}$ sparks and the $[\text{Ca}^{2+}]$, transient.** To the extent that the mitochondria may buffer $\text{Ca}^{2+}$ or actively take up and release $\text{Ca}^{2+}$, they may significantly affect $[\text{Ca}^{2+}]$, dynamics (Xu et al., 2002; Hidalgo et al., 2002; Terentyev et al., 2008).

### Table 1

**Changes in the sensitivity of RYR2 to be opened by $[\text{Ca}^{2+}]$,**

| A—Increase in RYR2 sensitivity | B—Decrease in RYR2 sensitivity |
|--------------------------------|--------------------------------|
| Increased $[\text{Ca}^{2+}]_{\text{SR}}$ | Decreased $[\text{Ca}^{2+}]_{\text{SR}}$ |
| (Lukyanenko et al., 1996; Györke and Györke, 1998; Györke et al., 2002) | (Lukyanenko et al., 1996; Györke and Györke, 1998; Györke et al., 2002) |
| RYR2 phosphorylation at PKA or CaMKII sites | Calstabin2 (FKBP 12.6) binding |
| (Valdivia et al., 1995; Li et al., 1998; Marx et al., 2000) | (Marx et al., 2000; Wehrens et al., 2003) |
| Caffeine | Tetracaine |
| (Rousseau and Meissner, 1989; O’Neill and Eisner, 1990; Venetucci et al., 2007) | (Xu et al., 1993; Overend et al., 1997) |
| RYR2 oxidation (ROS) | Anti-oxidation |
| (Hidalgo et al., 2002; Terentyev et al., 2008) | (Hidalgo et al., 2002; Terentyev et al., 2008) |
| RYR2 CPVT mutations | |
| (Marks et al., 2002; Wehrens et al., 2003) | |
| Calsequestrin mutations | |
| (Postma et al., 2002; Eldar et al., 2003; di Barletta et al., 2006; Knollmann et al., 2006) | |
| Triadin | |
| (Valdivia et al., 1995; Li et al., 1998; Marx et al., 2000; Györke and Terentyev, 2008; Chopra et al., 2009) | |
| RYR2 nitrosylation | |
Regulation of local Ca$^{2+}$ signaling in the heart

Dedkova and Blatter, 2008; Liu and O’Rourke, 2008; Andrienko et al., 2009; Kettlewell et al., 2009; O’Rourke and Blatter, 2009). The scale of this influence is potentially large because the mitochondria take up approximately one third of the intracellular volume of cardiac myocytes. The largest subset of cardiomyocyte mitochondria are the intermyofibrillar mitochondria that run from the jSR at one Z disk through the M-line structures to the jSR at the next Z disk. Each end of these sarcomere-spanning mitochondrial experiences Ca$^{2+}$ sparks near their source (both ends) and thus may influence the magnitude and kinetics of local [Ca$^{2+}$], at their ends and global [Ca$^{2+}$], changes in the middle. Although the potential for mitochondria to influence [Ca$^{2+}$], is enormous, their actual roles in modulating Ca$^{2+}$ signals are debated. Experimental evidence for a substantive role (Liu and O’Rourke, 2008, 2009), as well as a minimal role (Andrienko et al., 2009; Kettlewell et al., 2009), has been put forth. Cellular and molecular tools for these investigations are limited because even the primary sequence of virtually every functional structure (channel and transporters) that may influence mitochondrial Ca$^{2+}$ dynamics remains elusive, despite significant effort. Recently, one of the key transporters was identified, the mitochondrial Na$^+/Ca^{2+}$ exchanger (Palty et al., 2010), which may provide a fundamental tool that is needed to jump-start the investigation and help to resolve the dynamic role that mitochondria may play in cardiac Ca$^{2+}$ signaling. Cellular stretch, local Ca$^{2+}$ signaling dysfunction, and muscular dystrophies

**Stretch-dependent Ca$^{2+}$ sparks.** The recent discovery that physiological stretch increases the transient occurrence of Ca$^{2+}$ sparks (Iribe et al., 2009) reveals an important line of inquiry that can now be investigated for the first time. These findings are important in reexamination of normal cardiac Ca$^{2+}$ signaling as well as in the investigation of the pathophysiology of diverse diseases that appear to be linked to cellular contraction and shortening. Iribe et al. (2009) report that a stretch of 8% of the cellular diastolic length leads to a transient increase in the Ca$^{2+}$ spark rate, which can be blunted specifically by microtubule disruption, but not by inhibitors of L-type Ca$^{2+}$ channel flux, Na$^+$/Ca$^{2+}$ exchanger flux, stretch-activated channels, nor NO synthase. Although there was earlier evidence for spatial association of microtubules with the SR and mitochondria (Cartwright and Goldstein, 1985), no functional or contact evidence was provided. In contrast, Iribe and colleagues added to the functional connection noted above with EM tomography, demonstrating co-location of microtubules and the jSR. They thus provide strong evidence for a mechanism that can provide a stretch signal to the jSR. If such a signal were provided to the SR, it could affect all of the RyR2s in the jSR because they are organized in a paracrystalline array. These findings lead to the hypothesis that stretching the ventricular myocyte applies a distortion to the diastolic jSR and thereby increases the sensitivity of RyR2s in the jSR to [Ca$^{2+}$], (Iribe et al., 2009). After the stretch is a burst of Ca$^{2+}$ sparks that relax over seconds. This is the time scale needed for cellular contraction and shortening to influence local Ca$^{2+}$ signaling and the cardiac [Ca$^{2+}$], transient. Note too that such stretch-dependent changes in the open probability of the RyR2 should have analogous effects during all phases of cardiac Ca$^{2+}$ signaling, including during systole. These exciting and provocative results, still in need of much further investigation, provide us with a lens through which we can view one of the key “stretch-dependent” cardiac diseases, muscular dystrophy. We suspect that there may be important connections between specific kinds of muscular dystrophy and stretch-modulated Ca$^{2+}$ signaling because four interrelated components are involved in dystrophic cardiomyopathy: (1) Ca$^{2+}$ entry, (2) Ca$^{2+}$ release, (3) stretch-dependent modulation, and (4) molecular defects associated with some dystrophies.

**Duchenne muscular dystrophy (DMD).** DMD, the most common of the muscle dystrophies (1:3,500 males), is a fatal, X-linked disease characterized by progressive muscle weakness that most commonly leads to respiratory failure.
Dysregulated Ca$^{2+}$ signaling in dystrophic hearts. Dystrophic myocytes reveal elevated Ca$^{2+}$ spark activity and the appearance of Ca$^{2+}$ waves in response to mechanical stress (Yasuda et al., 2005; Jung et al., 2008; Fanchaouy et al., 2009). Of note, among the compensatory adaptations in the mdx heart is an ∼1.4-fold increase in β-tubulin (Wilding et al., 2005). Based on this cytoskeletal adaptation in conjunction with the findings of Iribe et al. (2009), it is attractive to ascribe altered mechano-transduction (via microtubules) to aberrant Ca$^{2+}$ signals in the DMD heart. In support of this notion, Prins et al. (2009) have discussed dystrophin as a giant “cytolinker” protein, one that stabilizes structures through the ability to link to actin filaments as well as to microtubules.

Work by Yasuda et al. (2005) suggests that the stretch-induced aberrant Ca$^{2+}$ signals in mdx myocytes depends on Ca$^{2+}$ (and possibly Na$^+$) entry. Use of the surfactant and putative “membrane sealant” poloxamer 188 (with support from experiments with the extracellular dye FM-143) has led to the hypothesis that “micro-ruptures” in the SL membrane may underlie Ca$^{2+}$ entry in stressed mdx myocytes (Yasuda et al., 2005; Fanchaouy et al., 2009). Other reports have linked stretch-activated channels to enhanced Ca$^{2+}$ influx in the dystrophic heart (Williams and Allen, 2007a). These channels have been shown to be overexpressed in the DMD heart, and their inhibition blunts the aberrant local Ca$^{2+}$ activity associated with stress (Fanchaouy et al., 2009).

Other mechanisms have been reported that could indirectly affect mechano-transduction pathways of altered SR Ca$^{2+}$ release. The dystrophic heart produces more reactive oxygen species (ROS) (Williams and Allen, 2007b), and mdx myocytes exhibit aggravated ROS production in response to osmotic shock (Jung et al., 2008; Fanchaouy et al., 2009), a commonly used, albeit non-physiological form of mechanical stress. Mechanically, oxidation sensitizes RYRs to Ca$^{2+}$ (Pessah et al., 2002), making excessive ROS production and RYR2 sensitization an appealing explanation for a greater degree of Ca$^{2+}$ instability in the DMD heart. Supporting this hypothesis is the apparent ability of ROS scavengers to protect mdx cells from the Ca$^{2+}$ signaling consequences of mechanical stress (Jung et al., 2008; Ullrich et al., 2009) and the up-regulation of NADPH oxidase in the DMD heart (Williams and Allen, 2007b). Furthermore, several additional pathways have been linked to dystrophic changes in skeletal muscle, such as RYR nitrosylation and RYR hyperphosphorylation (Bellinger et al., 2009). These pathways could also serve to increase the sensitivity of RYR2 in the DMD heart to [Ca$^{2+}$]. If so, this could result in an increase in the mechano-transduction–induced gain of CICR. Clearly, these provocative hypotheses must be tested experimentally.

Conclusions

The regulation of local Ca$^{2+}$ signaling is critically dependent on the sensitivity of RYR2 to [Ca$^{2+}$], and the subcellular anatomy of the heart cell. Physiological and pathophysiologic changes in local cardiac Ca$^{2+}$ signaling may depend on altered sensitivity of the RYR2s to [Ca$^{2+}$]. These critical signaling changes arise from many factors that influence the open probability of RYR2, including [Ca$^{2+}$], mutations of RYR2 itself, mutations of RYR2-linked proteins, RYR2 protein phosphorylation and nitrosylation, and other molecular and cellular features. Recently, cellular stretch and the activation of mechano-transduction pathways have been shown to contribute to this process. Quantitative molecular and biophysical investigations of the processes that regulate RYR2 behavior are needed to further elucidate cardiac Ca$^{2+}$ signaling in health and disease.

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