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REVIEW

Luminal Ca^{2+} activation of cardiac ryanodine receptors by luminal and cytoplasmic domains

Derek R. Laver

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Abstract The ryanodine receptors form the calcium release channel in the membrane of the sarcoplasmic reticulum (SR, the main intracellular Ca^{2+} store). The importance of ryanodine receptors (RyRs) to cardiac pacemaking and rhythmicity is highlighted by more than 69 mutations, RyR mutations, which underlie arrhythmias and sudden cardiac death. Although most of these mutations lie in cytoplasmic domains, they all cause increased RyR activation by Ca^{2+} in the SR lumen. Presented here is a review of the mechanisms by which cytoplasmic domains of the RyR can determine luminal activation.

Keywords Calcium release channels · Cardiac muscle · Calcium stores · Sudden cardiac death · Ryanodine receptor · Bilayer

Introduction

In muscle, the sarcoplasmic reticulum (SR) is the main intracellular Ca^{2+} store and the ryanodine receptors form the calcium release channel in the SR membrane. In the heart, RyR2 is the main RyR isoform. During normal function, the heart muscle cycles between periods of systole (contraction) and diastole (relaxation). Systole commences with the cardiac action potential which triggers a rise in cytoplasmic Ca^{2+} ($[Ca^{2+}]_C$), which is the signal for muscle contraction. The rise in $[Ca^{2+}]_C$ begins with the opening voltage-dependent, L-type Ca^{2+} channels in the sarcolemma. The inflow of Ca^{2+} into the cytoplasm activates the RyR2 via their cytoplasmic facing Ca^{2+}-activation sites. The subsequent release of Ca^{2+} from the SR further increases $[Ca^{2+}]_C$ and hence RyR2 activation. This process, known as Ca^{2+} induced Ca^{2+} release (CICR), provides a strong positive feedback on RyR2. In this way, Ca^{2+} release from the SR contributes to 95% of the Ca^{2+} entering the cytoplasm during excitation–contraction coupling in the frog (Fabiato 1985), 70% in rabbit and 92% in rat (Bers 2002). The heart then enters a period of diastole where $[Ca^{2+}]_C$ falls. For reasons which are yet to be fully understood the positive feedback cycle of CICR is broken and release of Ca^{2+} from the SR ceases. The excess Ca^{2+} in the cytoplasm is either pumped back into the SR by ATP-driven Ca^{2+} pumps in the SR membrane (SERCa) or extruded from the cell by the Na/Ca exchanger in the sarcolemma.

The Ca^{2+} release and uptake by the SR causes the free Ca^{2+} concentration in the SR lumen ($[Ca^{2+}]_L$) to cycle between 1 and 0.3 mM during diastole and systole, respectively (Bers 2002). Although it has been known for decades that RyR2 is stimulated by elevated $[Ca^{2+}]_L$ (Fabiato and Fabiato 1977), only recently was it proposed that fluctuations in $[Ca^{2+}]_L$ during the release/uptake cycle generate the cyclic stimulation/inhibition of RyR2, which underlies cardiac pacemaking and rhythmicity (Vinogradova et al. 2005) (see also (Van Helden 1993; van Helden and Imtiaz 2003)). The pacemaker Ca^{2+} oscillations occur in two phases: first, SERCa causes loading of the SR to a point where elevated $[Ca^{2+}]_L$ causes spontaneous opening of RyR2. Second,

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D. R. Laver (✉)
School of Biomedical Sciences,
Hunter Medical Research Institute,
University of Newcastle, Callaghan, NSW 2308, Australia
e-mail: Derek.Laver@newcastle.edu.au

CICR during Ca^{2+} release provides positive reinforcement of RyR2 activity, which continues until the stores sufficiently deplete to cause closure of RyR2 channels. Ca^{2+} release in turn activates the Na/Ca exchanger to extrude Ca^{2+} out of the cell causing a net depolarisation of the sarcolemma (i.e. 3 Na^+ enter for every Ca^{2+} extruded) and triggers an action potential. The importance of RyR2 to cardiac pacemaking and rhythmicity is highlighted by RyR2 mutations, which underlie arrhythmias and sudden cardiac death (SCD). So far, 69 mutations in RyR2 have been linked with a stress induced malignant ventricular tachycardia called catecholaminergic polymorphic ventricular tachycardia (CPVT) (George et al. 2007). All these mutations cause increased Ca^{2+} leak from the SR during diastole resulting in chronically elevated $[Ca^{2+}]_C$.

Measurements on isolated RyRs reveal that the RyR ion channel conducts both divalent and monovalent cations (Tinker et al. 1992). The opening of these channels is modulated by a wide variety of intracellular substances (Meissner 1994). However, RyR regulation by ATP, Ca^{2+} and Mg^{2+} plays a key role in excitation–contraction coupling (Lamb 2000). Cytoplasmic Ca^{2+} has a biphasic effect on RyR2, causing activation at μM concentrations (Meissner et al. 1986) and inhibition at mM concentrations (Laver et al. 1995). Luminal Ca^{2+} also shows a biphasic stimulation of RyRs that depends on the luminal to cytosolic Ca^{2+} flux (Sitsapesan and Williams 1994; Gyorke and Gyorke 1998; Xu and Meissner 1998). The Ca^{2+}-activation of RyR2 is substantially increased by ATP which produces a half-maximal effect at ~0.2 mM (Kermode et al. 1998) and near-maximal effect at physiological levels (~8 mM Godt and Maughan 1988). Intracellular Mg^{2+} (~1 mM) is a strong inhibitor of RyR2 from both the cytoplasmic (Xu and Meissner 1998) and SR luminal compartments (Laver and Honen 2008) at diastolic $[Ca^{2+}]_C$. Abnormal regulation of RyRs by these ligands has been shown to produce the changes in cardiac contractility that occurs in ischemia and a range of myopathies (George et al. 2007). In particular, SCD-causing mutations in RyR2 produce increased channel activation by both cytoplasmic Ca^{2+} (Jiang et al. 2002; Yang et al. 2006) and luminal Ca^{2+} (Jiang et al. 2005).

RyR2 is a homotetramer with total molecular weight of ~2.2 MDa, which makes it the largest ion channel protein identified to date (Meissner 1994). Cryo-electron micrograph (EM) images reveal the RyR2 to be comprised of four subunits that surround a central pore in which the C-terminal ~20% of its amino acids form a transmembrane ion channel (Samso et al. 2005) (Fig. 1). The remaining amino acids form a large cytoplasmic domain that transverses the 10 nm gap between the SR and the sarcolemma. More than two-thirds of the SCD causing mutations in RyR2 are believed to lie in its cytoplasmic

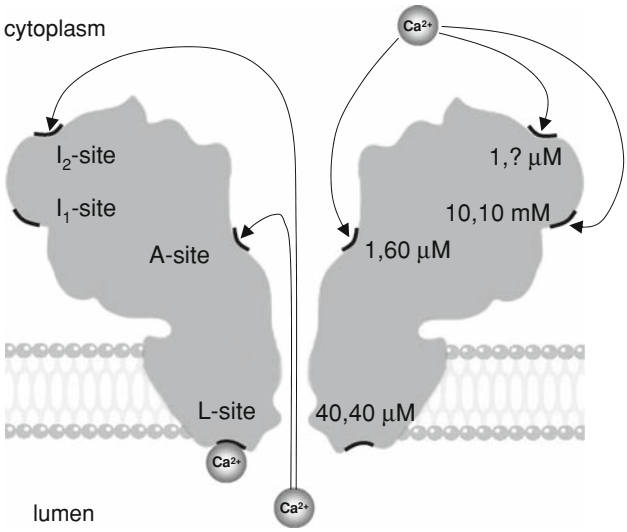

Fig. 1 Ca^{2+}/Mg^{2+} regulatory sites on the cardiac RyR (RyR2). The hypothetical locations of four divalent cation sites known to regulate the gating activity of RyR2 are shown on a structural silhouette obtained from Samso et al. (2005). The names given to these sites are indicated on the *left* and the corresponding Ca^{2+}–Mg^{2+} affinities of the sites are shown on the *right* (the Mg^{2+} affinity of the I_2-site is unknown (?)). Details of these sites are given in the text. The *arrows* indicate the ability of Ca^{2+} on the luminal and cytoplasmic sides of the membrane to access Ca^{2+} sites on the cytoplasmic domains of the channel

domain. Some of the clearest evidence for this has been derived from EM images of RyR2 constructs where green fluorescent protein (GFP) was included in the RyR2 amino acid sequence at sites within two separate clusters of CPVT mutation (hot spots), amino acids 167–466 (Wang et al. 2007) and 2246–2504 (Liu et al. 2005). Since most of the mutations lie in the cytoplasmic domain, it is puzzling that they all alter RyR2 response to luminal Ca^{2+} in addition to cytoplasmic Ca^{2+} (George et al. 2007). One possible reason for this is that cytoplasmic facing Ca^{2+} sites may sense luminal Ca^{2+} because Ca^{2+} can pass across the membrane through the RyR2 pore. The roles of the cytoplasmic and luminal domains of RyR2 in channel activation by luminal Ca^{2+} have been hotly debated since the initial observation that luminal Ca^{2+} activates RyRs, first made in the mid-1990s (Sitsapesan and Williams 1997; Gyorke et al. 2002). However, only recently has an experimentally derived, quantitative model for Ca^{2+} and Mg^{2+} regulation of RyR2 been developed, which assigns precise roles to cytoplasmic and luminal domains of the RyR2 (Laver 2007a; Laver and Honen 2008), and which also predicts an important role for luminal Mg^{2+} in shaping the luminal Ca^{2+} dependence of RyR2 activation. Here we review this functional model for RyR2 regulation by intracellular Ca^{2+} and Mg^{2+} with a focus on the roles of the cytoplasmic and luminal RyR2 domains in channel activation by Ca^{2+} in the SR.

Ca^{2+}/Mg^{2+} regulatory sites on RyR2 and its regulation by luminal triggered Ca^{2+} feed-through

Studies employing single channel recording of RyRs in artificial lipid bilayers have identified at least four, Ca^{2+}-dependent gating mechanisms on RyR2. Two of these cause channel activation (A- and L-sites) and two lead to inhibition (I_1- and I_2-sites). Figure 1 shows the approximate locations of these sites (their precise locations are unknown). Details of the properties of these sites are described in a previous review (Laver 2007b).

Cytoplasmic Ca^{2+}-activation of RyR2 is believed to occur in response to Ca^{2+} binding to a cytoplasmic facing, A-site with 1 μM affinity (Meissner et al. 1986; Meissner and Henderson 1987) which can trigger channel opening at rates up to ~100 s^{-1} (Laver and Honen, 2008). Luminal Ca^{2+}-activation was recently attributed, in part, to a Ca^{2+} site on the luminal facing portion of RyR2, the L-site (Laver 2007a). This site activates the RyR2 with a K_a of 40 μM and maximum opening rate of ~1 s^{-1} which is 100-fold slower than the A-site. Consequently, luminal Ca^{2+} produces much weaker activation of RyR2 than does cytoplasmic Ca^{2+}. The inhibitory effects of Mg^{2+} are due to competition between Mg^{2+} and Ca^{2+} at both the A- and L-sites. Mg^{2+} binds to the A-site (60 μM affinity (Meissner et al. 1986; Laver and Honen 2008)) to cause closure of the channel. At the L-site, Ca^{2+} and Mg^{2+} both have the same affinity (~40 μM) but unlike Ca^{2+}, Mg^{2+} binding fails to open the channel (Laver and Honen 2008).

Two Ca^{2+}-inhibitory mechanisms have been identified in RyR2. Millimolar concentrations of cytoplasmic Ca^{2+} cause inhibition of RyR2, which is believed to occur via a low affinity (10 mM) site on the cytoplasmic face of the channel (Laver et al. 1995) (I_1-site). More recently, a high-affinity (1 μM) Ca^{2+}-inactivation site was identified (I_2-site) (Laver 2007a) which causes brief (1 ms) channel closures at rates of up to 1,000 s^{-1}. The I_1-site does not distinguish between Ca^{2+} and Mg^{2+} and binds alkali metal ions with identical affinity. Efforts to detect any effect of Mg^{2+} at the I_2-site have, so far, been unsuccessful (Laver and Honen 2008).

The processes by which the four Ca^{2+} sensing sites regulate channel activation are complex and involve at least two independent mechanisms. Firstly, RyR2 activation by luminal Ca^{2+} occurs by a multi-step process called "luminal-triggered Ca^{2+} feed-through" in which Ca^{2+} binding to the luminal, L-site, initiates channel openings whereupon luminal Ca^{2+} can flow through to the cytoplasmic A-sites, producing prolongation of openings, and to the I_2-sites which inactivate the channel (Laver 2007a) (Fig. 1). The relative abilities of the A- and I_2-sites to detect flow through the pore (see below) indicate that they are ~16 nm and ~34 nm from the pore mouth, respectively (Laver and Honen 2008). Secondly, cytoplasmic and luminal Ca^{2+} have a synergistic action on the frequency of channel opening indicating that the triggering mechanism itself, also depends on both cytoplasmic and luminal domains of the protein, independently of Ca^{2+} feed-through. These two processes will be discussed in more detail below.

The experimental approach

Even though experiments on muscle cell preparations had demonstrated luminal Ca^{2+} as a stimulator of Ca^{2+} release over 30 years ago (e.g. (Ford and Podolsky 1972; Fabiato and Fabiato 1977)), it was not until isolated RyRs were studied in artificial lipid bilayers that the mechanisms for this process came to light. Artificial bilayer preparations allowed precise control of the membrane potential and the composition of the luminal and cytoplasmic solutions that bathe the RyR, a level of control that is not possible in cellular preparations. Moreover, the activity of RyRs could be directly measured and analysed in sufficient detail to dissect the complex regulation mechanisms that control channel gating (Laver 2001). However, even with the experimental power of single channel recording, the theory of luminal Ca^{2+} triggered feed-through has had a difficult birth (see reviews by Sitsapesan and Williams (1997), Gyorke et al. (2002) and Laver (2007b). Activation of RyRs by luminal Ca^{2+} was initially attributed exclusively to two different processes: activation either by Ca^{2+} sites on the *luminal* side of the RyR (Sitsapesan and Williams 1995; Ching et al. 2000) or by Ca^{2+} permeation to *cytoplasmic* sites (Herrmann-Frank and Lehmann-Horn 1996; Tripathy and Meissner 1996; Xu and Meissner 1998). The conceptual advance that led to the "luminal triggered feed-through" theory of luminal Ca^{2+}-activation centred on the way single channel recordings were analysed (Laver 2007a). Rather than focussing on the open probability of single channels, the analysis considered separately the durations of channel open and closed events. This offered one major advantage. By studying the regulation of channel closed events by luminal Ca^{2+}, one could be sure that Ca^{2+} only had access to luminal facing Ca^{2+} sites because Ca^{2+} cannot flow through a closed channel.

Synergistic triggering of channel openings by cytoplasmic and luminal Ca^{2+}

Opening of the cardiac RyR has an absolute requirement for Ca^{2+}. In the absence of both cytoplasmic and luminal Ca^{2+}, each channel opens, on average, less than once per minute (Laver and Honen 2008). Figure 2a shows the dependence of RyR2 opening frequency (F_o, defined as the

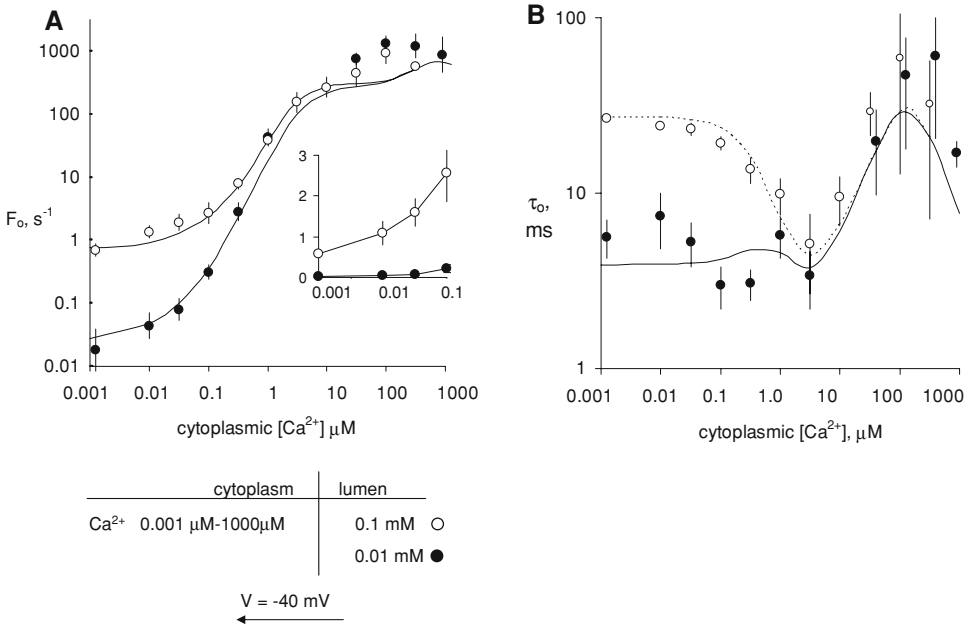

Fig. 2 Activation of RyR2 by luminal and cytoplasmic Ca^{2+}. Cytoplasmic Ca^{2+}-dependencies of opening frequency (**a**) and mean open time (**b**) in the presence of 2 mM ATP taken from Laver and Honen (2008). The experimental conditions are shown beneath each figure. The membrane potential (−40 mV) favours the flow of Ca^{2+} through the channel from the luminal to cytoplasmic baths. Data points show the mean ± standard error. The inset in A shows the same data plotted on a linear scale which shows that higher $[Ca^{2+}]_L$ causes larger activation of the channel by $[Ca^{2+}]_C$. The *curves* show predictions of the "luminal-triggered feed-through" model where the model parameters are given elsewhere (Laver and Honen 2008)

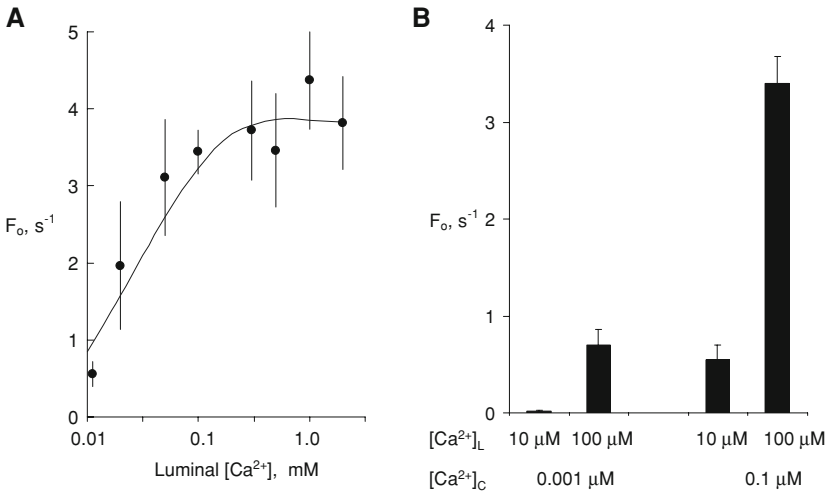

Fig. 3 Luminal Ca^{2+}-dependencies of opening frequency and its dependence on $[Ca^{2+}]_C$. (**a**) The hyperbolic profile luminal Ca^{2+}-dependence ($[Ca^{2+}]_C$ = 0.1 μM) and (**b**) and the magnitude of luminal Ca^{2+}-activation seen in different $[Ca^{2+}]_C$ taken from (Laver and Honen 2008). Note that higher $[Ca^{2+}]_C$ causes larger activation of the channel by $[Ca^{2+}]_L$. The experimental conditions are described in Fig. 2. The *solid curve* shows predictions of the "luminal-triggered feed-through" model

reciprocal of the mean closed duration, *i.e.,* measured in the absence of Ca^{2+} feed-through) on cytoplasmic $[Ca^{2+}]$ in the presence of sub-physiological luminal Ca^{2+} ($[Ca^{2+}]_L$ = 10 μM, filled circle). The corresponding mean open duration under these conditions is shown in Fig. 2b. Cytoplasmic Ca^{2+} increased F_o from 0.01 s^{-1} to a maximum of ~1,000 s^{-1} where the steepest $[Ca^{2+}]_C$-dependence occurred in the range 0.1–10 μM. Importantly, it is clear from Fig. 2a that F_o also depends on $[Ca^{2+}]_L$ but only over a limited range of $[Ca^{2+}]_C$ ($[Ca^{2+}]_C$ < 0.3 μM). The nature of the $[Ca^{2+}]_L$- dependencies of F_o is shown in Fig. 3a. F_o exhibits hyperbolic $[Ca^{2+}]_L$-dependencies with K_a's of 20 μM and upper limits (Fig. 3b) of 3.5 s^{-1} ($[Ca^{2+}]_C$ = 100 nM) and 0.6 s^{-1} ($[Ca^{2+}]_C$ = 1 nM). Figure 3b shows how cytoplasmic Ca^{2+} amplifies the activating effect of luminal Ca^{2+}. In other words, cytoplasmic and luminal Ca^{2+} have a synergistic triggering action on RyR2.

The $[Ca^{2+}]$- and $[Mg^{2+}]$-dependencies of channel opening frequency and mean open duration were accurately fitted by a model based on a tetrameric RyR structure with Ca^{2+} sensing mechanisms (L-, A-, I_1- and I_2-sites) on each subunit (Laver and Honen 2008). An example of this is

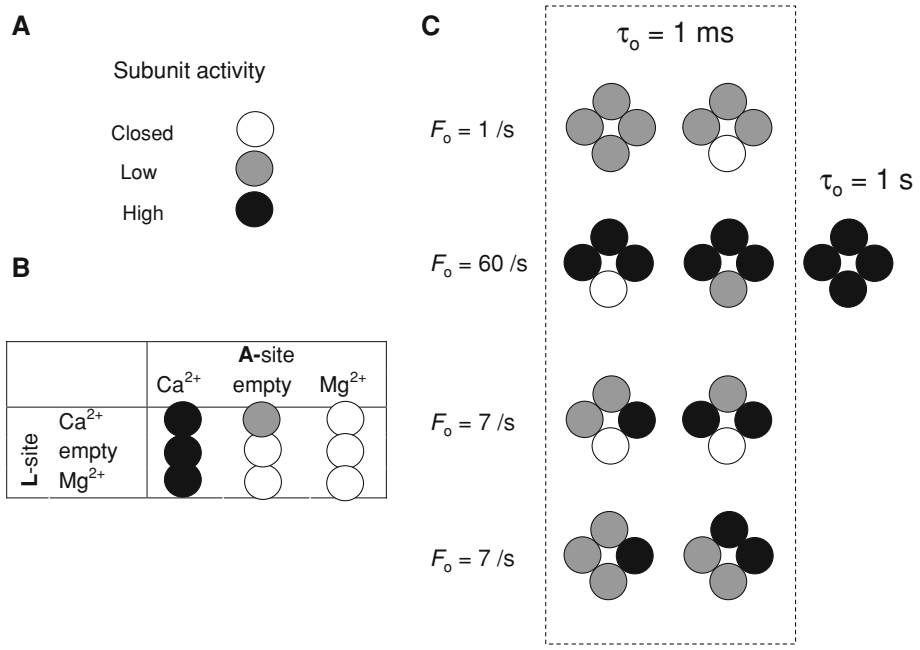

Fig. 4 The scheme for how luminal and cytoplasmic activation sites (A- and L-sites) regulate the gating of RyR2. (**a**) The key for the three different functional states of each of the four RyR2 subunits. (**b**) The contingency table showing the states of each subunit induced by combinations of Ca^{2+} and Mg^{2+} binding to the A- and L-sites. (**c**) The mean open times (τ_o) and opening frequencies (F_o) resulting from the various subunit stoichiometries. A high opening rate (60 s^{-1}) will occur when either 3 or 4 subunits are in the H-state, an intermediate opening rate (7 s^{-1}) when three subunits are not in the C-state *and* at least 1 subunit is in the H-state, and a low opening rate (1 s^{-1}) when no subunits are in the H-state but at least 3 are in the L-state. The mean open time of the channel is 1 ms when three or less subunits are in the H-state (the states enclosed by the *dashed line*). When all four are in the H-state the channel open configuration is stabilised such that the mean open time is extended 1,000-fold

shown in Fig. 2 (solid curves) where the model quantitatively predicts the Ca^{2+}-dependencies of F_o and mean open times over the full experimental range. In this model, it is envisaged that the RyR opens once a minimum of three of its subunits have been activated by combinations of the A- and L-sites (Figure 4c). Each subunit could independently undertake three activation states, namely, closed, low and high (Fig. 4a); so named after their relative contributions to the frequency of channel opening. The ability of the A- and L-sites to produce these three states is given in the contingency table in Fig. 4b. For example, Ca^{2+} bound to the A-site induces a high-activity conformation regardless of the ion occupancy of the L-site, whereas the L-site will only cause a low activity state provided the A-site is not occupied by Mg^{2+}. Figure 4c shows how the various subunit stoichiometries contribute to the opening frequency of the channel.

This model explains the synergistic activation of F_o by $[Ca^{2+}]_L$ and $[Ca^{2+}]_C$ in two ways; (1) Ca^{2+}-activation of subunits via the A-site will reduce the number of subunits that need to be activated by L-site to achieve a channel opening and (2) mixtures of subunits in low and high states (i.e., activated by L- and A-sites, respectively) cause higher opening frequencies (F_o) than what can be produced by subunits in the low state alone.

The fact that the RyR2 opening frequency depends on synergistic stimulation of the channel by cytoplasmic (A-site) and luminal (L-site) domains provides one explanation for how perturbations of the RyR2 cytoplasmic domains by CPVT linked mutations lead to malregulation of these receptors by both luminal and cytoplasmic Ca^{2+} (Yang et al. 2006). According to the above model, an increased response of the A-site to cytoplasmic Ca^{2+} will amplify the activating effect of luminal Ca^{2+} on RyR2 opening frequency.

Luminal-triggered Ca^{2+} feed-through

Ca^{2+} feed-through provides another means by which cytoplasmic domains of the RyR can regulate its activation by luminal Ca^{2+}. The idea is that Ca^{2+} ions in the SR lumen can stimulate RyR2 activity by permeating the channel and binding to Ca^{2+} regulatory sites on its cytoplasmic domains. Hence, altering the cytoplasmic sites should be able to alter RyR2 activation by luminal Ca^{2+}. The "feed-through" hypothesis for luminal Ca^{2+}-activation of RyRs from skeletal and cardiac muscle was first proposed by Meissner's group (Tripathy and Meissner 1996). The Ca^{2+} feed-through mechanisms provided an explanation for the correlation

between luminal Ca^{2+}-activation and the size of the Ca^{2+} flux through the channel. Initially, the feed-through hypothesis did not gain general acceptance because it did not explain why luminal Ca^{2+}-activation was altered by enzyme digestion of luminal domains of the RyR (Ching et al. 2000). However, the formulation of a quantitative theory for luminal Ca^{2+}-activation showed how luminal Ca^{2+} triggering sites and Ca^{2+} feed-through to cytoplasmic Ca^{2+}-activation sites both contribute to this phenomenon (Laver 2007a).

Analysis of RyR open durations (i.e., the times when there is Ca^{2+} feed-through) has provided five lines of evidence that luminal ions have access to cytoplasmic sites via the pore.

(1) The ability of luminal Ca^{2+} to activate or inhibit depends on the membrane potential. Positive membrane potentials which oppose Ca^{2+}-feed-through decrease RyR sensitivity to luminal activation and inhibition. This suggests that activation and inhibition by luminal Ca^{2+} relies on the Ca^{2+} flux and that these effects are mediated by the cytoplasmic A- and I_2-sites (Tripathy and Meissner 1996; Laver 2007a).
(2) The inhibitory action of luminal Ca^{2+} is reduced if strong Ca^{2+} buffering is present in the cytoplasmic bath suggesting that luminal ions must pass through the cytoplasmic solution to reach the inhibitory I_2-site (Tripathy and Meissner 1996).
(3) The combined effects of cytoplasmic and luminal Ca^{2+} on mean open duration are non-additive indicating that luminal and cytoplasmic ions compete for common activation sites (Laver 2007a).
(4) Since Mg^{2+} can inhibit RyRs by binding to the cytoplasmic Ca^{2+}-activation site (see above) then one would expect feed-through of luminal Mg^{2+} to cause a reduction in mean open duration (Mg^{2+} and Ca^{2+} have identical permeability in the pore (Tinker et al. 1992). This is just what has been reported (Xu and Meissner 1998; Laver and Honen 2008).
(5) Finally, what is particularly relevant to the present discussion is that changes in the response of RyR2 mean open time to cytoplasmic Ca^{2+} also cause corresponding changes in the channels response to luminal Ca^{2+} (Fig. 5). This is in accord with the luminal-triggered feed-through model which predicts that treatments that increase the durations of openings induced by Ca^{2+} binding to the cytoplasmic *A*-site will also increase the channels responsiveness to luminal Ca^{2+}. Figure 5a shows dependencies of mean open time, τ_o, of RyR2 on cytoplasmic Ca^{2+} in the presence of channel regulators that alter the sensitivity of the A-site to Ca^{2+}. The experimental conditions ($[Ca^{2+}]_L < 0.1$ mM, +40 mV) are set to minimise Ca^{2+} feed-through so that the RyR is responding primarily to cytoplasmic Ca^{2+}. Under these conditions and in the presence of ATP (2 mM), τ_o showed a monotonic increase with increasing $[Ca^{2+}]_C$. ATP plus Mg^{2+} (0.22 mM free) caused less activation than ATP alone because Mg^{2+} competes with Ca^{2+} for the A-site. However, ATP plus the volatile anaesthetic, halothane (20 mM) caused more activation than ATP alone. These effects can be explained by the model in terms of changes in channel activation mediated by the A-site alone (solid curves). Halothane changed the Ca^{2+} affinity of the A-site from 1 to 0.5 μM, whereas Mg^{2+} changed the apparent affinity to 7 μM. In Fig. 5b the luminal activation of RyRs is shown in the presence of the same cytoplasmic modulators except that the experimental conditions ($[Ca^{2+}]_C = 0.1$ μM, −40 mV) are set to *promote* Ca^{2+} feed-through. In the presence of ATP alone, τ_o shows activation at low $[Ca^{2+}]_L$ and inhibition at high $[Ca^{2+}]_L$ which, in the model (solid curves), correspond to the effects of the A- and I_2-sites. In the presence of ATP and Mg^{2+}, the activating phase of the $[Ca^{2+}]_L$-dependence is attenuated, whereas in the presence of halothane this activation is amplified. Both these effects are accurately explained by the model in terms of the same changes in the A-site affinity as derived from fitting to the model to data in Fig. 5a.

The observation that the response of RyR2 τ_o to cytoplasmic Ca^{2+} also causes corresponding changes in the channels response to luminal Ca^{2+} provides another explanation for how perturbations of the RyR2 cytoplasmic domains by CPVT linked mutations. In support of this idea, it was found that activation of RyR2 by DPc10 peptide, which is believed to mimic the effects CPVT mutations in RyR2 (Yamamoto and Ikemoto 2002), relied on a flow of Ca^{2+} through the channel from lumen to cytoplasm (Laver et al. 2008). In the absence of such a flow, DPc10 failed to activate RyRs. This study also showed that the effects of DPc10 could be explained almost entirely by an increase in the Ca^{2+} sensitivity of the cytoplasmic A-site.

Conclusion

The data show that changes in the Ca^{2+} sensitivity of the cytoplasmic A-site strongly influence the ability of luminal Ca^{2+} to activate the channel, thus providing an explanation for how luminal Ca^{2+}-activation of RyR2 is increased by mutations in their cytoplasmic domains. Firstly, the flow of luminal Ca^{2+} through the channel can regulate channel opening via the A-site. Secondly, Ca^{2+}-activation of subunits via the A-site will reduce the number of subunits that need to be activated by luminal Ca^{2+} at the L-site to achieve channel opening. This accounts for the synergy in the

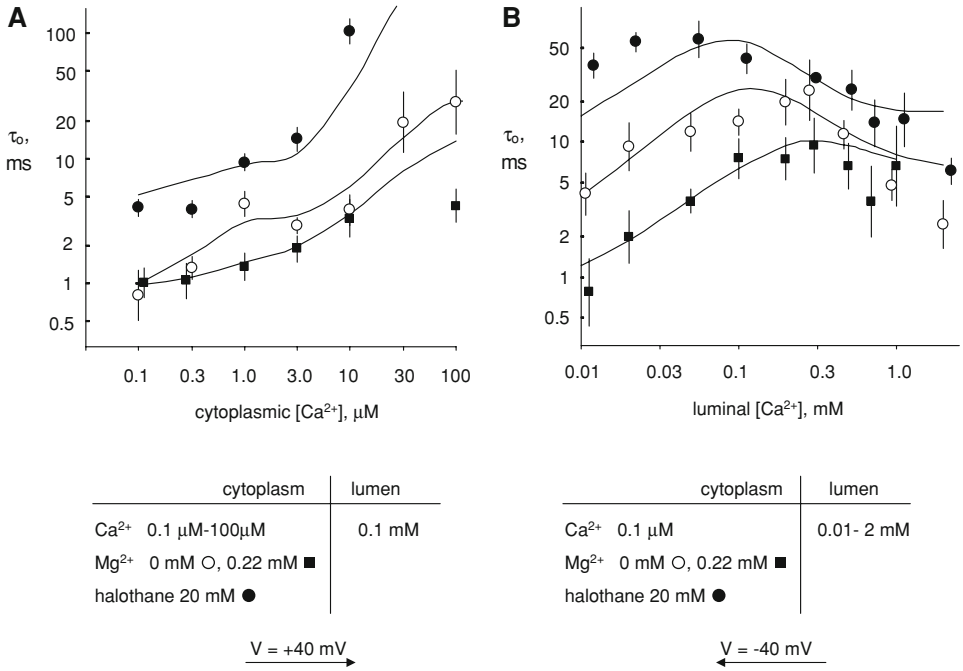

Fig. 5 The correlation between stabilisation of RyR2 openings due to binding of cytoplasmic Ca^{2+} to the A-site (**a**) and stabilisation of RyR2 openings due to luminal Ca^{2+} (**b**). The experimental conditions are shown beneath each figure. ATP (2 mM) was present in all the experiments. RyR mean open times were measured in the presence of three ligand conditions 2 mM ATP-(*open circle*), 2 mM ATP + 0.22 mM Mg^{2+} (free)-(*filled square*) and 20 mM halothane (*filled circle*). In A, the membrane potential of +40 mV prevents the flow of Ca^{2+} from the luminal to cytoplasmic sides of the channel so that channel open times are due almost entirely to the activating effect of cytoplasmic Ca^{2+} at the A-site. In B, the membrane potential of −40 mV favours the flow of Ca^{2+} from the luminal to the cytoplasmic sides. Halothane which increases the effect of $[Ca^{2+}]_C$ on τ_o amplifies the increase in τ_o in response to luminal Ca^{2+}, whereas Mg^{2+} which decreases the effect of $[Ca^{2+}]_C$ on τ_o reduces the rising phase of τ_o in response to luminal Ca^{2+}

$[Ca^{2+}]_L$-dependent and $[Ca^{2+}]_C$-dependent increases in channel opening frequency. Therefore, cytoplasmic CPVT mutations that increase the Ca^{2+}-sensitivity of the A-site will render the RyR more sensitive to the level of Ca^{2+} loading inside the SR, thereby explaining so-called 'store-overload Ca^{2+} release' and the aberrations underlying certain cardiac diseases.

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