Overview of the cardiac contractile cycle

Each contractile cycle in the heart, each heartbeat, requires an efflux of Ca\(^{2+}\) from the intracellular sarcoplasmic reticulum (SR) Ca\(^{2+}\) store through cardiac ryanodine receptor (RyR2) Ca\(^{2+}\) release channels. This release is followed by the reuptake of Ca\(^{2+}\) into the SR by the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) Ca\(^{2+}\) pump. The Ca\(^{2+}\) cycle is initiated by action potential depolarization, which allows Ca\(^{2+}\) entry into the cardiac myocyte from extracellular sources through L-type Ca\(^{2+}\) channels in the surface/transverse tubule membrane. The Ca\(^{2+}\) binds to Ca\(^{2+}\) activation sites located on the very large cytoplasmic domain of RyR2 and produces a massive increase in the open probability of this ligand-gated ion channel in the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Ca\(^{2+}\) flows through the open ion channel, down its concentration gradient, from the lumen of the SR into the cytoplasm where it binds to troponin C and activates the contractile machinery. After action potential repolarization, the myocardium relaxes as cytoplasmic Ca\(^{2+}\) falls. This is a net effect of a decline in the open probability of the RyR2 to very low levels, and Ca\(^{2+}\) is pumped back into the SR by SERCA, with a small amount of Ca\(^{2+}\) extrusion from the cell through the surface membrane Na\(^{+}\)–Ca\(^{2+}\) exchanger (NCX). Ca\(^{2+}\) enters the myocyte through NCX during the action potential depolarization and is extruded through NCX when the membrane potential is repolarized. Ca\(^{2+}\) movement through NCX contributes minimally to changes in cytoplasmic Ca\(^{2+}\) in normal muscle but can become dominant during heart failure. The Ca\(^{2+}\) cycle is generally divided into two periods: systole, when the ventricular myocardium contracts and ejects the contents of the ventricle, and diastole, where the ventricular muscle relaxes and the chamber refills. The systolic period encompasses surface depolarization and repolarization, Ca\(^{2+}\) entry through the L-type Ca\(^{2+}\) channels, release from the SR, and influx through NCX. During diastole, the membrane potential remains repolarized, and Ca\(^{2+}\) is taken back into the SR to be available for the next release phase and is extruded through NCX.

The pivotal role of RyR2 open probability in distole

The open probability of RyR2 must be high during systole for maximal Ca\(^{2+}\) release and very low during diastole for efficient Ca\(^{2+}\) accumulation in the SR. Even a small increase in open probability during diastole, leading to a “leak” of Ca\(^{2+}\) into the cytoplasm, can have major consequences for normal cardiac function. The coordinated activation of several RyRs in a cluster generates a local increase in cytoplasmic Ca\(^{2+}\) concentration that, when detected by a Ca\(^{2+}\) indicator, is seen as a “Ca\(^{2+}\) spark.” When RyR2 open probability is higher than normal during diastole, the frequency of sparks increases and each can in turn activate neighboring RyR2 clusters to produce a Ca\(^{2+}\) wave that is propagated throughout the cell (Bers, 2008). Ca\(^{2+}\) waves can increase the cytoplasmic Ca\(^{2+}\) concentration above normal diastolic levels, activating NCX and other surface membrane ion channels, leading to the generation of delayed afterdepolarizations (DADs) and certain types of early afterdepolarizations. These afterdepolarizations trigger uncoordinated action potentials and arrhythmia. Furthermore, because Ca\(^{2+}\) diffuses out of the SR at the same time as it is being accumulated by SERCA, the SR does not load to its optimal diastolic capacity and less Ca\(^{2+}\) is available for release during the subsequent systole.

The open probability of RyR2 during diastole depends on its interactions with many associated proteins and other intracellular factors, including Ca\(^{2+}\), Mg\(^{2+}\), and ATP, as well as modifications by phosphorylation and redox reactions, each of which either increases or decreases the probability of channel opening. The majority of the ligand-binding sites are on the massive cytoplasmic domain of the homotetrameric RyR2 channel, which is formed by ~80% of the mass of the protein. It was assumed that the activity of the ion channel is determined by the regulatory sites located on the cytoplasmic domain of RyR2. The very tiny luminal domain (<3% of the protein; Dulhunty et al., 2012) was thought to exist only to provide luminal linkers between the

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Regulation and dysregulation of cardiac ryanodine receptor (RyR2) open probability during diastole in health and disease

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transmembrane helices that form the ion channel. This idea was challenged by early findings that the Ca\(^{2+}\) concentration in the lumen of the SR can influence RyR2 channel activity, arguably by binding to residues on the luminal domain of the protein (Sitsapesan and Williams, 1997; Laver, 2007). In artificial lipid bilayer experiments, RyR2 channel activity increases when luminal Ca\(^{2+}\) concentration is increased from zero to amounts between 1 and 50 mM, as luminal Ca\(^{2+}\) binds to a luminal Ca\(^{2+}\) activation site (Laver, 2007; Altschafl et al., 2011; Dulhunty et al., 2012; Tencerová et al., 2012). Although observations of channel activity over this huge range provide an important biophysical profile of the channel, it is worth keeping in mind that changes in luminal Ca\(^{2+}\) concentration during the normal Ca\(^{2+}\) cycle are thought to be between \(~0.3\) mM during systole and up to \(1.5\) mM during diastole (Shannon et al., 2003). RyR2 channel activity does indeed increase steeply as luminal Ca\(^{2+}\) concentration rises within this much narrower physiological concentration range.

**Molecular factors in the SR that affect RyR2 open probability**

Several factors in the lumen of the SR, in addition to Ca\(^{2+}\) concentration, have a profound effect on RyR2 channel activity. Such factors include the cardiac isoform of the Ca\(^{2+}\)-binding protein calsequestrin (CSQ2), the histidine-rich Ca\(^{2+}\)-binding protein (HRC), as well as the triadin and junctin proteins. CSQ2 and HRC are located entirely within the SR lumen. Triadin and junctin are inserted into the SR membrane, with short cytoplasmic N-terminal domains and the bulk of each protein located inside the lumen of the SR. Triadin and junctin modify RyR2 channel activity when they bind to sites on the luminal loops of the channel and then bind to CSQ2 to further influence RyR2 activity (Dulhunty et al., 2012). The location of the luminal Ca\(^{2+}\) sensors on the RyR and associated luminal proteins is the subject of current research. It is thought that there are Ca\(^{2+}\)-sensitive sites on the luminal loops and transmembrane residues of RyR2 that are accessible to the luminal solution, because purified RyR2 channels respond to changes in luminal Ca\(^{2+}\) concentration, under conditions where Ca\(^{2+}\) passing through the pore has minimal impact on cytoplasmic Ca\(^{2+}\)-binding sites. The purified RyR2 protein lacks triadin, junctin, CSQ2, and other potential Ca\(^{2+}\) sensors and therefore must contain an intrinsic luminal Ca\(^{2+}\) sensor. However, the response of RyR2 to changes in luminal Ca\(^{2+}\) concentration is modified in the presence of CSQ2, junctin, triadin, and possibly the HRC in a Ca\(^{2+}\) concentration-dependent manner (Wei et al., 2009b; Altschafl et al., 2011; Dulhunty et al., 2012). Therefore, it appears that there are intrinsic luminal Ca\(^{2+}\) sensors on RyR2 but that the response of the protein to luminal Ca\(^{2+}\) is fine-tuned by its interactions with other Ca\(^{2+}\) sensors within the SR.

**SR factors affecting RyR2 open probability are critical to the normal cardiac rhythm**

CSQ2 is anti-arrhythmogenic in that CSQ2 knockout or mutation leads to arrhythmia and CSQ2 dampens the increase in RyR2 channel activity as luminal Ca\(^{2+}\) increases within the range experienced during diastole in lipid bilayer experiments (Dulhunty et al., 2012; Faggioni and Knollmann, 2012). Minimal Ca\(^{2+}\) leak into the cytoplasm through RyR2 during diastole ensures that the cytoplasmic Ca\(^{2+}\) concentration remains low and the likelihood of arrhythmia is reduced. Junctin is similarly anti-arrhythmogenic, as its deletion increases arrhythmia in animal models. In addition, the open probability of RyR2 channels in lipid bilayers increases more steeply as luminal Ca\(^{2+}\) increases in the absence of junctin (Altschafl et al., 2011), perhaps because CSQ2 can no longer modulate RyR2 through binding to junctin. This remains to be tested. Similarly, triadin deletion causes arrhythmia in addition to multiple changes in myocyte structure and excitation–contraction coupling (Faggioni and Knollmann, 2012; Roux-Buisson et al., 2012). These observations are recent and underline the fact that the very small luminal domain of the RyR2 has a critical role in facilitating the regulation of RyR2 ion channel activity. This is perhaps not surprising given the proximity of the luminal loops to the mouth of the ion channel pore and the ion selectivity filter.

The physiological importance of interactions within the lumen of the SR in maintaining normal cardiac function is indicated by the effects of the numerous (now exceeding 150), often point, mutations in RyR2 (Jiang et al., 2005). These mutations are associated with either catecholaminergic polymorphic ventricular tachycardia (CPVT) or with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2). With CPVT, arrhythmia is induced by catecholamines, either applied in a clinical setting or produced after \(\beta\)-adrenergic stimulation that occurs with exercise or stress. ARVD2 has a chronic phenotype associated with degeneration of the right ventricular myocardium and arrhythmia. A model of “store overload-induced Ca\(^{2+}\) release” (SOICR) was developed to explain both luminal Ca\(^{2+}\) activation of RyR2 and the disastrous effects of many of the RyR2 mutations linked to arrhythmias (Jiang et al., 2005). In essence, the model predicts that spontaneous Ca\(^{2+}\) release occurs when the luminal Ca\(^{2+}\) concentration increases above the threshold for RyR2 activation. SOICR occurs either (a) if the RyR2 threshold remains constant but store Ca\(^{2+}\) concentration increases above normal levels (Kashimura et al., 2010), or (b) if the threshold for RyR2 activation is reduced to lower than normal luminal Ca\(^{2+}\) concentrations. Several of the arrhythmogenic mutations produce RyR2 channels with enhanced sensitivity to luminal Ca\(^{2+}\) activation, causing an increase in spontaneous Ca\(^{2+}\) release and Ca\(^{2+}\) oscillations that could induce DADs, tachycardia, and sudden
death (Jiang et al., 2005). In addition, it is notable that in most animal models of heart failure, there is similar increased sensitivity of RyR2 to luminal SR Ca\textsuperscript{2+}, coupled with reduced store load, as assessed by the amplitude of the caffeine-induced Ca\textsuperscript{2+} transient (Eschenhagen, 2010). Reduced SR Ca\textsuperscript{2+} load during diastole is not unexpected with the more “leaky” RyR2 phenotype that results from enhanced sensitivity to changes in luminal Ca\textsuperscript{2+} associated with CPVT, ARVD2, and heart failure.

The role of interactions within the SR lumen in maintaining normal cardiac myocyte function is emphasized by the fact that in addition to the RyR2 mutations, mutations in CASQ2, triadin, and junctin, or changes in expression levels of the proteins, can cause arrhythmia and are associated with altered SR Ca\textsuperscript{2+} handling in heart failure. CPVT occurs with inherited mutations in CASQ2 (Faggioni and Knollmann, 2012) or with the absence of triadin (Roux-Buisson et al., 2012). Changes in the levels of triadin or junctin expression lead to increased arrhythmia in a variety of animal models (Fan et al., 2008; Roux-Buisson et al., 2012). Finally, triadin and junctin are down-regulated in human heart failure, whereas triadin and junctin knockout in mouse models are associated with increased susceptibility to ischaemia reperfusion injury (Cai et al., 2012). It is hard to escape the conclusion that normal protein–protein interactions within the lumen of the SR are essential for the precise regulation of RyR2 during diastole that maintains and fine-tunes the Ca\textsuperscript{2+} release processes essential for normal cardiac function.

Other factors affecting RyR2 function and dysfunction
The discussion so far simplifies a very complex situation, and the underlying cause of changes in RyR2 function resulting from mutations in the channel, or associated with heart failure, is hotly debated. There are several changes in RyR2 channels in addition to the enhanced RyR2 activation in response to increasing luminal Ca\textsuperscript{2+} concentration. Most notable are excess RyR2 phosphorylation, excess RyR2 oxidation, and increased dissociation of the 12.6- and/or 12.0-kD FK506 binding proteins (FKBP12.6 and FKBP12, respectively) from their cytoplasmic binding sites on RyR2. As with sensitivity to luminal Ca\textsuperscript{2+}, which is emphasized in some laboratories (discussed above), other investigators preferably implicate RyR2 phosphorylation, oxidation, and increased dissociation of FKBP12 and FKBP12.6 either individually or together in enhanced RyR2 activity during diastole. There are reports that phosphorylation of RyR2 by protein kinase A (PKA) causes dissociation of FKBP12.6, which leads to increased channel activity. This suggests that RyR2 proteins with mutations linked to CPVT may display enhanced channel activity on PKA phosphorylation as a result of FKBP12.6 dissociation. In contrast, other studies find that RyR2 channels with CPVT mutations have promoted enhanced Ca\textsuperscript{2+} release that is not associated with PKA phosphorylation or FKBP12.6 dissociation (Eschenhagen, 2010). Indeed, phosphorylation of RyR2 by PKA does not necessarily dissociate FKBP12.6 from RyR2 (Jiang et al., 2005). There is also a body of evidence implicating Ca\textsuperscript{2+} calmodulin kinase II in increases in RyR2 activity with β-adrenergic stimulation in heart failure and with RyR2 channels having CPVT mutations (Respress et al., 2012). It is yet to be determined which, if any, of phosphorylation of RyR2 or dissociation of FKBP12.6 or FKBP12, influences the sensitivity of RyR2 to the Ca\textsuperscript{2+} concentration in the SR lumen, induces SOICR, and increases Ca\textsuperscript{2+} leak from the SR.

The pathological increase in RyR2 activity during diastole may also be linked to RyR2 oxidation. RyR2 is oxidized by redox agents, such as GSSG, and by reactive oxygen species (e.g., superoxide anion, hydrogen peroxide, and hydroxyl radical) whose production is enhanced as part of the increased oxidative stress experienced during heart failure. In an established canine heart failure model, the ratio of reduced to oxidized glutathione, as well as the level of free thiols in RyR2, decreases relative to that found in healthy hearts (Terentyev et al., 2008). In myocytes from the same model, RyR2 Ca\textsuperscript{2+} leak is significantly enhanced and SR Ca\textsuperscript{2+} load is significantly reduced compared with levels in myocytes from control animals. Single RyR2 channels isolated from failing hearts demonstrate significantly increased sensitivity to activation by luminal Ca\textsuperscript{2+}. In this case, it appears that redox modification contributes to increased RyR2 luminal Ca\textsuperscript{2+} sensitivity, which could cause SOICR in heart failure. By analogy, excess oxidation may also occur in CPVT arising from RyR2 mutation.

Does pathologically elevated SR Ca\textsuperscript{2+} load increase SOICR?
To answer this question, the Ca\textsuperscript{2+} load in the SR must be directly measured. Much confusion has arisen from indirect measurements of Ca\textsuperscript{2+} load in experiments where the SR is overloaded with Ca\textsuperscript{2+} after a jump in extracellular Ca\textsuperscript{2+} (e.g., from 1 to 10 mM). The onset of Ca\textsuperscript{2+} oscillations is indicative of SOICR that occurs when the Ca\textsuperscript{2+} concentration reaches or exceeds the RyR2 threshold for activation by luminal Ca\textsuperscript{2+}. If oscillations occur sooner or are more frequent, the store is said to be more easily overloaded, and this has been reported in a variety of pathological conditions (cited in Tencercová et al., 2012). However, as discussed above, an increase in spontaneous Ca\textsuperscript{2+} release more often occurs as a result of an increased sensitivity of RyR2 to luminal Ca\textsuperscript{2+} concentration rather than to an actual increase in Ca\textsuperscript{2+} within the SR. There are very few studies where the Ca\textsuperscript{2+} load and actual Ca\textsuperscript{2+} concentration within the SR of cardiac myocytes have been directly measured, and even then, the measurements are complicated by...
numerous factors (Shannon and Bers, 1997). The most accurate measure of free Ca\textsuperscript{2+} within the SR is obtained with low affinity Ca\textsuperscript{2+} indicators such as fluo-5N and total releasable Ca\textsuperscript{2+} measured from caffeine transients (Shannon et al., 2003). Experiments in which free Ca\textsuperscript{2+} and Ca\textsuperscript{2+} load have been measured indicate that SR Ca\textsuperscript{2+} load can increase marginally, but that in most cases, the main factor influencing SOICR is the sensitivity of RyR2 to changes in luminal Ca\textsuperscript{2+} concentration.

An important consideration in addressing this question is that both fluorescence changes and caffeine transients measure averaged Ca\textsuperscript{2+} concentrations in the bulk SR and cannot determine Ca\textsuperscript{2+} concentrations in microdomains of the SR. Changes in Ca\textsuperscript{2+} concentration in the region of the SR immediately adjacent to the luminal loops of RyR2 are of greatest influence on RyR2 activity but would not be specifically measured using existing techniques. When a Ca\textsuperscript{2+} spark occurs, there is a depletion of Ca\textsuperscript{2+} from the release site inside the junctional SR. Measurement of these local depletions, or blinks, has revealed that diffusion of Ca\textsuperscript{2+} through the SR is relatively fast, limiting regions of Ca\textsuperscript{2+} depletion or accumulation under normal conditions. However, during spontaneous Ca\textsuperscript{2+} release, there is significant variability in Ca\textsuperscript{2+} diffusion at local release sites, and an arrhythmogenic substrate may develop (Picht et al., 2011). The microdomain adjacent to RyR2 is a confined space in which CSQ2, triadin, junctin, and possibly other proteins are concentrated, and local Ca\textsuperscript{2+} concentrations might deviate significantly from the overall SR or terminal cisternae concentrations. Non-uniform Ca\textsuperscript{2+} diffusion may cause microdomains of high or low Ca\textsuperscript{2+} concentration to develop around the RyR pore in some junctional release sites. Such variations may become more significant under pathological conditions where RyR2 sensitivity to Ca\textsuperscript{2+} is altered. The Ca\textsuperscript{2+} concentration within this microdomain is an important consideration in interpreting data obtained with isolated RyR2 channels in lipid bilayers where luminal Ca\textsuperscript{2+} concentration can be tightly controlled and varied over a large range (Laver, 2007; Altschafl et al., 2011; Tencerová et al., 2012). Such experiments reveal that RyR2 is regulated by luminal Ca\textsuperscript{2+} over a wide range of concentrations, and under a variety of conditions, that might be experienced in microdomains of the SR but are not reflected in the whole SR/terminal cisternae measurements.

The bulk measurements, using a variety of techniques, indicate that the nonpathological free luminal diastolic Ga\textsuperscript{2+} concentration in the SR is reported to be between 0.7 and 1.5 mM (Shannon et al., 2003). The concentration of total Ca\textsuperscript{2+} within the SR is set by the intraluminal Ga\textsuperscript{2+}-buffering capacity, which largely depends on the buffering ability of CSQ2. The normal Ca\textsuperscript{2+} load (taking into account Ca\textsuperscript{2+} binding to CSQ2) has been assessed to be of the order of 15 mM (Shannon and Bers, 1997). The important questions are how much more Ca\textsuperscript{2+} can be accumulated by the SR under pathological conditions, and indeed whether there is actually an increase in Ga\textsuperscript{2+} load in these conditions. Most recent studies report a decrease in SR Ca\textsuperscript{2+} load in animal heart failure models and in human heart failure (Guo et al., 2007). If the Ca\textsuperscript{2+} load does increase in some conditions, it is unlikely that the increase is large and has been estimated to be between 4% (Bassani et al., 1995) and 28% (Desantiago et al., 2008), which would allow an increase in the free Ga\textsuperscript{2+} concentration to \( \sim 1.9 \) mM at the most. In general, the conclusion that the SR Ca\textsuperscript{2+} load decreases in heart failure and CPVT is consistent with the fact that RyR2 leak is increased during diastole. Additional factors affecting SR Ca\textsuperscript{2+} load are changes in SERCA and NCX expression. In the failing heart, SERCA is down-regulated, further contributing to the reduced capacity of the SR to accumulate Ca\textsuperscript{2+}, and NCX is up-regulated. As a consequence, the NCX–SERCA extrusion balance is altered, and as such, a greater fraction of cytoplasmic Ca\textsuperscript{2+} is extruded from the cell via NCX (Bers, 2008). In some CSQ2-linked CPVT mutations, the buffering capacity of the SR is reduced or its ability to regulate RyR2 activity is compromised. In some circumstances, this would translate to an impairment in the ability of CSQ2 to function in an anti-arrhythmic manner to decrease the degree of RyR2 activation as luminal Ca\textsuperscript{2+} increases during diastole (Faggioni and Knollmann, 2012). The conclusion from these considerations is that there are few pathological situations in which the total Ca\textsuperscript{2+} load or free Ca\textsuperscript{2+} concentrations in the SR actually increase. What happens to Ca\textsuperscript{2+} concentrations in microdomains, and particularly in the region immediately adjacent to the luminal Ca\textsuperscript{2+} sensors for RyR2, remains to be determined.

The importance of studies of RyR2 channels in lipid bilayers to understanding the effects of changes in SR luminal Ca\textsuperscript{2+} concentration on RyR2 function

RyR2 channels inserted into lipid bilayers respond to changes in luminal Ga\textsuperscript{2+} concentration in a complex manner that depends in part on the fact that there are Ca\textsuperscript{2+} sensors on both the luminal and cytoplasmic sides of RyR2. Thus, luminal Ca\textsuperscript{2+} can activate RyR2 by binding to luminal sites, or it can diffuse through the channel to interact with sites on the cytoplasmic domain of the channel protein. The response to changes in luminal Ca\textsuperscript{2+} concentration is strongly dependent on the cytoplasmic Ca\textsuperscript{2+} concentration and on the concentration of Mg\textsuperscript{2+} in the cytoplasmic and the luminal solutions, as well as on the voltage across bilayer membrane (Laver, 2007). As mentioned above, the response to changes in luminal Ca\textsuperscript{2+} is strongly modulated by the presence of associated proteins including CSQ2, triadin, and junctin (Dulhunty et al., 2012). Therefore, results may differ depending on the degree of purity of the RyR2 protein. Purified RyR2 channels are not associated with CSQ2,
triadin, or junctin, in contrast to native RyR2 channels that are retained in SR vesicles that are incorporated into the artificial bilayer with associated triadin, junctin, and CSQ2. Thus, different results might be expected depending on the RyR2 preparation used in an experiment. Results from purified RyR2 channels best reflect the biophysical properties of the channel protein, whereas results obtained from native RyR2 retained in the vesicle membrane better reflect the response of RyR2 in a more intact situation that resembles the intracellular configuration.

An additional confounding factor in the interpretation of single-channel/lipid bilayer experiments is that the association of CSQ2 with RyR2 is profoundly influenced by the changes in luminal Ca\(^{2+}\) concentration that are commonly used to evaluate the sensitivity of RyR2 to luminal Ca\(^{2+}\). For example, as luminal Ca\(^{2+}\) increases above 3–5 mM, CSQ2 super-compacts and dissociates from triadin and junctin so that it can no longer regulate RyR2 (Wei et al., 2009b). In this bilayer situation, CSQ2 is unlikely to reassociate with RyR2 when luminal Ca\(^{2+}\) concentration is lowered because of the massive dilution of the dissociated CSQ2 in the 2-ml bilayer solution. Exogenous CSQ2 can be added if necessary to reform the native complex (Wei et al., 2009a), but this is not routinely done. On the other hand, when luminal Ca\(^{2+}\) is lowered to <0.1 mM, CSQ2 becomes less compact and the polymers dissociate into monomers and cannot reform without the addition of exogenous CSQ2, again because of dilution factors. CSQ2 depolymerization or dissociation from the RyR2 protein complex occurs over a period of several minutes. Depolymerization is an important consideration because the regulation of RyR2 by CSQ2 may depend on the polymeric state of CSQ2 in the same way as regulation of RyR1 by CSQ1. Thus, extreme caution is needed in designing and interpreting experiments in which the aim of the experiment is to determine the physiological response of RyR2 to changes in luminal Ca\(^{2+}\).

Despite the complexity, the lipid bilayer experiments have yielded important information and in fact the only molecular information that we have about the regulation of RyR2 channels by luminal and cytoplasmic Ca\(^{2+}\) and Mg\(^{2+}\) (Laver, 2007), as well as other cytoplasmic factors including ATP. Under normal conditions, ATP is the potentiation of the effect on open probability increased (to ~40-fold) as luminal Ca\(^{2+}\) was increased to 53 mM. It is highly unlikely that a free luminal Ca\(^{2+}\) concentration of 53 mM would ever be achieved in vivo, even in microdomains of the SR. This conclusion is based not only on the experimental observations described above but also on consideration of the Ca\(^{2+}\) affinity and binding capacity of CSQ2; the thermodynamic limitations on SERCA pumping in, working against a concentration gradient; and the significant Ca\(^{2+}\) leak through RyR2 channels as luminal Ca\(^{2+}\) concentrations increase. On the other hand, it is conceivable that concentrations approaching ~6 mM (where Tencerová et al., 2012, report a significant increase in ATP-dependent activation) may be achieved in the microdomain surrounding the luminal loops of RyR2, especially under some pathological conditions. It is also conceivable that, when the threshold for activation of RyR2 by luminal Ca\(^{2+}\) decreases in CPVT, ARVD2, and heart failure, the threshold for amplification of ATP by luminal Ca\(^{2+}\) may similarly fall to lower Ca\(^{2+}\) concentrations. These possibilities remain to be explored. However, the results as they stand once again underscore the importance of the luminal interactions of RyR2 in the overall activity of RyR2, especially during diastole.

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