Ca^{2+} stores regulate ryanodine receptor Ca^{2+} release channels *via* luminal and cytosolic Ca^{2+} sites

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Summary

1. In muscle, intracellular calcium concentration, hence skeletal muscle force and cardiac output, is regulated by uptake and release of calcium from the sarcoplasmic reticulum. The ryanodine receptor (RyR) forms the calcium release channel in the sarcoplasmic reticulum.

2. The free [Ca^{2+}] in the sarcoplasmic reticulum regulates the excitability of this store by stimulating the Ca^{2+} release channels in its membrane. This process involves Ca^{2+} sensing mechanisms on both the luminal and cytoplasmic side of the RyR. In the cardiac RyR these have been shown to be: a luminal Ca^{2+}-activation site (*L*-site, 60 μM affinity), a cytoplasmic activation site (*A*-site, 0.9 μM affinity) and a cytoplasmic Ca^{2+}-inactivation site (I_2-site, 1.2 μM affinity).

3. Cardiac RyR activation by luminal Ca^{2+} occurs by a multi-step process dubbed "luminal-triggered Ca^{2+} feed-through". Ca^{2+} binding to the *L*-site initiates brief (1 ms) openings at a rate of up to 10 per second. Once the pore is open, luminal Ca^{2+} has access to the *A*-site (producing up to 30-fold prolongation of openings) and to the I_2-site (causing inactivation at high levels of Ca^{2+} feed-through).

4. Reviewed here are: the evidence for the principal aspects of the "luminal-triggered Ca^{2+} feed-through" model, the properties of the various Ca^{2+}-dependent gating mechanisms and their likely role in controlling SR Ca^{2+} release in cardiac muscle.

5. The model makes the following important predictions: There will be a close link between luminal and cytoplasmic regulation of RyRs. Any cofactor that prolongs channel openings triggered by cytoplasmic Ca^{2+} will also promote RyR activation by luminal Ca^{2+}.

6. Luminal Mg^{2+} (1 mM) is essential for the control of SR excitability in cardiac muscle by luminal Ca^{2+}.

7. The different RyR isoforms in skeletal and cardiac muscle will be controlled quite differently by luminal milieu. For example, [Mg^{2+}] in the SR lumen (~1 mM) can strongly inhibit RyR2 by competing with Ca^{2+} for the *L*-site whereas RyR1 is not affected by luminal Mg^{2+}.

Introduction

Intracellular calcium stores are critical to the function of most cell types.[1] Ca^{2+} uptake and release controls contraction in muscle and has an important role in synaptic transmission and membrane excitability in neurons. The primary intracellular stores in excitable cells are the endoplasmic reticulum (ER) and its specialisation in muscle called the sarcoplasmic reticulum (SR). The free [Ca^{2+}] within the lumen of the ER/SR (0.3 to 1.0 mM[2,3]) is more than 1000-fold higher than in the cytoplasm. This large concentration gradient is maintained by the SERCA Ca^{2+} pump in the ER/SR membrane.

Ryanodine receptors (RyRs) mediate most of the Ca^{2+} release from the SR that occurs in muscle and neurons. Three mammalian isoforms have been cloned and sequenced. RyR1 and RyR2 are predominantly expressed in skeletal and cardiac muscle, respectively, and in most neurons RyR2 is the dominant isoform.[4] RyR3 is found to some extent in most tissues. RyRs are normally triggered by electrical depolarisation of the cell surface membrane. The different isoforms mediate different modes of transduction from the electrical signal in the surface membrane to the chemical signal in the ER/SR. RyR2 and RyR3 are triggered by Ca^{2+} influx through voltage sensitive Ca^{2+} channels in the surface membrane (L-type channels called dihydropyridine receptors, DHPRs) whereas RyR1s are triggered by a direct conformational link between the RyRs and the DHPRs.[5] Since cytoplasmic Ca^{2+} is a RyR agonist, the rise in cytoplasmic [Ca^{2+}] ([Ca^{2+}]$_C$) that accompanies Ca^{2+} release further activates RyRs, providing positive feedback for Ca^{2+} release (this phenomenon is known as Ca^{2+} induced Ca^{2+} release, CICR[6]). In respect of the regenerative nature of Ca^{2+} release the ER/SR is considered to be a chemically excitable organelle.[4]

The excitability of muscle ER/SR is substantially increased by increasing the luminal Ca^{2+} load (luminal [Ca^{2+}], [Ca^{2+}]$_L$).[4,7,8] Early studies on muscle cells and isolated SR vesicles found that the rate of Ca^{2+} release had a dependence on [Ca^{2+}]$_L$ that was too steep to be explained by an increase in the [Ca^{2+}] gradient across the SR membrane (see reviews 9,10). Thus, these studies indicated that [Ca^{2+}]$_L$ must somehow control the Ca^{2+} permeability of the membrane. [Ca^{2+}]$_L$-dependent Ca^{2+} release is believed to drive pacemaking and rythmicity in smooth and cardiac muscle[11-13] and in neurons.[4] In heart, the free [Ca^{2+}] within the SR most likely varies within the range ~0.3 to 1.0 mM during the normal beating cycle[2,3] but during SR overload it can increase to 5 mM.[14] The importance of [Ca^{2+}]$_L$ in cardiac function is highlighted by the cardiac arrhythmias associated with excessive [Ca^{2+}]$_L$.[15,16]

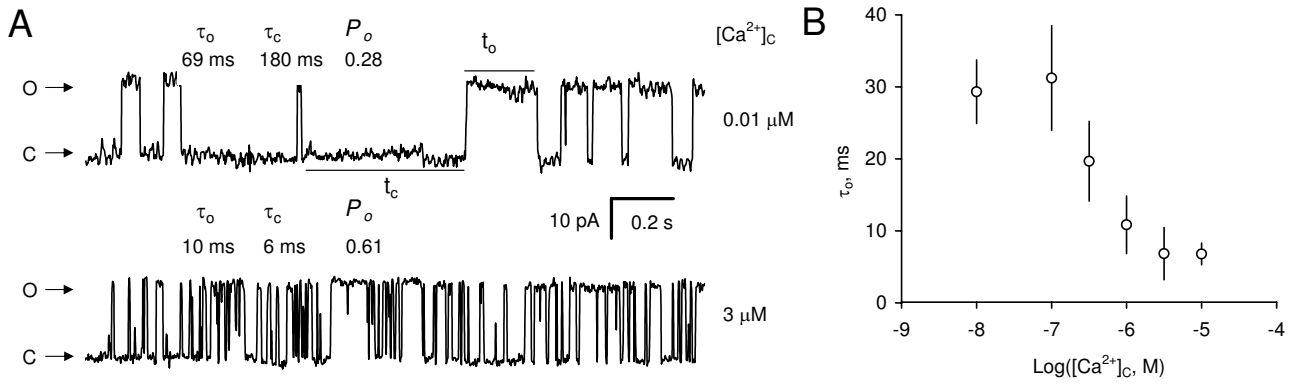

Figure 1. The effects of $[Ca^{2+}]_C$ on the activity of RyR2. (A) *Single channel recording of a RyR2 in the presence of 100 μM $[Ca^{2+}]_L$ and 2 mM cytoplasmic ATP (cytoplasm at -40 mV with respect to the lumen). Channel opening (labelled O) is marked by upward current steps from the closed current level (labelled C). Examples of channel dwell-times in the open and closed state are indicated by t_o and t_c, respectively. $[Ca^{2+}]_C$ is indicated at the right of each trace along with the mean open time, mean closed time and open probability of the channel. Increasing $[Ca^{2+}]_C$ caused activation of channel activity (measured by open probability, P_o) via a large reduction in the closed dwell-times (τ_c). In addition, $[Ca^{2+}]_C$ decreased the mean open dwell-times (τ_o) which is indicative of a channel inactivating process. The inactivation is minor compared to the activation so that activation dominates in the overall effect of $[Ca^{2+}]_C$.* ***(B)*** *Grouped data showing the $[Ca^{2+}]_C$–dependence of τ_o taken from Laver.[24]*

Insights into luminal Ca^{2+}-activation from single channel studies

Experiments on individual RyRs have obtained very detailed information about the activation and inhibition mechanisms associated with luminal Ca^{2+}. These measurements involved isolating SR vesicles from muscle and incorporating them into artificial lipid bilayers.[17] When SR vesicles fuse with the bilayer, the RyRs embedded in the vesicle membrane become incorporated into the bilayer. Once this happens it is possible to monitor their opening and closing (gating) by measuring the current through the membrane in response to an applied electrochemical gradient. Single RyR channel currents are essentially stochastic square waves that can be characterised by their amplitude and by the dwell-time of channel open and closed current levels (*e.g.* See Figure 1). An overall picture of channel gating is encapsulated in the mean open and closed dwell-times (τ_o and τ_c) and by the open probability (P_o, this is the fraction of time the channel is in a conducting state; *e.g.* $P_o = 1$, the channel never closes; $P_o = 0$, the channel is completely inactive, for further details see reference18). From these kinetic analyses, it has been possible to make inferences about the mechanisms underlying the gating processes of the RyR.

In the early 1990's, investigations on isolated RyRs in artificial bilayers found that the activity of RyRs was indeed modulated by $[Ca^{2+}]_L$[19] but just how luminal Ca^{2+} was doing this was not understood. Since Fabiato[6] had shown that efflux of Ca^{2+} from the lumen stimulates nearly all of the Ca^{2+} release from the cardiac SR, one could envisage that luminal Ca^{2+} could flow through one RyR and stimulate the opening of nearby RyRs in the cell. However, it was not clear how CICR would work in the context of an isolated RyR (Could the Ca^{2+} efflux through a channel further stimulate that channel to open even more?). Hence, in the following decade single-channel experiments were interpreted in two quite different ways. The "true luminal" hypothesis attributed luminal regulation to Ca^{2+} sites on the *luminal* side of the RyR[20] whereas the "feed-through" hypothesis proposed that luminal Ca^{2+} permeates the pore and binds to *cytoplasmic* Ca^{2+} sites.[21-23] In marshalling the arguments for and against these hypotheses[9,10] it appeared luminal regulation of RyRs somehow involved Ca^{2+} sensing mechanisms on both the luminal and cytoplasmic side of the channel.

Only recently has it been possible to unravel the complex interplay that exists between these mechanisms. Experiments by Laver[24] led to the proposal of a unified theory for activation of cardiac RyRs dubbed "luminal-triggered Ca^{2+} feed-through" which integrates the "feed-through" and "true luminal" hypotheses to provide a quantitative understanding of the Ca^{2+} permeability of the ER/SR. The proposal is that one luminal Ca^{2+} site (*L*-site) and two cytoplasmic Ca^{2+} sites (*A*- and I_2-sites) on RyR2 determine its regulation by luminal Ca^{2+} (Figure 2). The RyR2 can be opened by Ca^{2+} binding to either the *A*-site or the *L*-site whereas Ca^{2+} binding to the I_2-site closes the channel. In the virtual absence of cytoplasmic Ca^{2+}, as is the case during cardiac diastole, the binding of Ca^{2+} to the *L*-site on its own can activate channel openings of ~1 ms duration at rates up to 10 s^{-1}. Once the channel is open, the flux of Ca^{2+} from the luminal to cytoplasmic sides of the channel (Ca^{2+} feed-through) increases $[Ca^{2+}]_C$ in the vicinity of the *A*-site and produces up to 30-fold prolongation of channel openings. In addition, the

cytoplasmic Ca^{2+}-inactivation site (I_2-site) causes a reduction in channel open durations at high levels of Ca^{2+} feed-through. This review evaluates the evidence for the principal aspects of the "luminal-triggered Ca^{2+} feed-through" model, the properties of the various Ca^{2+}-dependent gating mechanisms and their likely role in controlling Ca^{2+} release from the SR.

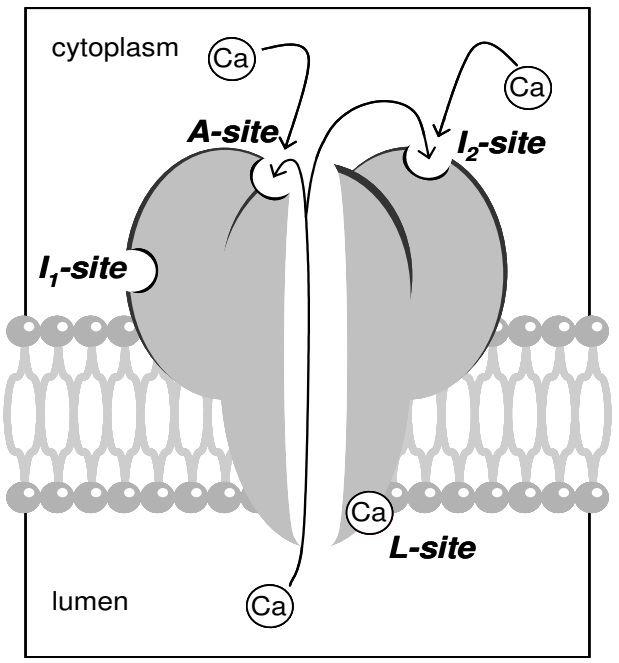

Figure 2. The process of "luminal-triggered Ca^{2+} feed-through" *(*after *Laver, 2007[24]). An illustration of the three Ca^{2+} sensing sites that have been linked to regulation of cardiac RyRs by luminal Ca^{2+}: the luminal activation site (*L*-site, 60 μM affinity), the cytoplasmic activation site (*A*-site, 1-10 μM affinity) and the cytoplasmic Ca^{2+}-inactivation site (*I_2*-site, 1 μM affinity). Ca^{2+} binding at the* L*-site is sufficient to activate channel openings whereupon Ca^{2+} flow through the channel (Ca^{2+} feed-through) causes either additional activation* via *the* A*-site or inactivation* via *the* I_2*-site. Also shown is the so-called Ca^{2+}/Mg^{2+} inhibition site (*I_1*-site, 10 mM affinity, previously referred to as the* I*-site). This site plays a role in cytoplasmic regulation of RyRs.*

A-site for $[Ca^{2+}]_C$-activation

At an early stage RyRs were found to be activated by μM $[Ca^{2+}]$ in the cytoplasm.[25,26] It is now generally recognised that this is mediated by a class of cytoplasmic facing Ca^{2+} sites referred to as *A*-sites.[27] In the absence of luminal Ca^{2+} or cytoplasmic cofactors such as ATP, $[Ca^{2+}]_C$ activates RyR2 from a basal P_o of approximately zero to P_o = 0.6 with a half-activating $[Ca^{2+}]_C$ (K_a) of ~5 μM (Figure 3A).[28,29] Activation is mediated primarily *via* a decrease in τ_c or an increase in channel opening rate (opening rate = $1/\tau_c$) whereas τ_o (~1 ms) showed only a minor dependence on $[Ca^{2+}]_C$ (Figure 3B, C). The *A*-site gating properties are strongly affected by cofactors such as ATP, caffeine and sulmazole.[19,30,31] On their own these cofactors do not trigger channel openings; instead they function to enhance RyR response to cytoplasmic Ca^{2+}. The result is an increase in both τ_o and opening rate which decreases the $[Ca^{2+}]_C$ needed for activation (this is shown for the case of ATP in Figure 3). In the presence of ATP, τ_o acquired a strong dependence on $[Ca^{2+}]_C$ (Figure 3B).

Although the precise location of the *A*-site is still unknown, mutation experiments carried out on RyR1 provide an indication of its general location. Truncated RyR1s comprised of only the C-terminal 1030 amino acids (RyRC; aa4007-aa5037) formed Ca^{2+} channels with *A*-sites[32] indicating that the *A*-site resides somewhere in the pore forming region of the RyR. Moreover, the aa4032 has been linked to the *A*-site gating mechanism because the E4032A substitution decreased RyR1 sensitivity to cytoplasmic Ca^{2+}-activation by four orders of magnitude[33] (but also see Fessenden *et al.*[34])

I_1-site for cytoplasmic Ca^{2+}/Mg^{2+}-inhibition

It has been long known that RyRs can be inhibited by mM cytoplasmic concentrations of divalent cations including Ca^{2+} and Mg^{2+} (~1 mM for RyR1 and ~10 mM for RyR2.[35]) This inhibitory action is mediated by low affinity non-specific divalent cation sites, previously dubbed *I*-sites.[27] In order to distinguish this process from the other inactivation process (see next section) the sites are now called I_1-sites and the process is referred to as inhibition rather than inactivation. The I_1-sites, together with the *A*-sites, produce a bell-shaped dependence of P_o on $[Ca^{2+}]_C$ (*e.g.* Figure 3A). Unlike the *A*-sites, the divalent cation affinity of the I_1-sites is unaffected by the cytoplasmic cofactors ATP and caffeine.[27,36] The precise location of the I_1-site is unknown. RyRC does not exhibit Ca^{2+}/Mg^{2+} inhibition[32] indicating that the I_1-sites reside somewhere in the N-terminal 4007 amino acids. Several studies have implicated aa1873-1903, aa1641-2437 and aa615 with the I_1-sites.[37] In cardiac muscle, the role of I_1-mediated inhibition is obscure because the affinity of the I_1-site is at least an order of magnitude above the intracellular $[Ca^{2+}]$ and $[Mg^{2+}]$. However, the skeletal muscle isoform is much more sensitive to I_1-mediated inhibition and the binding of cytoplasmic Mg^{2+} to this site has been proposed to be the primary restraint on Ca^{2+} release in resting skeletal muscle.[38]

I_2-site for $[Ca^{2+}]_C$-inactivation

Early studies of SR Ca^{2+} release detected a Ca^{2+}-inactivation mechanism that operates at μM $[Ca^{2+}]_C$. Measurements of global Ca^{2+} release in skeletal and cardiac muscle revealed a phenomenon[39-41] in which cytoplasmic Ca^{2+} causes rapid (< 100 ms) and partial inactivation of SR Ca^{2+} release with a half-maximal effect at 0.3 μM. The kinetics of localised Ca^{2+} release "Ca^{2+} sparks" in cardiac

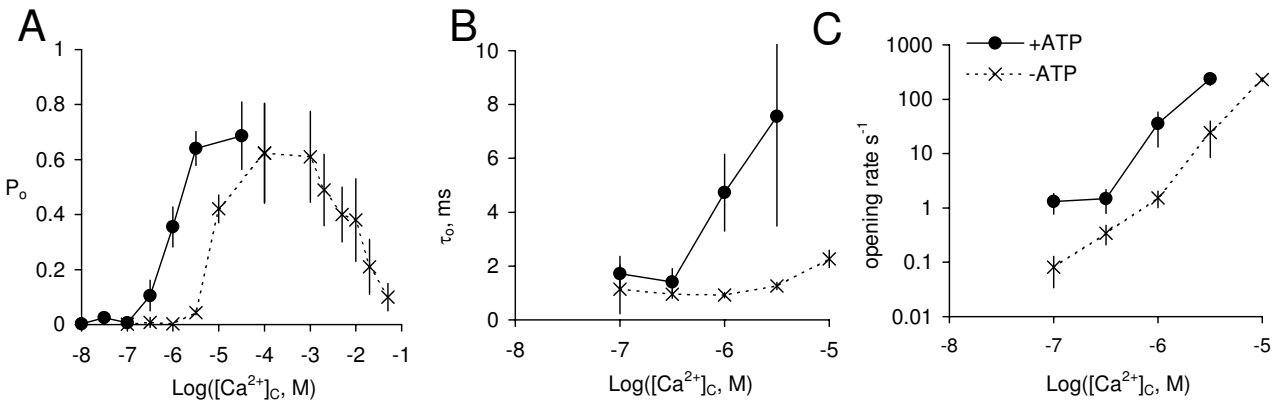

Figure 3. The effect of ATP and $[Ca^{2+}]_C$ on the activity of RyR2 *at +40 mV and 100 μM $[Ca^{2+}]_L$ (from Laver[24]).* P_o ***(A),*** τ_o ***(B)*** *and opening rate ($1/\tau_c$) **(C)** were measured in the presence (●) and absence (×) of ATP (2 mM). Data points show the means ± sem of 3-9 measurements. The $[Ca^{2+}]_C$–dependent increase in* P_o *at μM concentrations is due to the* A*-site whereas the inhibition seen at mM concentrations (A, ×) is due to the* I_1*-site. RyR2 gating properties associated with the* A*-site were strongly affected by ATP. ATP increases RyR2 activation in response to Ca^{2+} (K_a for $[Ca^{2+}]_C$ was decreased from 6 μM to 1 μM). Note that the* τ_o *values here where the membrane voltage opposes Ca^{2+} feed-through are much smaller than those in Figure 1B where the membrane voltage favoured Ca^{2+} feed-through.*

myocytes showed that termination of Ca^{2+} sparks was due to inactivation with a refractory period of 40 ms.[42] Curiously, the advent of single channel recording did not reveal a corresponding Ca^{2+}-inactivation mechanism in RyRs until recently when it was reported that under certain experimental conditions $[Ca^{2+}]_C$ could substantially decrease τ_o.[24] When RyR2 was activated by a combination of cytoplasmic ATP and Ca^{2+} feed-through, τ_o was relatively long (*e.g.* top trace in Figure 1A). Increasing $[Ca^{2+}]_C$ caused a decrease in τ_o (Figure 1A, bottom trace) with a half-maximal effect at ~1 μM (Figure 1B). These results pointed to a Ca^{2+}-dependent inactivation mechanism that operates *via* a cytoplasmic Ca^{2+} site (the I_2-site) with μM affinity. Moreover, the I_2-site produced only partial inactivation (measured as a 20-40% reduction in P_o) which is consistent with the inactivation properties of the SR Ca^{2+} release in cells. This Ca^{2+}-inactivation phenomenon seems to have escaped identification in single-channel studies for some time. A likely reason for this is that it is masked by Ca^{2+}-activation of RyR which occurs over the same range of $[Ca^{2+}]_C$. Reports from earlier single-channel studies, that activation by $[Ca^{2+}]_C$ caused shorter channel openings than activation by ATP or caffeine,[19,30,31] suggest that this phenomenon had already been unknowingly observed. As yet, the amino acid residues comprising the I_2-site are completely unknown.

L-site for luminal Ca^{2+}-activation

A clean measurement of the luminal-site mediated gating properties is difficult to achieve because the $[Ca^{2+}]_L$ required to open the channel can also sustain a level of Ca^{2+} feed-through that could produce *A*-site mediated gating phenomena. Therefore, the gating properties of the luminal sites have been measured under conditions where Ca^{2+} feed-through was either small or non-existent. Experimental strategies that have achieved this are: 1) to diminish or even reverse the lumen-to-cytoplasmic Ca^{2+} flux by applying high $[Ca^{2+}]_C$ or positive membrane voltages and 2) to restrict analysis to channel properties of the closed pore when Ca^{2+} feed-through is non-existent. Closed channel properties such as τ_c and opening rate should not be influenced by Ca^{2+} feed-through because the closed channel can't conduct Ca^{2+}.

The earliest demonstration of luminal facing Ca^{2+} sites was that luminal Ca^{2+}-activation could be abolished by tryptic digestion of the luminal side of RyR2.[43] There is now good evidence for at least two Ca^{2+} sensing mechanisms for RyR2 activation on the luminal side of the membrane. Gyorke and Gyorke[31] found that under conditions where the net Ca^{2+} flux was cytoplasmic-to-luminal, increasing luminal Ca^{2+} from 20 μM to 10 mM produced a 10-fold increase in P_o. A subsequent study showed that this could not be reversed by returning $[Ca^{2+}]_L$ to μM concentrations.[44] The nature of this Ca^{2+} sensing mechanism was revealed to be dissociation of the luminal Ca^{2+}-buffering protein, calsequestrin, from the RyR2 which occurs when $[Ca^{2+}]_L$ exceeds 2 mM. This mechanism could come into play when SR becomes overloaded in pathological situations of stress or cardiac ischaemia and reperfusion. In muscle, calsequestrin is trapped in the SR so that it can reassociate with the RyR once $[Ca^{2+}]_L$ returns to physiological levels.

A second sensor for luminal Ca^{2+} (*L*-site, Figure 2) has been identified which regulates RyR2 activity in the physiological range of $[Ca^{2+}]_L$ and at levels up to 2 mM.[24] In the absence of cytoplasmic Ca^{2+}, ATP and Ca^{2+} feed-through (analysis was restricted to opening rates), RyR2

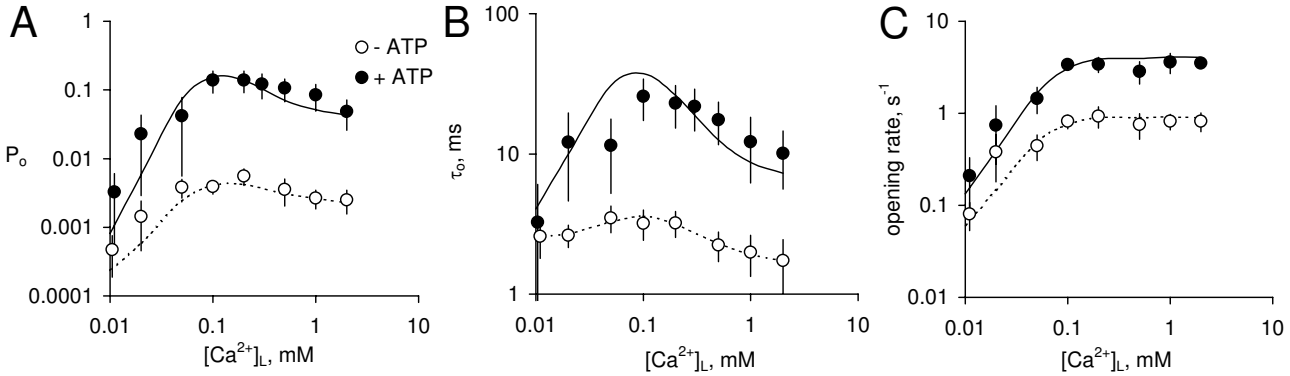

Figure 4. The effects of ATP on the $[Ca^{2+}]_L$-dependent gating of RyR2. *(*after Laver[24]*) **(A)** The open probability of RyRs (100 nM $[Ca^{2+}]_C$ and voltage = -40 mV) in the presence of 2 mM ATP (●) and in its absence (○). Also shown are the corresponding τ_o **(B)** and opening rates **(C)**. Solid and dashed curves show the fit to the data of the "luminal-triggered Ca^{2+} feed-through" using parameters given elsewhere.[24] The data points show mean ± sem of 3-18 measurements. Hill fits (not shown) to the opening rate reveal that 2 mM ATP increases the maximal opening rate from 0.8 ± 0.1 to 4.0 ± 0.4 without significantly changing K_a for $[Ca^{2+}]_L$ (K_a = 45 ± 8 μM and 60 ± 20 μM in the absence and presence of ATP, respectively).*

could be reversibly activated by luminal Ca^{2+} albeit to a much lesser extent than achievable with the *A*-site (*c.f.* maximal opening rates of 1 s^{-1} for luminal Ca^{2+} (Figure 4C) and 300 s^{-1} for cytoplasmic Ca^{2+} (Figure 3C)). Luminal activation was mediated primarily by an increase in channel opening rate with a K_a of 45 μM and Hill coefficient of 2 (Figure 4C). ATP increased the opening rate by 4-fold but did not significantly change the K_a (60 μM) or Hill coefficient (1.6). The K_a and Hill coefficient values suggested the involvement of multiple Ca^{2+} binding sites with an affinity of 60 μM which means that there could be one site on each RyR subunit. These values were also found to be independent of voltage indicating that ions did not cross the membrane to get to their effector site; further supporting the proposition that the *L*-site is indeed located on the luminal side of the membrane.

Ca^{2+} feed-through

There is now strong evidence that the Ca^{2+} flux through the RyR pore is sufficient to raise the local $[Ca^{2+}]_C$ enough to cause activation of RyRs. Bilayer studies have shown that Ca^{2+} feed-through couples RyR openings such that the opening of one channel can increase the opening rate of adjacent RyRs by up to 20-fold.[24,36,37] Coupling was abolished by either removal of luminal Ca^{2+} or by voltages that oppose Ca^{2+} feed-through. However, for isolated RyRs, it has been generally accepted that the effects of Ca^{2+} feed-through can only be observed in the channel open state properties such as τ_o.

Activation of RyRs by Ca^{2+} feed-through will be considered here first in channels that are modified by ATP because the effects of Ca^{2+} feed-through are nearly undetectable in the absence of stimulating cofactors. In the presence of ATP, caffeine and sulmazole, RyR activity has a bell-shaped dependence on $[Ca^{2+}]$.[19,21,22,24] In the case of ATP, increasing $[Ca^{2+}]_L$ from virtually zero to 100 μM (at -40 mV which favours Ca^{2+} feed-through) substantially increases the activity of the RyRs whereas a further increase to 1 mM decreases channel activity. This bell-shaped dependence was also reflected in τ_o which for RyR2, had values of 1 ms, 30 ms and 10 ms for $[Ca^{2+}]_L$ of 0, 100 and 1 mM, respectively (Figure 4B).

This bell-shaped $[Ca^{2+}]_L$-dependence is clear evidence for Ca^{2+}-dependent activation and inactivation mechanisms. Several pieces of evidence suggest that cytoplasmic structures mediate these effects. Firstly, both phenomena are closely correlated with the magnitude of Ca^{2+} feed-through.[21,24] Biasing the membrane voltage against Ca^{2+} feed-through shifts the bell-shaped $[Ca^{2+}]_L$-dependence to higher concentrations. Second, heavy Ca^{2+} buffering of the cytoplasmic bath alleviates inactivation indicating that luminal Ca^{2+} must be traversing the cytoplasmic solution to reach the inactivation site.[21] Finally, the activating and inactivating effects of $[Ca^{2+}]_L$ and $[Ca^{2+}]_C$ are not additive. Thus luminal Ca^{2+} does not affect τ_o at elevated $[Ca^{2+}]_C$ and *vice versa*,[24] indicating that luminal and cytoplasmic Ca^{2+} compete for the same activating and inactivating sites.

Since the *A*-site is the only site linked to RyR activation by $[Ca^{2+}]_C$ there is little doubt that activation by Ca^{2+} feed-through is caused by this site. It was originally thought that inactivation by $[Ca^{2+}]_L$ was mediated by the I_1-site[22] because until recently this was the only form of Ca^{2+}-dependent inhibition that had been clearly identified in RyRs. However, it is unlikely that the I_1-sites are involved in luminal inactivation because inactivation has a similar $[Ca^{2+}]_L$ sensitivity in RyR1 and RyR2[21,22] whereas the Ca^{2+} sensitivity of the I_1-sites differ 10-fold between the two isoforms. On the other hand, the I_2-site is at least 50-fold

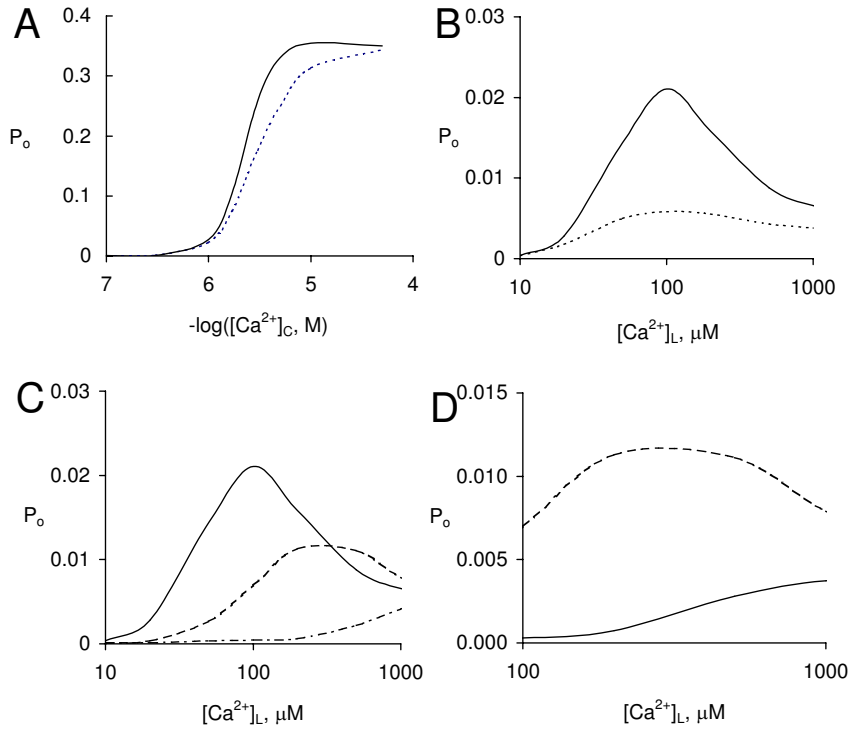

Figure 5. The dependence of $[Ca^{2+}]_L$-activation of RyR2 on A-site gating properties *predicted by the "luminal-triggered Ca^{2+} feed-through" model illustrated in Figure 2.* ***(A)*** *$[Ca^{2+}]_C$-activation of RyR2 under conditions of zero Ca^{2+} feed-through; (*short dashes*)- RyRs activated by $[Ca^{2+}]_C$ alone and (*solid*)- RyRs in the presence of a cofactor which increases channel open durations in a similar way to that shown for ATP in Figure 1B.* ***(B)*** *(*short dashes and solid*) The corresponding effects of the cofactor (panel A) on $[Ca^{2+}]_L$-activation of RyR2 under conditions that favor Ca^{2+} feed-through. (**C**) Biasing the voltage against Ca^{2+} feed-through by changing the membrane potential from (*solid*) -40 mV to (*long dashes*) 0 mV and (*alternating dashes*) +40 mV shifts the $[Ca^{2+}]_L$–dependence of RyR activity to higher levels. (*solid*) Ca^{2+}-activation seen between 10 and 100 μM is due to the action of the* A*–site whereas the decrease in RyR activity seen between 100 and 1000 μM is due to the inactivating action of the* I_2*-site. (**D**) (*long dashes*) The $[Ca^{2+}]_L$–dependence of* P_o *at zero membrane potential in the absence of luminal Mg^{2+} and (*solid*) in the presence of 1 mM $[Mg^{2+}]_L$.*

more selective for Ca^{2+} than Mg^{2+} which is consistent with the ion specificity of luminal inactivation (Laver *unpublished data*).

Accessibility of A- and I_2-sites to luminal Ca^{2+}

Even though the *A*- and I_2-sites have similar affinities for cytoplasmic Ca^{2+} the apparent affinities of these sites for luminal Ca^{2+} are more than 10-fold different. This difference is readily explained by the relative proximity of these sites to the Ca^{2+} pore.[21] Ca^{2+} emanating from the pore will diffuse into the cytoplasm and be sequestered by buffering molecules. This leads to a decline in $[Ca^{2+}]$ with distance from the pore.[37,45] Therefore Ca^{2+} sites that are further away from the pore will be less sensitive to the effects of Ca^{2+} feed-through than sites near the pore. By fitting the "luminal-triggered Ca^{2+} feed-through" model to experimental data, Ca^{2+} feed-through was estimated to increase $[Ca^{2+}]$ to ~100 μM at the *A*-site and ~2 μM at the I_2-site during Ca^{2+} release.[24] The calculations also placed the *A*- and I_2-sites at 11 nm and 26 nm from the RyR pore, respectively. Given that the furthest point on the RyR from the pore is ~20 nm (allowing for components of separation within and perpendicular to the plane of the membrane[46]) it would seem that the I_2-sites are located at the periphery of the protein or perhaps even on an adjacent inhibitory protein.

Cytoplasmic agonists and luminal Ca^{2+}-activation

As stated above, $[Ca^{2+}]_L$-dependent activation of RyRs is markedly increased by enhancers of $[Ca^{2+}]_C$-activation.[19,30,31] Early studies proposed that ATP, caffeine or sulmazole created a conformational change in the RyR protein that unmasked a luminal Ca^{2+} sensing site.[9] The "luminal-triggered Ca^{2+} feed-through" model points to an alterative explanation for the action of ATP. When fitting the model to data such as that shown in Figure 4 it was found that the ATP effects could be explained by three modes of action.[24] It increased the *L*-site mediated opening rate, increased τ_o in response to Ca^{2+} binding at the *A*-site and decreased the rate of inactivation *via* the I_2-site. A substantial fraction of the ATP effect on luminal Ca^{2+}-activation was due to the enhanced effect of Ca^{2+} feed-

through on *A*-site activation.[24] This highlights the intimate involvement of cytoplasmic and luminal regulation of RyRs that occurs as a result of Ca^{2+} feed-through.

More generally, the "luminal-triggered Ca^{2+} feed-through" model predicts that *any* cofactor that prolongs channel openings triggered by $[Ca^{2+}]_C$ will promote RyR activation by luminal Ca^{2+}. This is illustrated in Figure 5 (A & B) which shows predictions of the "luminal-triggered Ca^{2+} feed-through" model. In this example an agonist causes a slight increase in channel sensitivity to $[Ca^{2+}]_C$ (Figure 5A). This leads to a marked increase in the degree of channel activation by $[Ca^{2+}]_L$ (Figure 5B).

This is highly relevant to the effects of RyR2 mutations associated with Sudden Cardiac Death (SCD) which are known to enhance activation by luminal Ca^{2+}.[47] It was proposed that SCD mutations in RyR2 lead to cardiac arrhythmias because they promote spontaneous Ca^{2+} release from the SR when the store load is increased during periods of stress or exercise.[47] The model predicts that the enhanced luminal activation of mutant RyR2 can result from changes in gating associated with either luminal or cytoplasmic domains of the RyR.

The converse will be true for cytoplasmic antagonists that shorten channel openings by $[Ca^{2+}]_C$. Poly-unsaturated fatty acids (PUFAs) are RyR antagonists that reduce RyR2 activation by $[Ca^{2+}]_C$.[48,49] According to the "luminal-triggered Ca^{2+} feed-through" model, PUFAs will substantially reduce RyR2 activation by $[Ca^{2+}]_L$ and alleviate the affects of store overload hence protecting myocardium against overload-induced arrhythmias.

The role of luminal $[Mg^{2+}]$ in control of RyR2 by luminal Ca^{2+}

Even though the physiological potential across the SR membrane is approximately zero,[50] most measurements of $[Ca^{2+}]_L$–regulation of RyRs have been carried out at non-zero membrane potentials. This has been done to maintain a relatively large driving force on the conducting ions; thus ensuring that the current jumps signifying channel gating are large compared to the background electrical noise. The "luminal-triggered Ca^{2+} feed-through" model makes the rather interesting prediction that at zero volts the $[Ca^{2+}]_L$–dependence of P_o is nearly flat over the physiological range of $[Ca^{2+}]_L$ (0.3-1 mM, Figure 5C & D, long dashes) because the effects of the *A*- and I_2-sites roughly cancel. According to this prediction, variations in store load should have very little effect on store excitability; this is clearly not the case in cardiac muscle. This begs the question as to what causes SR excitability to increase with increasing $[Ca^{2+}]_L$? One intriguing possibility is that luminal Mg^{2+} is an essential cofactor for store load-dependent excitability in cardiac muscle. Although the free $[Mg^{2+}]$ in the lumen has not been measured, the total concentrations of Mg^{2+} and Ca^{2+} in the lumen are similar[51] and calsequestrin has similar buffering properties for both ions[52] so that their free concentrations should also be similar. Recent experiments (Laver *unpublished data*) have revealed that luminal Mg^{2+} inhibits RyR2 by competing with Ca^{2+} for the *L*-site. As $[Mg^{2+}]_L$ is increased from 0 to 1 mM, the competitive binding kinetics causes the half-activating $[Ca^{2+}]_L$ at the *L*-site to change from 60 μM to 500 μM which is in the physiological range of $[Ca^{2+}]_L$. Incorporating this effect into the model reinstates a substantial $[Ca^{2+}]_L$-dependence of P_o at zero volts (Figure 5D, solid line). Luminal Mg^{2+} does this because as $[Ca^{2+}]_L$ increases, luminal Mg^{2+} becomes a less effective antagonist of *L*-site activation.

Luminal control of skeletal and cardiac RyRs

There appears to be little awareness in the literature of the different ways that RyR1 and RyR2 could be regulated by luminal Ca^{2+} even though there are marked differences in how they are regulated by the cytoplasmic milieu.[25] Given the close link between cytoplasmic and luminal regulation of RyRs it would be surprising to find that luminal regulation of the two isoforms was indeed the same. One clear difference has recently come to light as a result of the different responses of RyR1 and RyR2 to ATP.[24] Even in the absence of activating Ca^{2+}, ATP can trigger the opening of RyR1 but not RyR2.[21,36] Hence, at the $[Ca^{2+}]_C$ and [ATP] present in resting muscle, RyR2 openings would be triggered mainly by Ca^{2+} binding to the *L*-site[24] whereas RyR1 openings would be triggered by cytoplasmic ATP,[21] thus bypassing the role of the *L*-site in channel activation. Preliminary data (Laver *unpublished data*) shows that physiological luminal $[Mg^{2+}]$ (~1 mM) can strongly inhibit RyR2 by competing with Ca^{2+} for the *L*-site whereas RyR1 is not affected by luminal Mg^{2+} at these levels. This indicates that RyRs in skeletal and cardiac muscle will be found to be controlled quite differently by luminal ions.

Concluding remarks

Luminal Ca^{2+} regulates RyR2 activity *via* Ca^{2+} binding to the luminal *L*-site and the cytoplasmic *A*- and I_2-sites. Alterations in channel gating associated with any of these sites will alter the regulation of RyRs by luminal Ca^{2+}. A unifying kinetic model has recently been developed that makes the first quantitative predictions of Ca^{2+} permeability of the ER/SR. This model demonstrates how luminal control of Ca^{2+} release can be changed by pharmacological agents such as ATP, caffeine and PUFAs as well as RyR2 mutations associated with sudden cardiac death. The model predicts that Ca^{2+} and Mg^{2+} are effectively the *yin* and *yang* of SR excitability.

Acknowledgments

Thanks to Katherine Bradley for helpful comments and proof reading of the manuscript. DRL was supported by a Senior Brawn Fellowship from the University of Newcastle. This work was supported by the Australian Research Council (grant number DP0557780) and by an infrastructure grant from NSW Health through Hunter Medical Research Institute.

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Received 8 January 2007, in revised form 24 January 2007.
Accepted 1 February 2007.

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