Regulation of the RyR channel gating by Ca^{2+} and Mg^{2+}

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Abstract

Ryanodine receptors (RyRs) are the Ca^{2+} release channels in the sarcoplasmic reticulum in striated muscle which play an important role in excitation-contraction coupling and cardiac pacemaking. Single channel recordings have revealed a wealth of information about ligand regulation of RyRs from mammalian skeletal and cardiac muscle (RyR1 and RyR2, respectively). RyR subunit has a Ca^{2+} activation site located in the luminal and cytoplasmic domains of the RyR. These sites synergistically feed into a common gating mechanism for channel activation by luminal and cytoplasmic Ca^{2+} . RyRs also possess two inhibitory sites in their cytoplasmic domains with Ca^{2+} affinities of the order of 1 μM and 1 mM. Magnesium competes with Ca^{2+} at these sites to inhibit RyRs and this plays an important role in modulating their Ca^{2+} -dependent activity in muscle. This review focuses on how these sites lead to RyR modulation by Ca^{2+} and Mg^{2+} and how these mechanisms control Ca^{2+} release in excitation-contraction coupling and cardiac pacemaking.

Keywords Ryanodine receptor · RyR1 · RyR2 · Excitation-contraction coupling · Cardiac pacemaking · Ca^{2+} activation · Mg^{2+} inhibition · Ca^{2+} release channels

Introduction

Muscle contracts in response to the electrical excitation of the surface membrane by a process called excitation-contraction (E-C) coupling whereby surface membrane depolarisation elicits calcium release from the sarcoplasmic reticulum (SR). The SR is the primary intracellular Ca^{2+} store and ryanodine receptors (RyR) are the Ca^{2+} release channels. Three RyR isoforms have been identified in mammals: RyR1 in skeletal muscle, RyR2 in cardiac and smooth muscle and RyR3 in many cell types (Ogawa 1994). Regulation of RyRs by intracellular Ca^{2+} and Mg^{2+} (Coronado et al. 1994; Meissner 1994) plays a major role in how the cell controls SR Ca^{2+} release. Single channel recordings have revealed a wealth of information about Ca^{2+} and Mg^{2+} regulation of RyR and have gone a long way to untangling their many interdependent RyR gating

mechanisms. This article reviews the current general picture of RyR regulation by Ca^{2+} and Mg^{2+} . A detailed review of functional models of RyR2 gating by Ca^{2+} and Mg^{2+} has been given elsewhere (Laver 2010).

RyR2 activation by cytoplasmic and luminal Ca^{2+}

Both RyR1 (Hymel et al. 1988; Smith et al. 1986) and RyR2 isoforms (Meissner et al. 1986) exhibit activation by micrometre concentrations of cytoplasmic Ca^{2+} . In the absence of any other channel activator, cytoplasmic Ca^{2+} can increase channel open probability (P_o) from virtually zero up to $P_o \sim 0.6$ with a half-maximal activation concentration (K_a) of 1–5 μM (Laver et al. 1995; Sitsapasan and Williams 1994a; Xu et al. 1996). RyR2 was shown to have a steep P_o dependence on cytoplasmic $[\text{Ca}^{2+}]$, typically exhibiting Hill coefficients of 2–4 (Sitsapasan and Williams 1994a) as would be expected if RyR2 activation resulted from cooperative involvement of one high-affinity ($\sim 1 \mu\text{M}$) Ca^{2+} binding site on each subunit of the homotetrameric channel (Zahradnik et al. 2005). The cytoplasmic Ca^{2+} activation site is referred to here as the A-site, a name originally coined by Balog et al. (2001) (A for Activation).

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Early studies gained clues to the distance of the A-sites from the channel pore from their sensitivity to Ca^{2+} flowing from the luminal side of the membrane into a cytoplasmic milieu containing a fast Ca^{2+} chelator such as BAPTA (Laver 2007; Tripathy and Meissner 1996). The dilution and sequestering of Ca^{2+} emanating from the pore creates a concentration gradient such that the $[\text{Ca}^{2+}]$ at the A-site will depend on its distance from the pore. Analytic solutions of the Ca^{2+} diffusion/buffering equations (Bers and Peskoff 1991) predicted a distance of 11 nm between the A-site and the cytoplasmic pore mouth. More recent mutagenesis experiments revealed that the RyR1 mutation, E4032A and the corresponding mutation E3987A in RyR2 impeded channel activation by ~ 1000 -fold, implicating this amino acid with the A-site (Fessenden et al. 2001; Li and Chen 2001). Recently, cryo-EM reconstructions of RyR1 identified the A-site Ca^{2+} binding pocket near, but not including, E4032. It is formed by the carboxylate side chains of E3893 and E3967 of the RyR1 core solenoid and the backbone carbonyl of T5001 in the C-terminal domain (des Georges et al. 2016). The approximate location of the A-site is shown in a RyR1 gating domain schematic in Fig. 1. Interestingly, the location of the site is ~ 9 nm from the pore gate, consistent with estimates from diffusion/chelation experiments (Laver 2007; Tripathy and Meissner 1996). Even though the nearby E4032 did not coordinate with Ca^{2+} , it was essential for the stability of the Ca^{2+} binding pocket comprising the A-site.

It has been long recognised that Ca^{2+} loading of the SR can trigger Ca^{2+} release (Fabiato 1985) but it was Sitsapesan and Williams (1994b) who first showed that luminal Ca^{2+} could directly activate RyR2. However, at that time, there was no consensus as to the location of the Ca^{2+} binding sites for this process. Based on the finding that luminal Ca^{2+} activation was abolished by tryptic digestion of the luminal domains of the RyR2 (Ching et al. 2000), Sitsapesan and Williams argued that luminal activation was due to luminal facing Ca^{2+} binding sites. Counter to this, Tripathy and Meisner (1996) provided evidence that luminal activation relies on the flow of Ca^{2+} through the pore to the cytoplasm (Ca^{2+} feedthrough) where it activates RyR1 via the A-site. By varying the electrochemical Ca^{2+} driving force (i.e., by varying both luminal $[\text{Ca}^{2+}]$ and transmembrane voltage), they showed that RyR1 activation increased with increasing Ca^{2+} flux through the channel. However, any firm assignment of luminal activation to either cytoplasmic or luminal domains of the RyR was confounded by the fact that the putative luminal and cytoplasmic Ca^{2+} sites were connected by a Ca^{2+} conducting pore. This problem was considerably simplified by performing separate analyses of channel open and closed durations associated with luminal activation (Laver 2007; Laver and Honen 2008; Laver et al. 2004). By studying channel closed events, it was possible to examine luminal activation of RyRs when no Ca^{2+} was flowing through the channel. Analysis of RyR2 opening rate

(the reciprocal of mean closed time) demonstrated the existence of a luminal facing Ca^{2+} binding site with an affinity of $40 \mu\text{M}$ (*L*-site). Separate analysis of channel open events provided information about the effects of Ca^{2+} feedthrough on channel gating. It was found that upon the triggering of a channel opening by the *L*-site, subsequent Ca^{2+} feedthrough to the A-site further stabilised the open channel state, thus enhancing channel activation (Laver 2007). Analysis of RyR1 opening rates demonstrated an *L*-site that not only triggered channel openings, but also decreased A-site-mediated Mg^{2+} inhibition through an allosteric interaction (Laver et al. 2004) (see below).

The location of the *L*-site has not yet been determined in cryo-EM reconstructions. The fact that the Ca^{2+} affinity of the *L*-site does not depend on voltage (Laver 2007) suggests that the site lies outside the transmembrane portion of the RyR. Mutagenesis experiments show luminal Ca^{2+} activation of RyR2 can be abolished by point mutations at E4872A in the S6 helix bundle crossing with relatively minor changes in cytosolic Ca^{2+} activation (Chen et al. 2014). This places the luminal Ca^{2+} site at the proposed gate of the RyR. Moreover, introducing histidine at this site converts RyR2 into a luminal Ni^{2+} -activated channel.

Recent measurements of RyR2 gating have shown that activation of RyR2 by luminal and cytoplasmic Ca^{2+} cannot be thought of as independent processes (Laver and Honen 2008). Opening rate increased in proportion to the third power of cytoplasmic $[\text{Ca}^{2+}]$ (in the μM range) and the 2nd power of luminal $[\text{Ca}^{2+}]$, peaking at ~ 1000 openings per second. By applying combinations of various cytoplasmic and luminal $[\text{Ca}^{2+}]$, Laver and Honen (2008) reported a marked synergy between A- and *L*-site activation of the RyR2. Increasing cytoplasmic $[\text{Ca}^{2+}]$ from 1 to 100 nM increased luminal Ca^{2+} activation by 4-fold without altering the apparent affinity of the *L*-site. Likewise, increasing luminal $[\text{Ca}^{2+}]$ from 1 nM to 0.1 mM increased cytoplasmic Ca^{2+} activation without altering the apparent affinity of the A-site. Therefore, Ca^{2+} activation is not uniquely attributable to either the A- or *L*-sites but rather is a combination of both. However, this synergy was only apparent at cytoplasmic $[\text{Ca}^{2+}]$ lower than $10 \mu\text{M}$. At higher concentrations, the RyR opening rate is insensitive to luminal $[\text{Ca}^{2+}]$. This synergy between A- and *L*-sites is also apparent in mutagenesis studies (Chen et al. 2014) where loss of either A- or *L*-site activation was always accompanied by partial loss of activation (opening rate) by the other site.

Cytoplasmic Ca^{2+} inhibition of RyR1 and RyR2

Millimolar cytoplasmic $[\text{Ca}^{2+}]$ inhibits RyR1 (Bezprozvanny et al. 1991; Meissner 1986) and RyR2 (Laver et al. 1995). In RyR1, P_o markedly declines when cytoplasmic $[\text{Ca}^{2+}]$ rises above $100 \mu\text{M}$ indicating the presence of low-affinity

Fig. 1 RyR1 structure reconstructed from cryo-EM images (des Georges et al. 2016) showing putative locations of key regulation sites on the RyR. Ca^{2+} sites are marked in red. *Top*—a distinctive sequence of 30 negative amino acids at positions 1873–1903 postulated to form the $\text{Ca}^{2+}/\text{Mg}^{2+}$

inhibitory; *I1*-site (Laver et al. 1997b) is shown on two of the four subunits. *Bottom*—a cutaway of the RyR1 structure showing activation sites for Ca^{2+} (A-site), caffeine and ATP (des Georges et al. 2016) and the L-site (Chen et al. 2014)

inhibitory sites on the RyR. RyR2 is 10-fold less sensitive to Ca^{2+} inhibition than RyR1 (Laver et al. 1995). In keeping with the nomenclature of Balog et al. (2001), this site is referred to here as the *II*-site (*I* for inhibition and *II* to distinguish it from a recently identified high-affinity inhibition site, *I2*-site, see below). At physiological concentrations of monovalent ions, the apparent Ca^{2+} affinity of the *II*-site in RyR1 was 100 μM and this increased as the 2nd power of monovalent ion concentration (Meissner et al. 1997; Shomer et al. 1993). Together, the *A*- and *II*-sites generate the well-known, bell-shaped cytoplasmic $[\text{Ca}^{2+}]$ dependence of P_o which characterises RyR activation at micrometre Ca^{2+} and inhibition at millimolar Ca^{2+} . Since Ca^{2+} levels in the bulk cytoplasm only reach $\sim 1 \mu\text{M}$ (Bers 2002c), this process was originally not seen as physiologically important (Bezprozvanny et al. 1991). Even taking into account the much higher levels of Ca^{2+} in the triadic cleft (200 μM (Soeller and Cannell 1997)), Mg^{2+} inhibition by the *II*-site will be more significant to muscle function than Ca^{2+} inhibition (see below).

The location of the *II*-site has not yet been determined in cryo-EM reconstructions. Mutated RyR1, which lacked amino acids 1641–2437 in the so-called junctional solenoid domain (des Georges et al. 2016) or Handle domain (Peng et al. 2016), required 10-fold higher Ca^{2+} than wt-RyR1 for inhibition but still had normal Ca^{2+} activation (Bhat et al. 1997). It has been noted (Laver et al. 1997b) that within this region of RyR1 is a distinctive sequence of 30 negative amino acids at positions 1873–1903, whereas in the corresponding region in RyR2 has far fewer negative amino acids. These would create a diffuse electrostatic binding region consistent with a low-affinity binding site that produces equal inhibition by Ca^{2+} and Mg^{2+} (Mg^{2+} is reviewed below). Also, the reduced negative charge in the corresponding region in RyR2 might explain their lower sensitivity Ca^{2+} inhibition.

Another cytoplasmic Ca^{2+} inhibition phenomenon has been identified in single channel recordings of RyR2 which has been attributed to a high-affinity, cytoplasmic facing site (*I2*-site) (Laver 2007). It is manifest as a cytoplasmic $[\text{Ca}^{2+}]$ -dependent reduction in channel open time in the sub micrometre range. Ca^{2+} diffusion/buffering experiments (described above for the *A*-site) estimate a 26-nm distance between the *I2*-site and the cytoplasmic mouth of the channel, somewhat further away than the *A*-site (Laver 2007). As yet, the physiological significance and precise location of this site are unknown.

Mg^{2+} inhibition of RyR1 and RyR2

Mg^{2+} is a strong inhibitor of RyRs that acts as a competitive Ca^{2+} antagonist at the *A*-site (Smith et al. 1986) with a binding affinity of $\sim 50 \mu\text{M}$ (Laver and Honen 2008; Meissner et al. 1986; Meissner and Henderson 1987). The effect of

physiological concentrations of Mg^{2+} (1 mM (Godt and Maughan 1988)) is to shift K_a for Ca^{2+} activation from 1 μM in the absence of Mg^{2+} to 50 μM (Cannell et al. 2013). Mg^{2+} binding at the *A*-site not only prevents Ca^{2+} activation but can also close the channels that are activated by luminal Ca^{2+} or ryanodine (Laver et al. 2004) which bind at other sites.

A biphasic $[\text{Ca}^{2+}]$ dependence of the Mg^{2+} IC_{50} revealed a second Mg^{2+} inhibitory mechanism due to the *II*-site (Laver et al. 1997a). At low cytoplasmic $[\text{Ca}^{2+}]$, the IC_{50} for Mg^{2+} inhibition increased in proportion to $[\text{Ca}^{2+}]$ as would be expected for $\text{Ca}^{2+}/\text{Mg}^{2+}$ competition at the *A*-site. However, at higher concentrations, the IC_{50} for Mg^{2+} plateaued as if another non-competitive mechanism dominated. These IC_{50} values for Mg^{2+} and Ca^{2+} were identical in each individual channel in spite of substantial channel-to-channel variations indicating that Mg^{2+} is a surrogate for Ca^{2+} , the *II*-site and both Ca^{2+} and Mg^{2+} cause channel closures (Laver et al. 1997a). This finding provided an explanation for the physiological significance of the *II*-site in skeletal muscle where the IC_{50} for the *II*-site is $\sim 0.1 \text{ mM}$. Even though physiological concentrations of Ca^{2+} are well below the IC_{50} , Mg^{2+} levels are 10-fold higher than this and will strongly inhibit RyR1 in the cell. However, in RyR2, the IC_{50} of the *II*-site is $\sim 10 \text{ mM}$ so it is unlikely that intracellular Mg^{2+} will have a sufficient inhibitory action at this site to make it a significant regulator in the heart.

Luminal Mg^{2+} was found to inhibit RyR2 by two mechanisms (Laver and Honen 2008). Firstly, Mg^{2+} flows through the channel and binds to the *A*-site and closes the channel (RyRs have the same permeability for both Mg^{2+} and Ca^{2+}). Secondly, luminal Mg^{2+} is a competitive antagonist for Ca^{2+} at the luminal *L*-site where Ca^{2+} and Mg^{2+} have the same affinity. Hence, Mg^{2+} shifts the K_a for luminal Ca^{2+} activation from 40 μM in the absence of Mg^{2+} to 1 mM at physiological $[\text{Mg}^{2+}]$. This is likely to be very important for the way luminal $[\text{Ca}^{2+}]$ regulates SR Ca^{2+} release. The physiological $[\text{Ca}^{2+}]$ in the junctional SR cycles between 0.3 and 1 mM (Bers 2002b) which, in the absence of Mg^{2+} , would be well above the K_a so that Ca^{2+} release would be virtually unregulated by SR Ca^{2+} loading. However, in the presence of 1 mM Mg^{2+} , the K_a lies within the physiological range will have a substantial effect on RyR2 activity and Ca^{2+} release.

RyR1 regulation during excitation-contraction coupling in skeletal muscle

In striated muscle, the *L*-type Ca^{2+} channels (DHPRs) located in the sarcolemma and transverse tubules are the voltage sensor. In skeletal muscle, the DHPRs directly interact with RyR1 and their depolarisation by an action potential induces

conformational changes that are relayed to RyR1 to cause it to open (Tanabe et al. 1990). E-C coupling in skeletal muscle does not rely on the influx of Ca^{2+} so that DHPRs can activate RyRs even though cytoplasmic $[\text{Ca}^{2+}]$ is 100 nM. Once RyR1 opens, SR Ca^{2+} release increases cytoplasmic $[\text{Ca}^{2+}]$ to 10 μM .

The properties of cytoplasmic Ca^{2+} , Mg^{2+} and ATP regulation of RyR1 give insight into the way DHPRs control Ca^{2+} release in skeletal muscle. In skeletal muscle, the *II*- and *A*-sites both have Mg^{2+} IC_{50} values of 50–100 μM so that both sites make similar contributions to Mg^{2+} inhibition. The fact that Mg^{2+} inhibition at the *II*-site is not alleviated by elevated cytoplasmic $[\text{Ca}^{2+}]$ (unlike the *A*-site) means that *II*-site-mediated Mg^{2+} inhibition provides an extremely strong brake on Ca^{2+} release. In fact, without some way of bypassing this Mg^{2+} inhibition, RyR1 would not release Ca^{2+} in skeletal muscle at all. However, experiments on skinned muscle fibres have shown that depolarisation of DHPRs leads to a loss of Mg^{2+} inhibition of RyR1 (Lamb and Stephenson 1994). Though the particular Mg^{2+} inhibition mechanism being modulated has not been determined, it would need to involve the *II*-site for it to be effective in de-repressing RyR1.

ATP can substantially activate RyR1 even when cytoplasmic $[\text{Ca}^{2+}]$ is at resting levels (~ 100 nM). This explains why RyR1 can be opened without there being any influx of extracellular Ca^{2+} to act as a trigger. Therefore, in resting muscle, RyR1 is primed for activation and once the activation of DHPRs alleviates Mg^{2+} inhibition, the RyR1 will open. Removal of ATP stimulation of RyR1 ‘deprimes’ RyR1 resulting in a loss of E-C coupling, i.e. the E-C coupling mechanism in skeletal muscle absolutely requires upregulation of RyR1 by ATP (Blazev and Lamb 1999a, 1999b). Adenosine, a non-agonist competitor for the ATP site, inhibited voltage sensor control of Ca^{2+} release in rat skinned muscle fibres. Therefore, it is likely that the DHPR triggers SR Ca^{2+} release simply by removing the inhibitory effect of Mg^{2+} exerted at the *II*-site, possibly by lowering its affinity for Mg^{2+} as suggested previously (Lamb 2000). ATP would then be allowed to activate RyR1 and the released Ca^{2+} could then reinforce further activation.

Decreasing SR luminal Ca^{2+} from resting levels of 1 to 0.1 mM decreases the K_i for Mg^{2+} at the *A*-site in RyR1 from 72 to 20 μM without altering the K_i for the *II*-site (Laver et al. 2004). Therefore, depletion of the SR will impose a stronger Mg^{2+} inhibition by the *A*-site which could explain why chemical stimulation of RyR1 (i.e. lowering Mg^{2+} and application of agonists such as caffeine) is unable to fully deplete the SR (Fryer and Stephenson 1996; Lamb and Stephenson 1994). However, activation of DHPRs can completely empty the SR of Ca^{2+} , even in the presence of physiological $[\text{Mg}^{2+}]$ (Kurebayashi and Ogawa 2001; Posterino and Lamb 2003) which has led to the proposal that DHPRs

alleviate Mg^{2+} inhibition by ‘commandeering’ the *L*-site/*A*-site allosteric interaction (Laver et al. 2004).

RyR2 regulation during excitation-contraction coupling in cardiac muscle

In the presence of diastolic concentrations of Mg^{2+} (1 mM) and Ca^{2+} (100 nM), the open probability of RyR2 has been measured at $\sim 10^{-6}$ (Laver *unpublished*). Hence, during diastole, RyR2 are inactive because of insufficient activation by the *A*-site. RyR2 is not as strongly activated by ATP as RyR1 and so ATP is not the primary RyR2 agonist. Instead, Ca^{2+} influx through DHPRs increases $[\text{Ca}^{2+}]$ in the dyadic cleft to ~ 100 μM (Soeller and Cannell 1997) which provides the activating stimulus to RyR2 via their *A*-sites, even in the presence of 1 mM Mg^{2+} . The subsequent release of Ca^{2+} from the SR further increases dyadic $[\text{Ca}^{2+}]$ to ~ 200 μM (Laver et al. 2013) to cause further RyR2 activation. This process, known as *Ca^{2+} -induced Ca^{2+} release* (CICR (Fabiato 1983)), provides a strong positive feedback that should sustain RyR2 activation until the SR is substantially depleted of Ca^{2+} (Zima et al. 2008). However, in myocytes, Ca^{2+} release can undergo rapid termination of CICR in spite of only moderate SR depletion as measured by fluo-5N (Picht et al. 2011; Shannon et al. 2003). This is clearly seen in localised SR Ca^{2+} release events (Ca^{2+} sparks) that exhibit CICR lasting only 10 ms, after which the positive feedback cycle of CICR is broken and SR Ca^{2+} release ceases (Cheng et al. 1993). It was a long-standing problem that single channel studies have not found any RyR2 regulation mechanisms that could terminate CICR this rapidly (see reviews by Cannell and Kong 2012; Stern and Cheng 2004). Recently, two theoretical studies and one experimental study have provided a plausible explanation for a negative feedback mechanism (Cannell et al. 2013; Guo et al. 2012; Laver et al. 2013). Modelling of RyR2-mediated Ca^{2+} release by simulating Ca^{2+} diffusion and buffering within a 3D spatial model of a dyad accurately reproduced the experimental values of Ca^{2+} spark duration, refractory period and SR depletion. The model also produced reliable and spontaneous RyR2 closure without any form of RyR2 inactivation (Cannell et al. 2013; Laver et al. 2013), by a mechanism called ‘induction decay’ (Laver et al. 2013). In this process, depletion of the junctional SR leads to a decay of CICR and subsequent deactivation of RyR2. The junctional SR depleted by $\sim 90\%$ with virtually no depletion of the longitudinal SR due to the limited connection between the two SR compartments. Model simulations of confocal images revealed that the inability of confocal microscopes to resolve fluo-5N fluorescence in junctional and longitudinal SR leads to an underestimation of junctional SR depletion and so reconciled model and experimental estimates of SR depletion. Experimental support for

the idea of ‘induction decay’ came from the observation that reducing RyR2 conductance increased the rate of Ca^{2+} spark termination (Guo et al. 2012).

RyR2 regulation during cardiac pacemaking

In cardiomyocytes, SERCA2a and RyR2 carry Ca^{2+} uptake and release, respectively, across the SR membrane while DHPRs and Na/Ca exchangers (NCXs) carry Ca^{2+} uptake and release across the sarcolemma, respectively (Dibb et al. 2007). These transporters generate the large oscillations in free $[\text{Ca}^{2+}]$ in the cytoplasm ($[\text{Ca}^{2+}] \sim 0.1$ to $1 \mu\text{M}$) and SR lumen ($[\text{Ca}^{2+}] \sim 1$ to 0.3 mM) between diastole and systole, respectively (Bers 2002b). Spontaneously, cyclic SR Ca^{2+} uptake and release have been observed as Ca^{2+} waves in intact cardiomyocytes under conditions of Ca^{2+} overload (Bers 2002a). This process was first shown to generate a ‘clock cycle’ that could entrain pacemaker action potentials in lymphatic smooth muscle (Van Helden 1993) and more recently in cardiac pacemaking in the sinoatrial node (Ju and Allen 1998;

Ju and Allen 2007; Lakatta and DiFrancesco 2009; Rigg and Terrar 1996). Although this process depends on many ion transport and buffering mechanisms (Bers 2002a), the role of the RyR2 luminal and cytoplasmic activation sites in cyclic Ca^{2+} uptake and release is envisaged as follows. First, SERCA2a sequesters cytoplasmic Ca^{2+} , loading the SR to a point where elevated luminal $[\text{Ca}^{2+}]$ causes opening of RyR2 via the *L*-site, a process dubbed store overload-induced Ca^{2+} release (SOICR (Jiang et al. 2005)). Second, during Ca^{2+} release, CICR provides positive reinforcement of RyR2 activity via the *A*-site which continues until the SR lumen depletes and the decaying CICR can no longer support RyR2 activation and RyR2 close. Subsequently, the SR reloads and the cycle is repeated, producing a cyclic, bistable oscillation analogous to the motion of a tipping urn (Fig. 2). By this analogy, one can see how cycling rate is influenced by *L*-site sensitivity of RyR2 (pivot point), SERCA2a activity (filling rate) and Ca^{2+} capacity/buffering of the SR (urn capacity). SR Ca^{2+} cycling is coupled to sarcolemmal pacemaking by the NCX. Ca^{2+} released during RyR2 activation phase of each cycle promotes Ca^{2+} extrusion from the cell via NCX leading to

Fig. 2 Two schematics illustrating how SERCA2a and RyR2 produce cycling of Ca^{2+} uptake and release in cardiac SR. The left shows an idealised SR with SERCA2a driven uptake and RyR2-mediated release which is controlled by luminal and cytoplasmic facing Ca^{2+} activation sites (*L*-sites—red; *A*-sites—blue). Store filling with Ca^{2+} is signified by dark blue. As the store fills (middle left), the *L*-sites trigger the opening of RyR2 (bottom left) and the resulting Ca^{2+} flow to the *A*-sites reinforce

channel opening until the stores are nearly depleted SERCA2a (top left). The right side shows the analogy of a ‘tipping urn’ that undergoes bistable oscillations by similar mechanisms. The thick arrows indicate water flow. Analogous features are (1) SERCA2a transport rate and water flow rate; (2) SR capacity and urn capacity; and (3) effective Ca^{2+} affinity of the *L*-site and the pivot point

depolarization of the sarcolemma (i.e. 3 Na⁺ enter for every Ca²⁺ extruded) and enhanced sarcolemmal excitability with the effect of entraining pacemaker action potentials (Bers 2002a).

An example of how this would work within a physiological setting is the case of the sympathetic ‘fight or flight’ response that increases heart rate and contractility. Increased catecholamine concentrations stimulate cardiac β -adrenergic receptors (β -AR), resulting in phosphorylation and increased activity of key Ca²⁺ transporters including SERCA2a and RyR2 (Bers 2002b). Many studies have demonstrated increased phosphorylation of RyR2 at S2808, S2814 and S2030 (Benkuský et al. 2007; Carter et al. 2006; Currie et al. 2004; Ferrero et al. 2007; Huke and Bers 2008; Li et al. 2013; Rodríguez et al. 2003; Xiao et al. 2006). Detailed analysis of RyR2 regulation of channels isolated from adrenergic-stimulated hearts showed that adrenergic stimulation increased their *L*-site-mediated luminal Ca²⁺ activation without affecting *A*-site RyR2 activation (Li et al. 2013). Such an increase in *L*-site sensitivity would cause earlier onset of the Ca²⁺ release phase in the cardiac cycle which would increase Ca²⁺ cycling rate and so provide a driver for increasing pacemaking frequency.

Concluding remarks

Thus far, four Ca²⁺ regulation sites have been identified in RyRs. They have two Ca²⁺ activation sites located in the luminal and cytoplasmic domains of the RyR (*L*- and *A*-sites). These sites feed into a common gating mechanism and produce synergistic activation by luminal and cytoplasmic Ca²⁺. The cytoplasmic domains also possess two inhibitory sites (*I1*- and *I2*- sites) with Ca²⁺ affinities of the order of millimolar and micromolar, respectively. Magnesium competes with Ca²⁺ at these sites and plays an important role in inhibiting RyRs and shaping the Ca²⁺-dependent activation of RyRs in muscle. Recent analysis of RyR1 structure has identified one *A*-site in each subunit at the interfaces between the C-terminal domain and the core solenoid, at a distance from the cytoplasmic pore mouth that was predicted by functional studies.

RyR2 and RyR1 share common Ca²⁺/Mg²⁺ regulation mechanisms albeit with different sensitivities and these differences lead to quite different types of cellular control over SR Ca²⁺ release in skeletal and cardiac muscle (Lamb 2000; Laver et al. 2001). In resting skeletal muscle, RyR1 would be activated by ATP if it were not for Mg²⁺ which strongly inhibits the channels via the *I1*-site. RyR1 activation by voltage sensor depolarisation is due to a conformational coupling between the voltage sensor and RyR1 that alleviates this Mg²⁺ inhibition thereby de-repressing RyR1. Once the voltage sensor deactivated upon membrane repolarisation, Mg²⁺ inhibition is restored and this will close RyR1 even in the presence of high [Ca²⁺]. Thus, cessation of Ca²⁺ release is under

voltage sensor control. In cardiac muscle during diastole, RyR2 inhibition by Mg²⁺ is weak because the *I1*-site is ~ 100-fold less sensitive than in RyR1 and Mg²⁺ inhibition that occurs at the *A*-site is competitively overcome by the Ca²⁺ stimulus. Hence, RyR2 inactivity in diastole is due to a lack of RyR2 stimulation rather than inhibition. During E-C coupling, Ca²⁺ entry through the voltage sensor is sufficient to stimulate SR Ca²⁺ release but cessation of Ca²⁺ release is not under voltage sensor control. Rather, it is controlled by restricted Ca²⁺ diffusion between the junctional and longitudinal SR leading to ‘induction decay’ of RyR2.

Finally, RyR2 activation by *L*- and *A*-sites serves different roles in SR Ca²⁺ release. The *A*-site stimulates RyR2 opening during cardiac E-C coupling where the trigger for Ca²⁺ release is the Ca²⁺ influx through voltage sensors, whereas the *L*-site stimulates RyR2 opening in response to store loading which forms part of the cardiac pacemaking mechanism.

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Compliance with ethical standards

Conflict of interest Derek R. Laver declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the author.

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