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REVIEW

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Coupled calcium release channels and their regulation by luminal and cytosolic ions

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Abstract Contraction in skeletal and cardiac muscle occurs when Ca^{2+} is released from the sarcoplasmic reticulum (SR) through ryanodine receptor (RyR) Ca^{2+} release channels. Several isoforms of the RyR exist throughout the animal kingdom, which are modulated by ATP, Ca^{2+} and Mg^{2+} in the cytoplasm and by Ca^{2+} in the lumen of the SR. This review brings to light recent findings on their mechanisms of action in the mammalian isoforms RyR-1 and RyR-2 with an emphasis on RyR-1 from skeletal muscle. Cytoplasmic Mg^{2+} is a potent RyR antagonist that binds to two classes of cytoplasmic site, identified as low-affinity, non-specific inhibition sites and high-affinity Ca^{2+} activation sites (A-sites). Mg^{2+} inhibition at the A-sites is very sensitive to the cytoplasmic and luminal milieu. Cytoplasmic Ca^{2+}, Mg^{2+} and monovalent cations compete for the A-sites. In isolated RyRs, luminal Ca^{2+} alters the Mg^{2+} affinity of the A-site by an allosteric mechanism mediated by luminal sites. However, in close-packed RyR arrays luminal Ca^{2+} can also compete with cytoplasmic ions for the A-site. Activation of RyRs by luminal Ca^{2+} has been attributed to either Ca^{2+} feedthrough to A-sites or to Ca^{2+} regulatory sites on the luminal side of the RyR. As yet there is no consensus on just how luminal Ca^{2+} alters RyR activation. Recent evidence indicates that both mechanisms operate and are likely to be important. Allosteric regulation of A-site Mg^{2+} affinity could trigger Ca^{2+} release, which is reinforced by Ca^{2+} feedthrough.

Keywords Ryanodine receptor · Magnesium · Calcium · Skeletal muscle · Lipid bilayer

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Ryanodine receptors in skeletal muscle

Skeletal and cardiac muscle contract in response to depolarisation of the surface membrane, during an action potential. The action potential propagates along the surface membrane and along a network of transverse and longitudinal tubules, which are continuous with the surface membrane (i.e., the transverse tubular system, t-tubule). The transverse tubules permit penetration of the action potential deep into the muscle fibre and contribute to its propagation along the fibre (Posterino et al. 2000). Depolarisation of the t-tubule membrane causes release of Ca^{2+} from the sarcoplasmic reticulum (SR), which leads to an increase in cytoplasmic Ca^{2+} concentration, which, in turn, is the signal for contraction. Excitation–contraction coupling (EC coupling) describes the link between depolarisation of the t-tubule and the release of calcium from the SR. Two types of calcium channels are central to this process. The dihydropyridine receptors (DHPR, L-type calcium channels) in the t-tubules are activated by membrane depolarisation and these trigger the opening of the calcium release channels in the SR (ryanodine receptors, RyRs). Electron micrographs reveal specialised regions of the t-tubules and the SR membrane that form close junctions often referred to as triad junctions (Franzini-Armstrong 1970). Within these regions, RyRs form a rhombic lattice in the SR, which is apposed by an array of DHPRs in the t-tubule such that every second RyR is linked with four DHPRs (Protasi et al. 1997). In skeletal muscle, the RyRs are stimulated by a direct coupling between the DHPRs and RyRs (Tanabe et al. 1990; Melzer et al. 1995), whereas in cardiac muscle, it is the influx of extracellular Ca^{2+} through the DHPR that activates RyRs.

The RyR is a homotetramer of 560-kDa subunits containing approximately 5,035 amino acids which form the largest known ion channel protein. Reconstructed images from electron micrographs (Samso and Wagenknecht 1998) of the RyR reveal a square cytoplasmic

domain approximately 29 nm on edge, rising approximately 12 nm from the membrane, and a relatively small transmembrane region which is presumably the calcium channel. The cytoplasmic domain seems to correspond to the foot region seen in early electron micrographs of the triad junction (Franzini-Armstrong 1970). The transmembrane pore is composed of the c-terminal amino acids between positions 4,000 and 5,000 in the sequence (Bhat et al. 1997), while the remaining amino acids form the foot region. The RyR is ubiquitous in the animal kingdom and exists in a number of isoforms (Coronado et al. 1994). In mammals, three isoforms have been cloned and sequenced. RyR-1 is the main isoform found in skeletal muscle and RyR-2 is the main isoform in cardiac muscle, while RyR-3 is found in many cell types (Ogawa 1994). In amphibian, fish and avian muscle two isoforms have been sequenced, RyR-α and RyR-β, which are similar to RyR-1 and RyR–3, respectively (Sutko and Airey 1996).

Regulation of RyRs by cytoplasmic ATP, Ca^{2+} and Mg^{2+}

In order to understand the mechanisms of Ca^{2+} release, SR membranes containing RyRs have been extracted from muscle and studied in isolation. This is done because cellular function is complex and it is often difficult to identify underlying mechanisms from experiments on intact cells. RyR activity in SR vesicles has been measured by ryanodine binding and Ca^{2+} release assays. SR vesicles have been fused with artificial lipid bilayers to provide a powerful system for recording single RyRs (Laver 2001). Although RyRs are regulated by a wide range of substances (see Table 2 in Coronado et al. 1994), it is recognised that Ca^{2+} Mg^{2+} and ATP are key regulators of the RyR (Meissner 1994). RyR-1 and RyR-2 are regulated differently by these ligands and it has been argued that these differences underlie the different characteristics of EC coupling in skeletal and cardiac muscle (Laver et al. 2001; Lamb 2002). Measurements of isolated RyR-1 show that they are activated at approximately 1 μM Ca^{2+} and inhibited at approximately 1 mM Ca^{2+} in the cytoplasm (Meissner 1994). Mg^{2+} is believed to inhibit RyRs by two mechanisms (the dual-inhibition hypothesis). Mg^{2+} can inhibit RyRs by competing with Ca^{2+} for the activation sites (A-sites, Dunnett and Nayler 1978; Meissner et al. 1986) or Mg^{2+} can close RyRs by binding to low-affinity, non-selective divalent cation inhibition sites (I-sites) that also mediate Ca^{2+} inhibition (Meissner et al. 1986; Soler et al. 1992; Laver et al. 1997). Regulation of cardiac RyRs by Ca^{2+} and Mg^{2+} shows the same basic properties as skeletal RyRs with the exception that the I-sites have a tenfold lower affinity for divalent ions than RyR-1 (Laver et al. 1995, 1997). The dual-inhibition model predicts marked differences in Mg^{2+} inhibition of RyR-1 and RyR-2 at elevated cytoplasmic Ca^{2+} (approximately 10 μM) in which the I-sites produce potent Mg^{2+} inhibition of RyR-1 and relatively little inhibition of RyR-2.

ATP is an activator of RyRs. ATP strongly activates RyR-1 in the virtual absence of cytoplasmic Ca^{2+}, and in conjunction with Ca^{2+} can cause almost full activation. Activation of skeletal RyRs by ATP and its non-hydrolysable analogue AMP-PCP has been reported to have a K_a in the range 0.3–1 mM (Meissner 1984; Meissner et al. 1986; Jona et al. 2001; Laver et al. 2001) so that in muscle (ATP concentration approximately 8 mM, Godt and Maughan 1988) ATP activation of RyRs is near maximal. Cardiac RyRs are not appreciably activated by ATP in the absence of Ca^{2+}, but ATP augments their activation by Ca^{2+} (Meissner et al. 1988; Kermode et al. 1998).

The properties of RyR-1 measured in isolation are consistent with the calcium permeability of the SR in resting muscle. However, during contraction of skeletal muscle the regulation of RyRs by cytosolic ligands appears to be substantially altered. At rest Mg^{2+} is the primary inhibitor of Ca^{2+} release from the SR. This was highlighted in experiments by Lamb and Stephenson (Lamb and Stephenson 1994; Owen et al. 1997) which showed that a reduction of cytoplasmic free Mg^{2+} from physiological levels (approximately 1 mM) to 0.2 mM or lower in skinned muscle preparations released Ca^{2+} from the SR. During t-tubule depolarisation and activation of the RyR by the DHPR, the sensitivity of RyRs to inhibition by Mg^{2+} is reduced by more than tenfold (Lamb and Stephenson 1991, 1992, 1994). SR calcium release can be substantially inhibited by reduced ATP concentration (0.5 mM) or by ATP antagonists such as adenosine (Kermode et al. 1998; Laver et al. 2001; Dutka and Lamb 2004). This indicates that the DHPR's stimulation of RyRs is not sufficient on its own for Ca^{2+} release and that stimulation of RyRs by intracellular ligands is necessary for EC coupling (Laver et al. 2001). Although Ca^{2+} is thought to augment RyR activation it is not believed to be the major RyR activator during muscle contraction. Thus, an overall picture emerges in which, during EC coupling, DHPRs somehow relieve Mg^{2+} inhibition and thus permit RyR activation by ATP and Ca^{2+} (the Mg^{2+} de-repression hypothesis, Lamb and Stephenson 1992). In accord with this hypothesis, synthetic peptides with amino acid sequences corresponding to Glu^{724}–Pro^{760} of the skeletal DHPRs (the C region of the II–III loop which is believed to mediate EC coupling) interact with isolated RyRs and decrease their Mg^{2+} inhibition at the I-sites (Haarmann et al. 2005).

Until recently, it was not clear if Mg^{2+} acts as a competitive non-agonist at the A-sites (i.e., merely preventing Ca^{2+} from activating the channel) or as an antagonist in its own right. The importance of this distinction was realised by Laver et al. (1997). Skeletal RyRs can be activated by ATP, even in the absence of Ca^{2+} (Meissner 1986; Smith et al. 1986; Laver et al. 2001) so that at physiological levels of ATP, Mg^{2+} could not be inhibiting RyRs simply as a competitive non-agonist because the channels do not require Ca^{2+} at the A-site in order to open. Only recently, measurements of

Mg^{2+} inhibition at the A-site showed that Mg^{2+} is indeed an antagonist that inhibits in the absence of Ca^{2+} (Laver et al. 2004). Thus, if DHPRs do indeed activate RyRs by de-repression, both the A-sites and the I-sites must be involved.

Luminal Ca^{2+}

The Ca^{2+} load of the SR is an important stimulator of Ca^{2+} release in skeletal (Ford and Podolsky 1972) and cardiac muscle (Fabiato and Fabiato 1977). Increased store Ca^{2+} load increases the sensitivity of Ca^{2+} release in skeletal muscle to cytoplasmic Ca^{2+} (Ford and Podolsky 1972; Endo 1985; Meissner et al. 1986), caffeine (Lamb et al. 2001) and ATP (Morii and Tonomura 1983; Donoso et al. 1995). In bilayer experiments, increasing luminal Ca^{2+} concentration augments the activity of RyRs that are activated by cytosolic ligands such as ATP (RyR-1) (Sitsapesan and Williams 1995; Tripathy and Meissner 1996; Beard et al. 2000; Laver et al. 2004) (Fig. 1a), sulmuzol (Sitsapesan and Williams 1994) or caffeine (RyR-2) (Xu and Meissner 1998).

The action of luminal Ca^{2+} on skeletal RyRs at sub-activating cytoplasmic Ca^{2+} depends on the membrane potential difference (PD). An example of this is shown in Fig. 1a, which shows the voltage-dependence of luminal Ca^{2+} activation RyRs in the presence of cytoplasmic ATP (2 mM) and in the virtual absence of cytoplasmic Ca^{2+} (approximately 1 nM). At +40 mV the luminal Ca^{2+} activated RyRs at a higher concentration than it did at −40 mV. Similar results have been reported by Sitsapesan and Williams (1995) and Tripathy and Meissner (1996), who also showed that further increasing luminal Ca^{2+} to greater than 5 mM at negative PD causes 30% decrease in RyR activity. These three studies also show that increasing luminal Ca^{2+} concentration has a relatively weak stimulating effect on the activity on RyRs that are sub-maximally activated by cytoplasmic Ca^{2+} alone. Moreover, at higher cytoplasmic Ca^{2+} concentration, luminal Ca^{2+} has been shown to have either no effect (Laver et al. 2004) or a slightly inhibiting effect on RyRs (Tripathy and Meissner 1996).

Overall the picture for cardiac RyRs is similar although there is some disagreement between studies. At negative membrane PD, increasing luminal Ca^{2+} from less than 10 μM to approximately 100 μM increased RyR activity, while a further increase in Ca^{2+} to millimolar levels caused approximately 50% decrease in activity. This was shown using a range of membrane preparations and cytoplasmic Ca^{2+} concentrations: SR vesicles from sheep heart at 100 pM Ca^{2+} (Sitsapesan and Williams 1994) and 100 nM Ca^{2+} (D.R. Laver, unpublished data); purified RyR-2 from dog heart at 1 μM Ca^{2+} (Xu and Meissner 1998). At higher cytoplasmic Ca^{2+}, increasing luminal Ca^{2+} slightly decreased the activity of RyR-2 (Xu and Meissner 1998). However, these studies are at odds with findings using SR vesicles from dog heart that showed that luminal Ca^{2+} caused a monotonic increase in activity over the range 0–5 mM that was seen at low and high cytoplasmic Ca^{2+} and which did not depend on membrane PD (Gyorke and Gyorke 1998). The source of these discrepancies is unclear but they might be due to membrane preparation-dependent differences in the protein complexes formed between RyR-2 and calsequestrin, tradin, junctin and calmodulin since these are known to have major effects on RyR regulation by luminal Ca^{2+} (Gyorke et al. 2004; Xu and Meissner 2004).

As yet there is no consensus on how the Ca^{2+} load in the SR alters RyR activation (Sitsapesan and Williams 1997). Two quite different mechanisms have been used to explain the activation of RyRs by luminal Ca^{2+}. The "true luminal regulation" hypothesis attributes luminal Ca^{2+} activation to Ca^{2+} regulatory sites on the luminal side of the RyR (Sitsapesan and Williams 1995), while the "feedthrough" hypothesis proposes that luminal Ca^{2+} permeates the pore and binds to the cytoplasmic

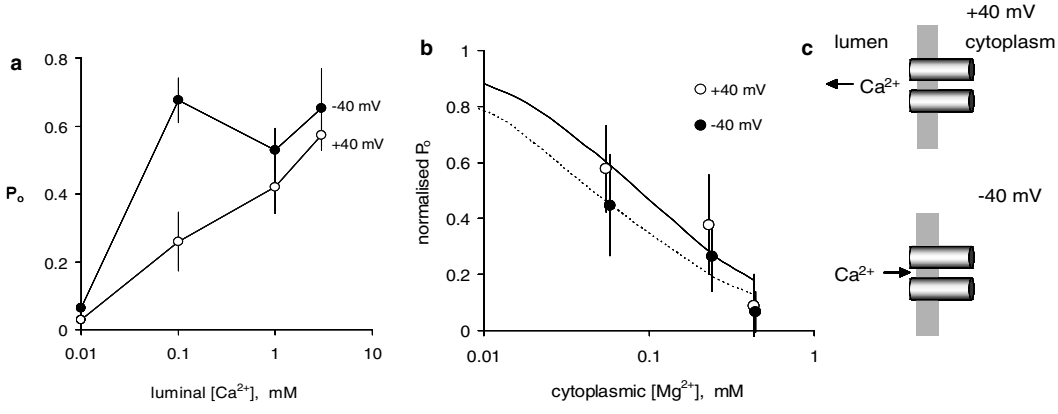


Fig. 1 The effects of membrane potential (−40 mV, *filled circles*; +40 mV, *open circles*) on the regulation of ryanodine receptors (*RyRs*) by **a** luminal Ca^{2+} and **b** cytoplasmic Mg^{2+}. **c** Schematic of RyR channels in a bilayer illustrating how at −40 mV the voltage favours Ca^{2+} flow from lumen to cytoplasm, while +40 mV opposes it. Increasing luminal Ca^{2+} concentration markedly increased the channel open probability (P_o) and changing the membrane potential from −40 to +40 mV shifted this Ca^{2+} dependence to lower luminal Ca^{2+} concentration but had no significant effect on RyR sensitivity to Mg^{2+} inhibition. **a** and **b** The cytoplasmic bath contained 2 mM ATP and 1 nM Ca^{2+}. **b** The luminal bath contained 1 mM Ca^{2+}

A-sites and I-sites (Tripathy and Meissner 1996; Xu and Meissner 1998). The latter hypothesis is supported by the close correlation between open probability and Ca^{2+} flux (lumen to cytoplasm), which is seen with a wide range of experimental conditions in both cardiac and skeletal RyRs. An example of this is shown in Fig. 1, which shows that luminal Ca^{2+} activation of RyRs is enhanced at a membrane PD which favours the flow of Ca^{2+} from the luminal to cytosolic sides of the RyR.

Several experimental observations are more difficult to reconcile with the Ca^{2+} feedthrough model; however, most of these are observations on cardiac RyRs and it is not yet clear if the same luminal Ca^{2+} sensing mechanisms apply to both skeletal and cardiac RyRs (see before). In skeletal RyRs, luminal Ca^{2+} activation is susceptible to tryptic digestion from the luminal side of the membrane (Ching et al. 2000) which points to Ca^{2+} sensors on the luminal face of the RyRs. The existence of a luminal Ca^{2+} sensor in cardiac RyRs is supported by more specific manipulations of the luminal face. It is known that the luminal proteins, calsequestrin, triadin and junctin are associated with RyR-1 (Beard et al. 2004) and RyR-2 (Gyorke et al. 2004) and modulate their activity. Dissociation of calsequestrin, triadin and junctin from RyR-2 abolished regulation of RyR-2 by luminal Ca^{2+} (Gyorke et al. 2004).

Ca^{2+} A-sites as a probe for luminal Ca^{2+} feedthrough

The A-sites of the RyRs have been shown to bind monovalent and divalent ions, exhibiting specificity in binding and efficacy even among ions of the same valency (Rousseau et al. 1992; Meissner et al. 1997; Laver et al. 2004). These properties make the A-sites useful probes for the ionic environment near the RyR pore. Recently, the ''feedthrough'' hypothesis was re-examined in RyR-1 by measuring the effects of competition between luminal Ca^{2+} and cytoplasmic cations at the A-sites of the RyR-1 (Laver et al. 2004). Increased luminal Ca^{2+} decreased the RyR inhibition by cytoplasmic Mg^{2+} by reducing the apparent affinity of the A-sites for Mg^{2+}. Increasing the luminal Ca^{2+} concentration from 0.1 to 1 mM produced a fourfold decrease in the Mg^{2+} affinity of the A-sites. Whilst this finding might seemingly be explained by the Ca^{2+} feedthrough mechanism, other findings weighed against this and indicated an allosteric mechanism involving Ca^{2+} binding sites on the luminal face of the RyR-1. Firstly, the regulation of Mg^{2+} inhibition in RyRs by a range of luminal and cytoplasmic ions could not be described by a competitive model but could be described by non-competitive models (Table 1). Secondly, the sensitivity of RyRs to Mg^{2+} inhibition did not depend on membrane PD, as one would expect if Ca^{2+} was permeating the channel and competing with Mg^{2+} for the A-sites (Fig. 1b). Theoretical concentration profiles of Ca^{2+} emanating from a single RyR predict that the Ca^{2+} flow at −40 mV can increase cytoplasmic Ca^{2+} concentration in the vicinity of the RyR by more than 10 μM (Stern 1992; Xu and Meissner 1998). The effect of a global rise in cytoplasmic Ca^{2+} concentration from approximately 1 nM to more than 10 μM has been found to decrease the apparent affinity of Mg^{2+} at the A-site tenfold (Laver et al. 2004). However, at +40 mV the Ca^{2+} flux through the RyR is tenfold lower than at −40 mV and so will have a much smaller, and probably insignificant, effect on the apparent Mg^{2+} affinity.

Thus, it appears that, at least in RyR-1, luminal Ca^{2+} alleviates Mg^{2+} inhibition of RyRs via an allosteric mechanism that involves luminal Ca^{2+} sensing sites on the RyR, which modulate the regulation of RyRs by cytoplasmic ligands. However, the apparent lack of competition between luminal Ca^{2+} and cytoplasmic Mg^{2+} does not tally with our current understanding of how isolated RyRs are regulated by cytoplasmic ligands. Theoretical calculations (see before) predict that RyRs can sustain a Ca^{2+} flux sufficient to markedly decrease the apparent affinity of the A-sites. This puzzling lack of competition between luminal Ca^{2+} and cytoplasmic Mg^{2+} at the A-sites might be a result of limited temporal access between cytoplasmic and luminal baths (i.e., there is access only when the channel is open). This is highlighted by the different effects of luminal Ca^{2+} on isolated RyRs as compared with RyR clusters as discussed later.

Luminal Ca^{2+} modulation of Mg^{2+} inhibition in normal and ryanodine-affected muscle

The discovery that luminal Ca^{2+} can directly activate RyRs (Sitsapesan and Williams 1994) provided the first plausible molecular mechanism for the regulation Ca^{2+} release by store load. Recently, the finding that luminal Ca^{2+} alleviates Mg^{2+} inhibition (Laver et al. 2004) points to an additional mechanism by which Ca^{2+} release in muscle can be stimulated by luminal Ca^{2+}. In resting muscle, the presence of physiological free Mg^{2+} (1 mM) suppresses RyR activity even if the SR is well loaded with Ca^{2+} (Mg^{2+} is present at 4 times its half-inhibitory concentration at the I-site and 10–50 times that at the A-site). Models describing Mg^{2+} inhibition predict that changes in the SR load could change the degree of Mg^{2+} inhibition by up to 25-fold (Laver et al. 2004). Hence, modulation of Mg^{2+} inhibition by luminal Ca^{2+} is likely to be a significant regulator of Ca^{2+} release from the SR.

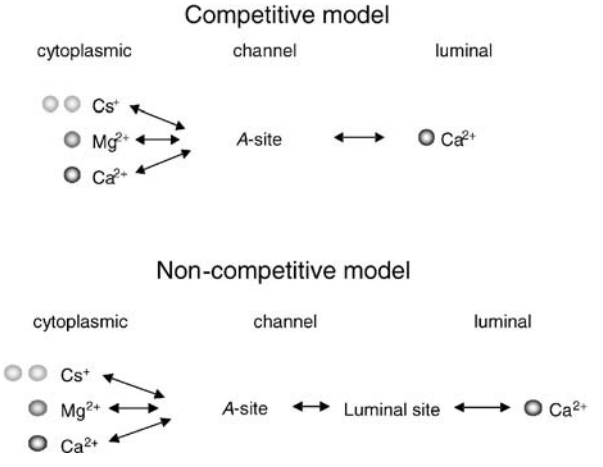
Luminal Ca^{2+} has also been shown to modulate Mg^{2+} inhibition of isolated RyRs that have been modified by ryanodine and this may explain some of the ryanodine-induced changes in Ca^{2+} handling observed in muscle cells. Ryanodine has been one of the most widely used pharmacological probes for elucidating the mechanisms of muscle contraction. Ryanodine produces rigour in skeletal muscle and loss of contractility in

cardiac muscle (Procita 1956; Hillyard and Procita 1959). Its most potent action was found to be on the calcium-handling properties of the SR (Jenden and Fairhurst 1969) by enhancing calcium release (Fleischer et al. 1985; Meissner 1986; Lattanzio et al. 1987) as a result of high-affinity, specific binding of ryanodine to the calcium release channel, now known as the RyR (Inui et al. 1987; Rousseau et al. 1987; Lai et al. 1988; Smith et al. 1988). Ryanodine irreversibly modifies the conductance and gating properties of RyRs. At nanomolar concentrations ryanodine "locks" the channel into a long-lived sub-conductance state, while at concentrations above 100 μM it inhibits Ca^{2+} release from SR vesicles (Meissner 1986; Lattanzio et al. 1987; Humerickhouse et al. 1993) and closes RyR channels in lipid bilayers (Lai et al. 1989; Buck et al. 1992). Ryanodine preferentially binds with high affinity to RyRs in their open state (Pessah et al. 1986) thus making ryanodine binding a widely used assay for RyR activity. Ryanodine has a profound effect on the regulation of RyRs by intracellular constituents. Ryanodine-modified RyRs are inhibited by Mg^{2+} (Masumiya et al. 2001; Laver et al. 2004) and low pH (Ma and Zhao 1994; Laver et al. 2000) albeit with reduced sensitivity. The effect of ryanodine on Ca^{2+} regulation of RyRs is not clear. Three studies show that ryanodine-modified RyR-1 and RyR-2 incorporated into bilayers from SR vesicles are insensitive to cytoplasmic Ca^{2+} and adenine nucleotides (Rousseau et al. 1987; Laver et al. 1995, 2004). Two other studies report that purified RyR-2 is sensitive to ATP and becomes hypersensitive to Ca^{2+} activation (half-activation, K_a, ranging from 0.1 to 10 nM) when modified by ryanodine (Du et al. 2001; Masumiya et al. 2001). Analysis of Ca^{2+}–Mg^{2+} competition at the A-sites indicates that ryanodine-modified channels have $K_a = 100$ nM (Laver et al. 2004) and are fully open at Ca^{2+} concentrations down to 1 nM, indicating that ryanodine can open RyRs independently of Ca^{2+} binding to the A-sites.

In spite of the pharmacological importance of ryanodine, the mechanisms underlying its effect on intracellular Ca^{2+} handling is not clear. Because ryanodine can either irreversibly activate or inhibit RyRs (see before), loss of Ca^{2+} release in ryanodine-modified cells can be attributed to either ryanodine inhibition of RyRs or to depletion of Ca^{2+} stores by chronic activation of RyRs (Gilchrist et al. 1992). The idea that ryanodine inhibits RyRs in cells is difficult to reconcile with the ryanodine concentrations needed to inhibit Ca^{2+} release, which are typically an order of magnitude lower than that required to inhibit RyRs in bilayer studies. The data shown in Table 1 point to another mechanism by which ryanodine may inhibit intracellular Ca^{2+} release. Initial application of ryanodine to resting muscle fibres will strongly activate RyRs because ryanodine at the high-affinity binding site (1) bypasses the Ca^{2+} requirement at the A-sites for channel activation, (2) removes Mg^{2+} inhibition at the I-sites and (3) raises the half-inhibitory Mg^{2+} concentration at the A-sites from 40 μM to approximately 1–2 mM. Actions 2 and 3 together effectively alleviate the inhibition of Ca^{2+} release by cytoplasmic Mg^{2+} (approximately 1 mM). The massive ryanodine-induced Ca^{2+} release will eventually deplete the store load and lead to an increase in the Mg^{2+} affinity of the A-sites and an increase in RyR inhibition by cytoplasmic Mg^{2+}. This form of RyR inhibition would be readily distinguished from non-reversible ryanodine inhibition because it can be reversed by an increase in store load or by a decrease in cytoplasmic Mg^{2+}.

Table 1 The effect of cytoplasmic Cs^+ and luminal Ca^{2+} on Mg^{2+} concentration required for half inhibition of ryanodine receptors (*RyRs*) [K_i (Mg^{2+})]. RyRs were modified by exposure to 10 μM ryanodine (+40 mV, 1 nM cytoplasmic Ca^{2+}, data from Laver et al. 2004). The data were interpreted in terms of two alternative models. In these models the channel is open in its unbound form (ryanodine opens the RyR in the absence of Ca^{2+}) or when bound to Ca^{2+} or Cs^+ and the binding of Mg^{2+} closes the channel. In the competitive model cytoplasmic Ca^{2+}, Cs^+, Mg^{2+} and luminal Ca^{2+} all compete at the activation site (*A-site*). In the non-competitive model, cytoplasmic ions compete at the A-site while luminal Ca^{2+} ions bind at another site (presumably a luminal site) and modulate the affinity of the A-site for cytoplasmic Mg^{2+}. The data for three of the ionic conditions (*normal text*) were fitted exactly by adjusting three parameters of the models (i.e., affinities for Cs^+, Mg^{2+} and luminal Ca^{2+}). Predictions of each model for K_i (Mg^{2+}) at 1 mM luminal Ca^{2+} and 250 mM cytoplasmic Cs^+ (*two last rows*) are compared with the data obtained under this condition (*bold*). The data were predicted by the non-competitive model but were inconsistent with predictions of the competitive model.

Luminal Ca^{2+}	10 μM	1 mM
[Cs^+], mM	K_i (Mg^{2+}), mM	
50	90 ± 40 (4)	400 ± 100 (3)
250	1050 ± 150 (7)	**3900 ± 900 (10)**
Competitive model		**1306**
Non-competitive model		**4439**



Coupled RyRs in bilayers

The bilayer method has allowed the study of RyR function in considerable detail (Laver 2001). However,

one of the perceived shortcomings of the bilayer technique has been that RyRs could only be studied in isolation and that even when several RyRs were present in a bilayer they were probably too widely separated to mimic the close packing of RyRs in the triad junctions. Therefore detailed investigations of mechanisms underlying Ca^{2+}-induced Ca^{2+} release and sparks were considered to be outside the scope of bilayer studies. However, in recent years a number of studies have reported that not all RyRs in bilayers are isolated channels. Evidence for this comes from the fact that when several RyRs are present, the opening of some RyRs depends on the opening of others (i.e., the gating of these RyRs is coupled), indicating that RyRs can form relatively stable, closely packed aggregates in the bilayer. Coupling of RyRs was first identified in purified RyRs (RyR-1 and RyR-2, Marx et al. 1998, 2001). Those authors found that protein fractions enriched in RyR multimers produced synchronously gated channels in approximately 10% of instances when reconstituted with lipid bilayers. Functional coupling required FK506 binding proteins (FKBP12 and FKBP12.6) but was independent of luminal Ca^{2+}, suggesting that coupling arose from a direct physical link between RyRs. A similar coupled gating phenomenon has since been reported with RyR-2 incorporated from cardiac SR vesicles into bilayers (Ondrias and Mojzisova 2002).

There is another, distinct phenomenon where multiple RyR-1 or RyR-2 channels are clearly coupled but do not open in synchrony (Copello et al. 2003; Laver et al. 2004; Porta et al. 2004). These authors proposed that coupled gating of RyRs occurred in bilayers when Ca^{2+} flow (luminal to cytoplasm) through one channel raised the local cytoplasmic Ca^{2+} concentration sufficiently to activate neighbouring RyRs. Their conclusions were based on the finding that coupled gating was promoted under conditions which favoured the flow of luminal Ca^{2+} through the channel (i.e., negative potential and high luminal Ca^{2+} concentration) and by the presence of sub-activating cytoplasmic Ca^{2+}. The latter condition is necessary because if the ambient cytoplasmic Ca^{2+} concentration is high enough to activate RyRs, then increasing the local Ca^{2+} concentration by Ca^{2+} feedthrough is not likely to further stimulate RyRs and so produce coupled gating. Coupled gating by Ca^{2+} feedthrough occurs even when the cytoplasmic bath is heavily buffered by Ca^{2+} chelators that confine the raised cytoplasmic Ca^{2+} concentration to a very small region near the pore (Laver et al. 2004). The separation of these RyRs in the bilayer may be estimated from theoretical concentration profiles of the Ca^{2+} concentration in the vicinity of a pore (Stern 1992). The Ca^{2+} concentration near an open channel falls off sharply with distance, because Ca^{2+} emerging from the pore is rapidly chelated by 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (4.5 mM BAPTA) in the bath. At −40 mV, 1 mM luminal Ca^{2+} generates sufficient cytoplasmic Ca^{2+} (1 μM) to activate other channels within a radius of 30 nm. Reversing the polarity of the potential or reducing the luminal Ca^{2+} concentration to 0.1 mM (conditions that prevent coupling) decreases this distance to approximately 20 nm. This indicates that the A-sites of the RyRs lie between 20 and 30 nm from neighbouring pores. This is consistent with the dimensions of RyR spacing in triads. Freeze-fracture electron micrographs show that RyRs within the triad junction are organised into rhombic, two-dimensional arrays with the pores of nearest and second nearest neighbours being separated by 31 and 43 nm, respectively (Protasi et al. 1997). This suggests that during isolation and reconstitution, the RyR arrays in muscle are not completely disrupted and that rafts containing three to ten RyRs remain stable in lipid bilayers throughout the experiments. In fact, Ca^{2+} feedthrough mediated coupling seems to occur whenever a single SR vesicle fusion incorporates many RyRs into the bilayer (Laver et al. 2004), which is in about 20% of instances. It has been reported that the number of RyRs incorporated into a bilayer with a single vesicle fusion event follows a bimodal distribution in which there tends to be a few (one or two) or many RyRs (5–30) incorporated into the bilayer (O'Neill et al. 2003). The different types of fusion events may reflect the different types of SR vesicles present in the membrane preparations, namely, vesicles containing fragments of close-packed triad arrays (5–30 RyRs) and vesicles containing isolated extrajunctional RyRs.

Analysis of coupled channels

Fusion of multiple RyRs into the bilayer produced records like those shown in Fig. 2a, which shows the activity of six ATP-activated RyRs at negative bilayer potentials. The current trace shows transitions between the current baseline (labelled C) and six equally spaced levels (O1–O6) corresponding to one to six open channels. At positive potentials, the weighting of each current level followed a binomial distribution expected from the gating of independent channels in the bilayer (not shown). At negative potentials, downward current steps frequently "bypassed" some of the current levels, indicating that several channels frequently opened in near synchrony. The weighting of the current levels in these records markedly deviated from a binomial distribution. There have been a number of approaches to analysing recordings of coupled channels, and these were reviewed by Laver and Gage (1997). The only detailed analysis of coupled RyRs (Laver et al. 2004) employed a hidden Markov model (HMM) approach first developed by Chung et al. 1990. The HMM was used to calculate the idealised, multilevel, current time course (i.e., background noise subtracted, Fig. 2b) and the transition probability matrix from the raw signal using maximum likelihood criteria. The mean channel opening and closing rates associated with each current level in the recordings (Fig. 3) were calculated from the transition probability matrix. This analysis revealed that the cou-

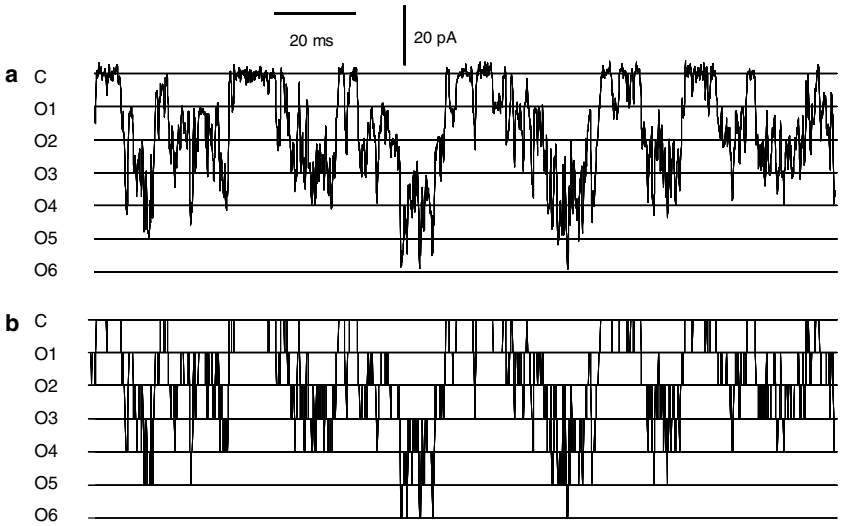


Fig. 2 **a** A current recording of six RyRs in a bilayer showing coupled gating at −40 mV. The current trace shows transitions between the current baseline (*C*) and equally spaced levels (*O1–O6*) corresponding to one to six open channels. The cytoplasmic bath contained 2 mM ATP, 1 nM Ca^{2+} and 60 μM Mg^{2+} (free). The luminal bath contained 1 mM Ca^{2+}. Both cytoplasmic and luminal baths contained symmetric 250 mM Cs^{+} solutions. **b** An idealised representation of the record in **a** produced by the hidden Markov model (*HMM*) algorithm (Chung et al. 1990). The HMM finds the maximum likelihood estimate of the channel current transitions present in the record and provides for the determination of current transition rates. It is based on the assumption that the channel current signal is the sum of a first-order finite-state Markov process and white, uncorrelated, Gaussian noise of known variance.

pling between RyRs was primarily due to the opening rate, which for each channel could be increased fourfold by the presence of one or more open channels in the raft. The closing rates were independent of the open states of other channels in the group.

Coupled and single RyRs are regulated differently by luminal Ca^{2+}

The flow of luminal Ca^{2+} through a pore has a very different effect on the receptor containing that pore than on the neighbouring RyRs. This is highlighted by the different effects of luminal Ca^{2+} on Mg^{2+} inhibition in isolated and coupled RyR-1 shown in Fig. 4. Cytoplasmic Mg^{2+} (220 μM) produced 70–80% reduction in the rate of single RyR openings in the presence of 1 mM luminal Ca^{2+} at both positive and negative PD. Mg^{2+} had a similar effect on the first RyR openings within a cluster but had a markedly reduced effect on subsequent channel openings provided that the membrane potential favoured Ca^{2+} feedthrough (i.e., −40 mV). This means that the flow of luminal Ca^{2+} through a pore produces no apparent competition with cytoplasmic Mg^{2+} for the A-sites on the receptor containing that pore while producing a substantial competitive effect on the neighbouring RyRs. Thus, it appears that RyRs are effectively "immune" to Ca^{2+} emanating from their own pore but are sensitive to Ca^{2+} from neighbouring channels.

The reason for this curious phenomenon probably lies in the different ways that the A-sites of single RyRs and RyR rafts access luminal Ca^{2+}. With a single RyR, the A-sites are only accessible to luminal Ca^{2+} while the channel is open, but with a raft, even when the channel is closed, the A-sites can still have access to luminal Ca^{2+} via adjacent open channels. With a single RyR, it is likely that luminal Ca^{2+} feedthrough will not affect closed dwell times since the A-sites are inaccessible to luminal Ca^{2+} when the channel is closed. However, luminal Ca^{2+} would be able to modulate both open and closed durations of a RyR in a raft. Therefore, there is more scope for luminal Ca^{2+} to have an effect on RyRs in rafts than on individual RyRs. This difference should become quite significant under circumstances where changes in activity are mediated almost entirely through changes in closed dwell time [regulation of RyR activity does occur primarily via closed durations when the activity of either RyR-1 or RyR-2 is low ($P_o < 0.2$, Kermode et al. 1998; Laver et al. 2001)]. In this situation, luminal Ca^{2+} would only have access to A-sites during channel openings but would not affect channel open duration, thus having no observable effect on channel activity.

Concluding remarks

The precise roles of Ca^{2+} feedthrough and direct luminal activation mechanisms in regulation of single, isolated RyRs are not yet determined. There is clear evidence that luminal Ca^{2+} reduces Mg^{2+} inhibition at the A-site and that this effect is mediated by an allosteric mechanism. Since it has been shown that Mg^{2+} inhibition of RyRs is effectively "immune" to Ca^{2+} emanating from their own pores, it is entirely possible that Ca^{2+} activation is similarly immune so that Ca^{2+} feedthrough

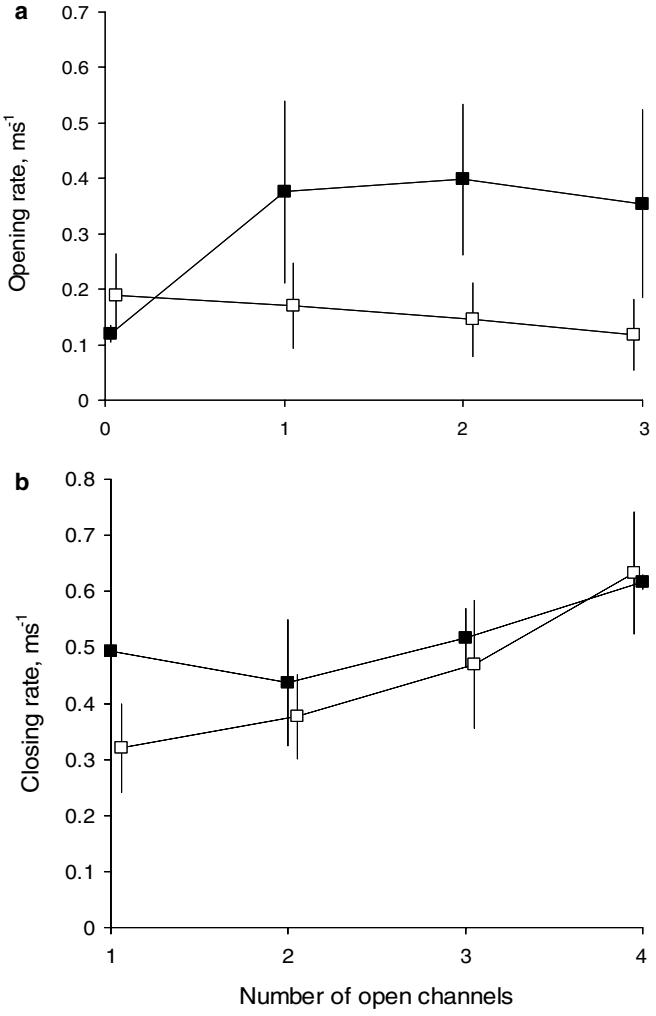


Fig. 3 The dependence of channel opening rates (**a**) and closing rates (**b**) on the number of channels open in the bilayer at positive and negative membrane potentials (mean ± standard error from seven experiments). HMM analysis of current records like that shown in Fig. 2 shows that the mean RyR-1 opening rate associated with transitions between the current baseline and first open level was significantly slower than opening rates associated with transitions between higher levels. Thus, the opening rate of an RyR could be increased by the opening of other RyRs in the bilayer. However, the closing rates did not significantly depend on the number of open channels. The cytoplasmic bath contained 2 mM ATP, 1 nM Ca^{2+} and 60–230 μM Mg^{2+} and the luminal bath contained 1 mM Ca^{2+}. Both luminal and cytoplasmic baths contained 250 mM Cs^{+} (data from Laver et al. 2004).

will not contribute to RyR activation. However, in the context of the cell, this might be a moot point, because RyRs operate in clusters that behave differently from isolated RyRs. RyR clusters in bilayers exhibit the clear effects of Ca^{2+} feedthrough. Therefore, both mechanisms have a part to play in RyR activation in muscle. The direct effect of luminal Ca^{2+} on the Mg^{2+} affinity of the A-sites may well be the trigger for Ca^{2+} release from internal stores while feedthrough of luminal Ca^{2+} to the cytoplasmic A-sites would further promote Ca^{2+} release.

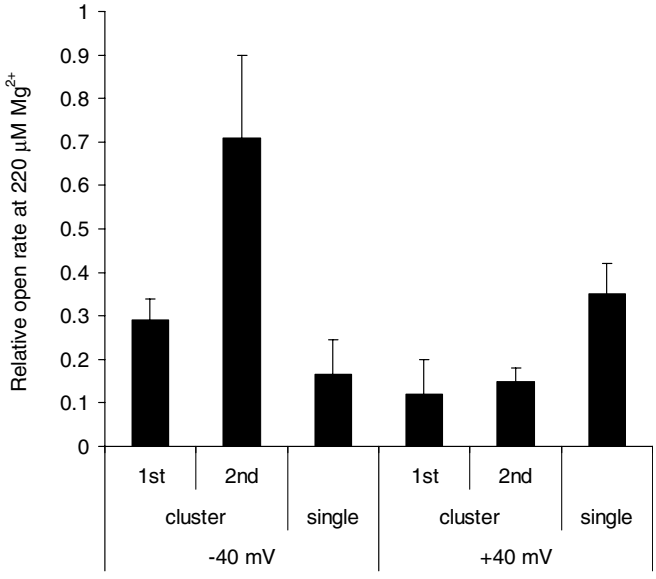


Fig. 4 The relative effects of cytoplasmic Mg^{2+} on the channel opening rate of single RyRs and coupled clusters of RyRs at bilayer potentials of +40 and −40 mV. The data show the decrease in the channel opening rate caused by 220 μM cytoplasmic Mg^{2+} relative to values obtained in the absence of Mg^{2+} (luminal Ca^{2+} concentration 1 mM). Inhibition of the first channel opening in a cluster is similar to that seen for single channels, while inhibition of the second channel opening is markedly different, being less sensitive to cytoplasmic Mg^{2+} at −40 mV but not at +40 mV.

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