Luminal Ca^{2+}–regulated Mg^{2+} Inhibition of Skeletal RyRs Reconstituted as Isolated Channels or Coupled Clusters

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ABSTRACT In resting muscle, cytoplasmic Mg^{2+} is a potent inhibitor of Ca^{2+} release from the sarcoplasmic reticulum (SR). It is thought to inhibit calcium release channels (RyRs) by binding both to low affinity, low specificity sites (*I*-sites) and to high affinity Ca^{2+} sites (*A*-sites) thus preventing Ca^{2+} activation. We investigate the effects of luminal and cytoplasmic Ca^{2+} on Mg^{2+} inhibition at the *A*-sites of skeletal RyRs (RyR1) in lipid bilayers, in the presence of ATP or modified by ryanodine or DIDS. Mg^{2+} inhibits RyRs at the *A*-site in the absence of Ca^{2+}, indicating that Mg^{2+} is an antagonist and does not simply prevent Ca^{2+} activation. Cytoplasmic Ca^{2+} and Cs^{+} decreased Mg^{2+} affinity by a competitive mechanism. We describe a novel mechanism for luminal Ca^{2+} regulation of Ca^{2+} release whereby increasing luminal [Ca^{2+}] decreases the *A*-site affinity for cytoplasmic Mg^{2+} by a noncompetitive, allosteric mechanism that is independent of Ca^{2+} flow. Ryanodine increases the Ca^{2+} sensitivity of the *A*-sites by 10-fold, which is insufficient to explain the level of activation seen in ryanodine-modified RyRs at nM Ca^{2+}, indicating that ryanodine activates independently of Ca^{2+}. We describe a model for ion binding at the *A*-sites that predicts that modulation of Mg^{2+} inhibition by luminal Ca^{2+} is a significant regulator of Ca^{2+} release from the SR. We detected coupled gating of RyRs due to luminal Ca^{2+} permeating one channel and activating neighboring channels. This indicated that the RyRs existed in stable close-packed rafts within the bilayer. We found that luminal Ca^{2+} and cytoplasmic Mg^{2+} did not compete at the *A*-sites of single open RyRs but did compete during multiple channel openings in rafts. Also, luminal Ca^{2+} was a stronger activator of multiple openings than single openings. Thus it appears that RyRs are effectively "immune" to Ca^{2+} emanating from their own pore but sensitive to Ca^{2+} from neighboring channels.

KEY WORDS: ryanodine receptor • magnesium • calcium • skeletal muscle • lipid bilayer

INTRODUCTION

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. In striated muscle, excitation–contraction coupling describes the link between depolarization of the transverse tubule and calcium release from the SR. Upon depolarization of the transverse tubule membrane, dihydropyridine receptors (DHPRs, L-type calcium channels) are activated, which trigger the RyRs. In skeletal muscle, the RyRs are stimulated by a direct coupling with the DHPRs (Tanabe et al., 1990; Melzer et al., 1995), while in cardiac muscle, it is the influx of extracellular Ca^{2+} through the DHPR that initiates Ca^{2+} release.

Calcium release is modulated by a variety of substances, including small diffusible molecules such as ATP, Ca^{2+}, Mg^{2+}, and protons (pH) (Meissner, 1994). RyRs are activated at μM [Ca^{2+}] and inhibited at 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). In addition, Mg^{2+} can close RyRs by binding to low affinity, nonselective divalent cation inhibition sites that also mediate Ca^{2+} inhibition (*I*-sites; Meissner et al., 1986; Soler et al., 1992; Laver et al., 1997). However, with the former mechanism, it is not clear if Mg^{2+} acts as a competitive nonagonist (i.e., prevents Ca^{2+} from activating the channel) or as an antagonist in its own right. This distinction becomes important when one considers cytoplasmic ATP. Physiological levels of ATP (8 mM) strongly activate skeletal RyRs even in the absence of cytoplasmic Ca^{2+} (Meissner et al., 1986; Laver et al., 2001). Therefore if Mg^{2+} merely prevents Ca^{2+} activation, it would not inhibit RyRs that are activated by ATP (Laver et al., 1997). Here we measure the effects of

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A-site Mg^{2+} inhibition on RyRs that are activated by ATP in the absence of Ca^{2+}. The first major finding of this work is that Mg^{2+} is an RyR antagonist that inhibits in the absence of cytoplasmic and luminal Ca^{2+}.

Ryanodine and the disulfonic stilbene derivatives are widely used pharmacological probes for elucidating the mechanisms of muscle contraction. Diisothiocyanostilbene-2′,2′-di-sulfonic acid (DIDS) has two isothiocyanate groups that can form covalent bonds with several amino acid residues. Modification of RyRs by ryanodine and DIDS is used here to help dissect the two mechanisms of Mg^{2+} inhibition. In the presence of ryanodine and DIDS, the Ca^{2+} sensitivities of the *A*- and *I*-sites are more disparate, producing wider separation of the two Mg^{2+} inhibition mechanisms and a much clearer manifestation of Mg^{2+} inhibition at the *A*-sites (Laver et al., 1997). DIDS has been shown to activate RyRs by reversible and nonreversible mechanisms (Kawasaki and Kasai, 1989; Zahradnikova and Zahradnik, 1993; Sitsapesan, 1999). Two independent mechanisms for nonreversible activation have been distinguished by their different kinetics (referred to as slow and fast) and their different specific effects (O'Neill et al., 2003). The fast mechanism increased the degree of Ca^{2+} activation with no significant change in sensitivity (*A*-sites) and reduced RyR sensitivity to Ca^{2+}/Mg^{2+} inhibition (*I*-sites) by 10-fold. The slow mechanism activated RyRs in the absence of Ca^{2+} and ATP.

Ryanodine has a profound effect on the regulation of RyRs by intracellular constituents. Ryanodine-modified RyRs are insensitive to cytoplasmic Ca^{2+} and adenine nucleotides (Rousseau et al., 1987; Laver et al., 1995), though they are still inhibited by Mg^{2+} (Masumiya et al., 2001) and low pH (Ma and Zhao, 1994; Laver et al., 2000), albeit with reduced sensitivity. In spite of the pharmacological importance of ryanodine, its effects on RyR function are not well understood. Earlier studies report that ryanodine-modified RyRs are active (i.e., open probability ~ 1) in the virtual absence of Ca^{2+} (Rousseau et al., 1987; Laver et al., 1995). However, two recent studies on recombinant cardiac RyRs (RyR2) report that ryanodine shifts their Ca^{2+} activation response to lower concentrations by four orders of magnitude (Du et al., 2001; Masumiya et al., 2001). We investigate the effects of cytoplasmic Ca^{2+} and Cs^+ on *A*-site Mg^{2+} inhibition in ryanodine-modified RyRs to probe the competitive ion binding kinetics at the *A*-site. Our second major finding is that ryanodine increases the Ca^{2+} sensitivity of the *A*-sites by 10-fold but this is insufficient to explain the level of activation of ryanodine-modified RyRs at nM Ca^{2+}.

The Ca^{2+} load of the SR is an important stimulator of Ca^{2+} release (Fabiato and Fabiato, 1977). It has been shown to regulate Ca^{2+} release in response to 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). Raised luminal Ca^{2+} increases channel activity in both purified (Tripathy and Meissner, 1996) and native RyRs (Sitsapesan and Williams, 1995; Beard et al., 2000). Activation of RyRs by luminal Ca^{2+} has been attributed to two quite different mechanisms, and there is as yet no consensus on just how the Ca^{2+} load in the SR alters RyR activation (Sitsapesan and Williams, 1997). 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). This is supported by the fact that luminal Ca^{2+} activation is susceptible to tryptic digestion from the luminal side of the membrane (Ching et al., 2000). The "feedthrough" hypothesis proposes that luminal Ca^{2+} permeates the pore and binds to the cytoplasmic activation sites (Tripathy and Meissner, 1996; Xu and Meissner, 1998). The latter is supported by the close correlation between open probability and Ca^{2+} flux (lumen to cytoplasm), which is seen under a wide range of experimental manipulations in both cardiac and skeletal RyRs. Here we investigate the effects of luminal Ca^{2+} on Mg^{2+} binding to the *A*-site in order to detect competition between luminal and cytoplasmic ions resulting from Ca^{2+} feedthrough. Our third and most important finding is a novel mechanism for regulation of Ca^{2+} release by luminal Ca^{2+}, whereby increasing luminal $[Ca^{2+}]$ decreases the affinity of the *A*-sites for Mg^{2+}. We show that luminal Ca^{2+} activates RyRs by both the true luminal regulation and Ca^{2+} feedthrough mechanisms in a way that depends on whether they are in close proximity to other open RyRs. Finally, we present an ion binding model of Mg^{2+} inhibition at the *A*-sites that predicts that modulation of Mg^{2+} inhibition by luminal Ca^{2+} is a significant regulator of Ca^{2+} release from the SR.

MATERIALS AND METHODS

Lipid Bilayers, Chemicals, and Solutions

SR vesicles were prepared from the back and leg muscles of New Zealand rabbits killed by captive bolt before muscle removal. The procedure was performed by the holder of a current license granted under ACT State legislation. Native SR vesicles were isolated using techniques based on those of Chu et al. (1988), as previously described by Laver et al. (1995).

Unless otherwise stated, lipid bilayers were formed from phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (8:2) (Avanti Polar Lipids) dissolved in 20 μl *n*-decane (50 mg/ml). The bilayers were formed across an aperture of 100–200 μm diameter in a Delrin cup. The bilayer separated two solutions: cis and trans ($\sim$1 ml). Vesicles were added to the cis solution and vesicle incorporation with the bilayer occurred as described by Miller and Racker (1976). Due to the orientation of RyRs in the SR vesicles, RyRs added to the cis chamber incorporated into the bilayer with the cytoplasmic face of the channel orientated to the cis solution.

The cesium salts were obtained from Sigma-Aldrich and $CaCl_2$ from BDH Chemicals. Unless otherwise stated, solutions were

pH buffered with 10 mM *N*-tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid (TES, obtained from ICN Biomedicals) and solutions were titrated to pH 7.4 using CsOH (optical grade from ICN Biomedicals). During SR vesicle incorporation the cis (cytoplasmic) solution contained 230 mM $CsCH_3O_3S$ and 20 mM CsCl (250 mM Cs^+ solution) with 1.0 or 0.1 mM $CaCl_2$, while the trans (luminal) solution contained 30 mM $CsCH_3O_3S$ and 20 mM CsCl (50 mM Cs^+ solution) and 0–1.0 mM $CaCl_2$. The osmotic gradient across the membrane and the Ca^{2+} in the cis solution also aided vesicle fusion with the bilayer. In experiments using symmetric [Cs^+], vesicle fusion was performed using cis and trans baths containing 250 mM Cs^+ solution with 500 mM mannitol in the cis bath to produce the necessary osmotic gradient. Cs^+ was used as the current carrying ion rather than K^+ in order to extinguish interfering signals from the SR K^+ channels. During measurements, the composition of the cis solution was altered either by addition of aliquots of stock solutions or by continuous local perfusion of the bilayer via a tube placed in close proximity (O'Neill et al., 2003).

The required free [Ca^{2+}] was attained by buffering with 4.5 mM BAPTA (1,2-bis[o-aminophenoxy]ethane-*N,N,N′,N′*-tetra-acetic acid, obtained as a tetra potassium salt from Molecular Probes) and titrated with $CaCl_2$. Free [Ca^{2+}] in excess of 0.1 µM was measured using a Ca^{2+} electrode (Fluka). At lower concentrations, free [Ca^{2+}] was estimated using published association constants (Marks and Maxfield, 1991) and the program Bound and Determined (Brooks and Storey, 1992). Solutions, which mimicked zero Ca^{2+}, were made with 4.5 mM BAPTA and no added Ca^{2+}. In these solutions, the total [Ca^{2+}] arising from impurities was measured to be 15 µM so that with addition of 4.5 mM BAPTA the free [Ca^{2+}] was calculated to be 1 nM.

In solutions containing ATP, which buffers Mg^{2+}, the required free [Mg^{2+}] was determined using the fluorescent magnesium indicator, Mag-fura-2 (tetra potassium salt from Molecular Probes). The ratio of fluorescence intensities at 340 and 380 nm was calibrated in the experimental solutions (50 and 250 mM Cs^+ solutions, see above) also containing 5 µM Mag-fura-2, 4.5 mM BAPTA, (free [Ca^{2+}] 1 nM–1 µM) and $MgCl_2$ from aliquots of a calibrated stock. This allowed determination of the ATP purity and effective Mg^{2+} binding constants under experimental conditions, which in turn allowed calculation of free [Mg^{2+}] in solutions containing >1 µM Ca^{2+}, where Mag-fura-2 is not a suitable indicator of [Mg^{2+}].

ATP was obtained in the form of sodium salts from Sigma-Aldrich. Unless otherwise stated, DIDS (sodium salt) was prepared as a stock solution in DMSO (dimethyl sulfoxide) at concentrations between 5 and 50 mM. Care was taken to ensure that DMSO in the bath solutions did not exceed 1% since DMSO concentrations >2% inhibited RyR activity (O'Neill et al., 2003).

Acquisition and Analysis of Single Channel Recordings

Bilayer potential was controlled and currents recorded using an Axopatch 200B amplifier (Axon Instruments). The cis chamber was electrically grounded to prevent electrical interference from the perfusion tubes, and the potential of the trans chamber was varied. However, all electrical potentials are expressed here using standard physiological convention (i.e., cytoplasmic side relative to the luminal side at virtual ground). Measurements were performed at 23 ± 2°C.

During the experiments, the channel current was recorded after low pass filtering at 5 kHz and sampling at 50 kHz. The data was stored on computer disk using a data interface (Data Translation DT301) under the control of in-house software written in Visual Basic. For measurements of P_o, the current signal was digitally filtered at 1 kHz with a Gaussian filter and sampled at 5 kHz.

Unitary current and time-averaged currents were measured using Channel2 software (P.W. Gage and M. Smith, Australian National University, Canberra, Australia). To calculate P_o from single channel records, a threshold discriminator was set at 50% of channel amplitude to detect channel opening and closing events. For experiments in which bilayers contained several RyRs, the time-averaged current was divided by the unitary current and the number of channels. The number of channels in each experiment could be determined during periods of strong activation (in the absence of Mg^{2+}). Both methods of calculating P_o gave similar results.

For measurements of channel open and closed dwell times, the current signal was filtered at 2 kHz and sampled at 10 kHz. Open and closed durations were extracted from single channel recordings using the 50% threshold (see above). Channel gating in multichannel recordings were analyzed using the Hidden Markov Model (HMM; Chung et al., 1990). The algorithm calculated the idealized, multilevel, current time course (i.e., background noise subtracted) and the transition probability matrix from the raw signal using maximum likelihood criteria. The mean channel opening and closing rates in the presence of n_o open channels, k_o^+ and k_o^-, respectively, were calculated from the transition probability matrix, P, by Eq. 1:

$$k_o^+ = \frac{-\ln(1 - P_{n_o,n_0+1})}{\Delta t} / (n_T - n_o) \qquad (1)$$

$$k_o^- = \frac{-\ln(1 - P_{n_o,n_0-1})}{\Delta t} / (n_o),$$

where n_T is total number of channels in the bilayer and Δt is the sample interval.

Dissecting Mg^{2+} Inhibition at A-sites and I-sites

RyRs are inhibited when Mg^{2+} is bound to either *A*- or *I*-sites so that the open probability of the channel is the product of the Mg^{2+} occupancies of each site (Laver et al., 1997). Consequently, the half-inhibiting [Mg^{2+}], $K_i(Mg^{2+})$ depends on the Mg^{2+} affinities of both *A*- and *I*-sites (K_A and K_I, respectively). Provided the $K_A << K_I$ then $K_A \approx K_i(Mg^{2+})$, with a relative error of $(K_A/K_I)^2$ (the second power in this equation stems from the Hill coefficient of ~2 for Mg^{2+} inhibition at the *I*-sites). Since *I*-sites are nonselective between divalent ions (Soler et al., 1992; Laver et al., 1997; Meissner et al., 1997), then one can estimate K_I from the half-inhibitory [Ca^{2+}] for RyR inhibition, $K_i(Ca^{2+})_c$. The subscripts *c* here, and *l* later, refer to cytoplasmic and luminal solutions, respectively. For example, in the case of ATP-activated RyRs in this study, $K_i(Ca^{2+})_c$ is 2.5 mM (Table I) so that $K_A \approx K_i(Mg^{2+})$ with <15% error when $K_i(Mg^{2+}) < 1$ mM. Since the experimental conditions were always chosen to keep the error in $K_A < 15\%$, we denote the Mg^{2+} affinity of the *A*-sites by either K_A or $K_i(Mg^{2+})$. We found that DIDS and ryanodine are useful pharmacological tools for studying the *A*-sites because they markedly increased $K_i(Ca^{2+})_c$ and so increased the range of K_A that could be determined from $K_i(Mg^{2+})$.

Modeling Mg^{2+} Inhibition at the A-sites

Here we consider competitive and noncompetitive models to explain the modulation of Mg^{2+} inhibition by the other major ion species present. Within the competitive model, $K_i(Mg^{2+})$ is related to the Mg^{2+} binding affinity, $K_m(Mg^{2+})$, and the concentrations, C_j, and binding affinities $K_m(j)$ of other ions, *j*, by Eqs. 2 and 3.

$$K_i(Mg^{2+}) = K_m(Mg^{2+}) \cdot F \qquad (2)$$

where

$$F = 1 + \sum_{j} (C_j / K_m(j))^{n_j} \qquad (3)$$

The parameter n_j is equal to the number of ions of species, j, that can bind at that site. The ions considered to compete with Mg^{2+} in this study are Cs^+ and Ca^{2+} in the cytoplasm and Ca^{2+} in the lumen. When these ions are explicitly included Eq. 2 becomes

$$F = 1 + ([Ca^{2+}]_c / K_m(Ca^{2+})_c) + \qquad (4)$$
$$([Ca^{2+}]_l / K_m(Ca^{2+})_l) + ([Cs^+]_c / K_m(Cs^+))^2.$$

The term for Cs^+ is raised to the second power because it is likely that two monovalent ions can bind to a divalent cation site (Meissner et al., 1997). Examination of Eqs. 2–4 reveals that the relative effect of a competing ion on Mg^{2+} inhibition is significant when its concentration exceeds the value of its affinity and the ratio of its concentration to affinity exceeds that of the other ions present.

In the noncompetitive model, each ion regulates channel activity at independent sites. A specific example of this, used in this work, is where the binding of luminal Ca^{2+} prevents the binding of cytoplasmic Mg^{2+} or Ca^{2+} (denoted by X^{2+}) by an allosteric effect. The apparent affinities for cytoplasmic Ca^{2+} and Mg^{2+}, $K_{app}(X^{2+})_c$, are related to the affinity $K_m(X^{2+})_c$ in the absence of luminal Ca^{2+} by Eq. 5:

$$K_{app}(X^{2+})_c = K_m(X^{2+})_c (1 + ([Ca^{2+}]_l / K_m(Ca^{2+})_l)). \qquad (5)$$

The Ca^{2+}_c dependence of Mg^{2+} inhibition is analyzed in this study in terms of apparent affinities for Ca^{2+}_c and Mg^{2+}, $K_{app}(Ca^{2+})_c$, and $K_{app}(Mg^{2+})$, respectively (see Fig. 4). For this analysis Eqs. 2–4 are recast into the following form:

$$K_i(Mg^{2+}) = K_{app}(Mg^{2+}) \cdot (1 + ([Ca^{2+}]_c / K_{app}(Ca^{2+})_c)). \qquad (6)$$

It can be seen from Eq. 6 that the value of $K_i(Mg^{2+})$ at low $[Ca^{2+}]_c$ approximates the value of $K_m(Mg^{2+})$ and the value of $K_m(Ca^{2+})_c$ determines the $[Ca^{2+}]$ threshold where $K_i(Mg^{2+})$ becomes Ca^{2+} dependent.

Statistics and Curve Fitting

Unless otherwise stated the data are presented as mean $\pm$ SEM obtained from N bilayers and n RyRs. Theoretical curves were fitted to the data using the criteria of least squares. The open probability (P_o) of RyRs, activated or inhibited by a compound with concentration, C, was fitted by Hill equations, Eqs. 7 and 8, respectively:

$$P_o = P_i + \frac{(P_{max} - P_i)}{(1 + (K_a / C)^{n_a})} \qquad (7)$$

$$P_o = \frac{P_{max}}{(1 + (C / K_i)^{n_i})}, \qquad (8)$$

where P_i and P_{max} are RyR open probabilities in the absence of the compound and at maximal activation, respectively. K_a and K_i are half-activation and inhibition concentrations, and n_a and n_i are the associated Hill coefficients. Ion binding models of the *A*-site were fitted to the logarithm of the data by minimizing the residuals weighted by the experimental error on each datum. Statistical uncertainty in the elements of the transition probability matrix produced by HMM analysis ranged from 5 to 25% relative error. This was estimated by the SEM of transition probability matrices derived from four subsections in four representative experiments.
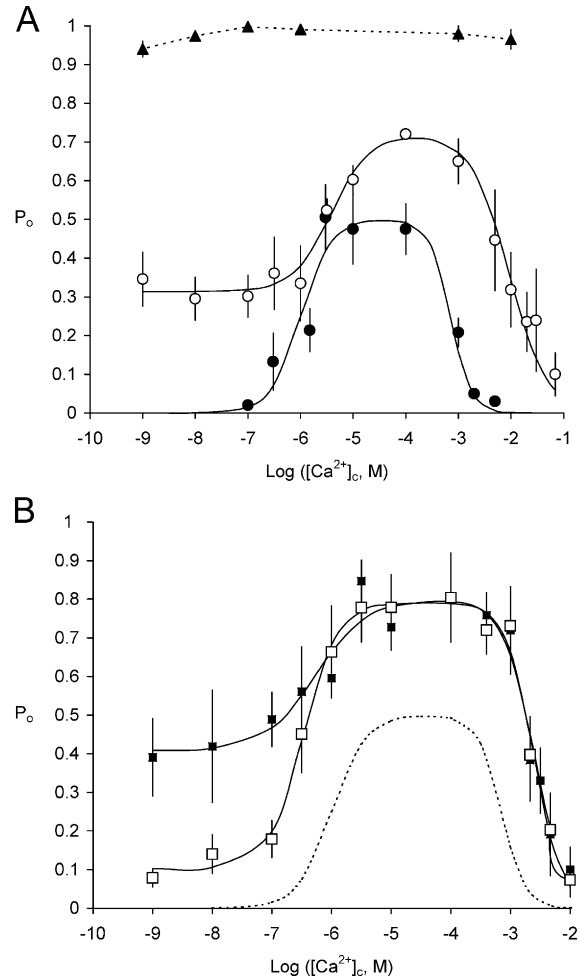

FIGURE 1. The dependence of RyR open probability, P_o, on cytoplasmic $[Ca^{2+}]$. (A) RyRs activated by cytoplasmic Ca^{2+} alone ($\bullet$, $N = 30$), modified by 10 μM ryanodine ($\blacktriangle$) or 4-min exposure to 100 μM DIDS ($\bigcirc$, $N = 12$, $n = 64$). Luminal $[Ca^{2+}] = 1$ mM. (B) In the presence of 2 mM ATP with luminal $[Ca^{2+}] = 1$ mM ($\blacksquare$, $N = 9$, $n = 49$) or 0.01 mM ($\square$, $N = 15$, $n = 89$). The membrane potential was +40 mV. The data points represent means of 2–20 measurements. The solid curves are Hill fits to the data and the parameter values are shown in Table I. The dashed curve is the Hill fit to the circles in A shown again for comparison.

R E S U L T S

RyR Ca^{2+} Dependencies

RyRs in the absence of ATP and the modifying agents DIDS and ryanodine (i.e., native RyRs) are inactive in the absence of cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_c$). They are activated by μM Ca^{2+} and inhibited by mM Ca^{2+}, thus producing the characteristic bell-shaped Ca^{2+} dependence in open probability (P_o) (Fig. 1 A, closed circles).

Consistent with previous findings (Smith et al., 1986), cytoplasmic ATP (2 mM) amplified the bell-shaped Ca^{2+} dependence without greatly altering the half-activating $[Ca^{2+}]_c$, $K_a(Ca^{2+})_c$ (Fig. 1 B and Table I), and activated RyRs in the absence of cytoplasmic Ca^{2+}. We also found

The Apparent Affinities of the Ca^{2+} and Mg^{2+} Binding Sites on the RyRs under Various Experimental Conditions

	Conditions		P_{max}	P_i	$K_a(Ca^{2+})_c$	$K_i(Ca^{2+})_c$	$K_{app}(Ca^{2+})_c$	$K_{app}(Mg^{2+})$
	mM				μM	*mM*	μM	μM
Unmodified		$[Ca^{2+}]_l = 1$	0.55 ± 0.07	0	1.1 ± 0.4^{a}	0.7 ± 0.3^{a}	–	–
ATP	2	$[Ca^{2+}]_l = 1$	0.86 ± 0.05	0.40 ± 0.06	0.4 ± 0.1	2.5 ± 0.4	0.51 ± 0.07	54 ± 4
	2	$[Ca^{2+}]_l = 0.01$	0.86 ± 0.05	0.1 ± 0.07	0.3 ± 0.1	2.5 ± 0.6	–	–
	2	$[Ca^{2+}]_l = 1$	0.71 ± 0.07	–	–	0.25 ± 0.05	–	–
		$[Cs^+] = 100$						
DIDS	0.5	$[Ca^{2+}]_l = 1$	0.73 ± 0.02	0.3 ± 0.05	4.0 ± 0.6	9.2 ± 1.1	0.9 ± 0.2	120 ± 17
Ryanodine	0.01	$[Ca^{2+}]_l = 1$	–	–	–	–	0.10 ± 0.008	4000 ± 160

Unless otherwise stated the cytoplasmic $[Cs^+] = 250$ mM. $K_a(Ca^{2+})_c$ and $K_i(Ca^{2+})_c$ are the cytoplasmic $[Ca^{2+}]$ for half-activation and inhibition of RyRs, respectively. These values were obtained from fits of the Hill equation (Eqs. 7 and 8) to the P_o vs. $[Ca^{2+}]_c$ data in Fig. 1. $K_{app}(Ca^{2+})_c$ and $K_{app}(Mg^{2+})$ are affinities of the *A*-sites for Ca^{2+} and Mg^{2+}, respectively. These values were obtained from fits of Eq. 6 to the $K_i(Mg^{2+})$ vs. $[Ca^{2+}]_c$ data in Fig. 4.
[a]Data obtained from Laver et al. (1997).

that ATP increased the half-inhibitory $[Ca^{2+}]$, $K_i(Ca^{2+})_c$, by approximately threefold (Table I). Luminal Ca^{2+} ($[Ca^{2+}]_l$) increased channel activity in 2 mM ATP and 1 nM $[Ca^{2+}]_c$ (P_i; Fig. 2, top). The mean P_i increased from 0.25 ± 0.07 in 0.1 mM luminal Ca^{2+} (number of bilayers, $N = 7$, and number of channels, $n = 21$) to 0.50 ± 0.06 in 1 mM ($N = 15$, $n = 37$) and 0.63 ± 0.08 in 3 mM ($N = 12$, $n = 43$). However, luminal Ca^{2+} had no effect on the maximum of bell-shaped Ca^{2+} dependence, P_{max} (Table I).

Ryanodine (10 μM) produced nonreversible activation of RyRs by "locking" them into a conductance sub-state. Once RyRs were modified by ryanodine in this way, the nonbound ryanodine was washed away from the RyR by local perfusion (see MATERIALS AND METHODS) before measurements. Ryanodine-modified channels were relatively insensitive to $[Ca^{2+}]_c$ (Fig. 1, triangles). Ryanodine-modified channels were not inhibited by high $[Ca^{2+}]_c$, neither were they inhibited by low $[Ca^{2+}]_c$ in the presence of 1 mM luminal Ca^{2+}. In the absence of both cytoplasmic and luminal Ca^{2+}, the distribution of RyR activity appeared to be bimodal. Most had P_o values ~1, while a minority had substantially lower P_o as follows. In ~1 nM $[Ca^{2+}]_c$ (impurity Ca^{2+}
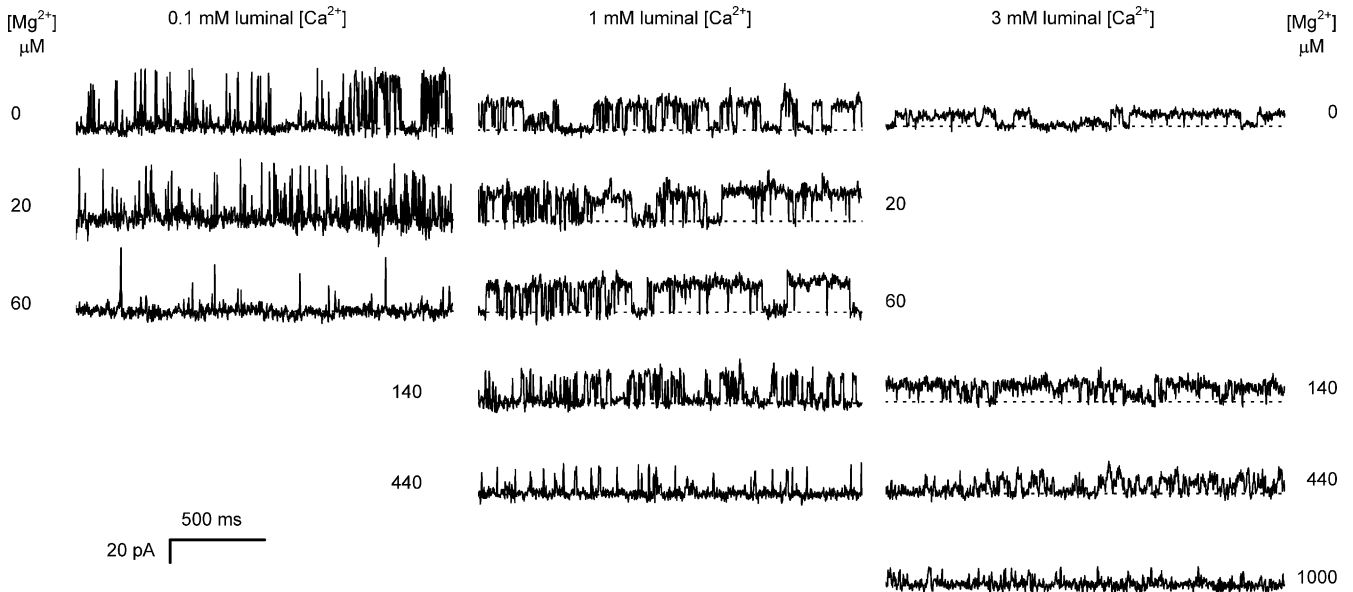

FIGURE 2.   Recordings from one experiment on a single skeletal RyR (RyR1) showing the effects of luminal $[Ca^{2+}]$ and cytoplasmic $[Mg^{2+}]$ on channel gating. Increasing luminal $[Ca^{2+}]$ markedly increased the channel open probability and decreased the channel sensitivity to Mg^{2+} inhibition. The cytoplasmic solution contained (in mM) 250 $CsCH_3O_3S$, 10 TES (pH 7.4), 4.5 BAPTA (~1 nM free Ca^{2+}), 2 ATP plus the various $[Mg^{2+}]$. The free Mg^{2+} is indicated at the left and right of each row. The luminal solution contained (in mM) 30 $CsCH_3O_3S$, 20 CsCl, 10 TES, and the indicated $[Ca^{2+}]$. Under these conditions, Mg^{2+} is thought to inhibit primarily by binding at the high affinity Ca^{2+} activation site. Membrane potential was held at +40 mV. The current baselines are shown by dashed lines.

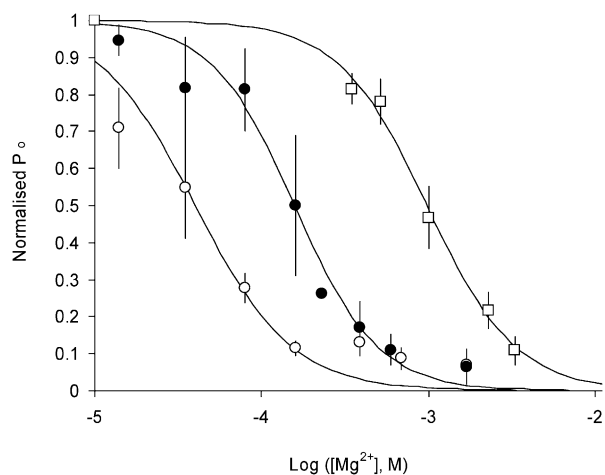FIGURE 3. Mg^{2+} inhibition of RyR P_o in the presence of 2 mM ATP and $[Ca^{2+}]_c = 10$ nM ($\bigcirc$, $N = 7$, $n = 20$), 1 μM ($\bullet$, $N = 6$, $n = 13$), and 10 μM ($\square$, $N = 7$, $n = 35$). Luminal $[Ca^{2+}] = 1$ mM. The data points represent means of two to seven measurements. The solid curves are Hill fits to the data. Half-inhibiting Mg^{2+} concentrations, $K_i(Mg^{2+})$, are summarized in Fig. 4 and Table III.

FIGURE 4. The $[Mg^{2+}]$ required to half-inhibit RyRs, $K_i(Mg^{2+})$, at the indicated cytoplasmic $[Ca^{2+}]$. Mg^{2+} inhibition was measured in RyRs that were modified by 10 μM ryanodine ($\blacksquare$) or 100 μM DIDS ($\bigcirc$) or activated by 2 mM ATP ($\blacktriangle$). Luminal $[Ca^{2+}] = 1$ mM and the membrane potential was +40 mV. The data points represent means of 4–11 measurements. The solid curves are generated by Eq. 6. The parameters of the fits are given in Table I.

plus 4.5 mM BAPTA), P_o were 0.71 and 0.66 in 2 of 9 experiments. In ~0.1 nM $[Ca^{2+}]_c$ (impurity Ca^{2+} plus 5 mM EGTA), we observed 2 out of 11 RyRs where P_o was <0.5. The decrease in activity was associated with an increase in the frequency of short channel closures.

DIDS produces both reversible and nonreversible activation of RyRs (O'Neill et al., 2003). Here, the effects of permanent RyR modification were measured by applying DIDS (100–500 μM) to the cytoplasmic bath for 4 min. DIDS was then removed by local perfusion before measurements commenced. DIDS modification activated RyR in the absence of cytoplasmic Ca^{2+} (Fig. 1, open circles) and shifted Ca^{2+} activation and inhibition to higher concentrations by ~10-fold and by ~3-fold, respectively (Table I).

Regulation of Modified RyRs by Mg^{2+}

We investigated the possibility that Mg^{2+} inhibits RyRs by occluding Ca^{2+} from its activation sites on the protein. We did this by measuring the effect of Mg^{2+} on RyRs activated by ATP, DIDS, or ryanodine in the absence of cytoplasmic Ca^{2+}. In all three cases, Mg^{2+} could totally and reversibly inhibit RyRs (e.g., see Figs. 2 and 3 for the case of ATP-activated RyRs). An increase in $[Ca^{2+}]_c$ produced a corresponding increase in the $[Mg^{2+}]$ required to inhibit the channels (Figs. 3 and 4). Therefore, Ca^{2+} and Mg^{2+} compete for a common site (presumably the *A*-site) where Mg^{2+} is an antagonist and not merely an inhibitor of Ca^{2+} activation.

The Mg^{2+} dependencies of P_o for RyRs were fitted with the Hill equation (Eq. 8). Examples of such fits are shown for ATP-modified RyRs in Fig. 3. The half-inhibiting Mg^{2+} concentration, $K_i(Mg^{2+})$, in the pres-
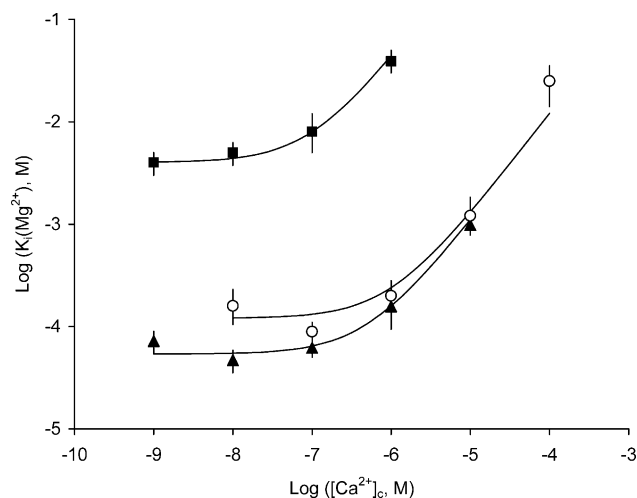
ence of 1 mM luminal Ca^{2+} is plotted against $[Ca^{2+}]_c$ in Fig. 4. There is a range of $[Ca^{2+}]_c$ over which $K_i(Mg^{2+})$ is relatively insensitive to Ca^{2+}. At higher concentrations, $K_i(Mg^{2+})$ increases in proportion to $[Ca^{2+}]_c$. The data is fitted with Eq. 6, which describes Mg^{2+} inhibition in terms of competition between Mg^{2+} and Ca^{2+} at a common binding site. The apparent binding affinities for Ca^{2+} and Mg^{2+} associated with Mg^{2+} inhibition are given in Table I. The values for $K_{app}(Ca^{2+})_c$ obtained from Mg^{2+} inhibition and $K_a(Ca^{2+})$ for Ca^{2+} activation are the same order of magnitude,
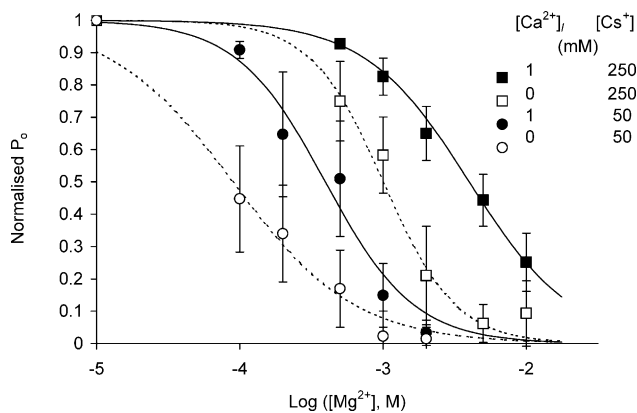

FIGURE 5. The dependence of Mg^{2+} inhibition of ryanodine-modified RyRs on cytoplasmic $[Cs^+]$ and luminal $[Ca^{2+}]$. Recordings were made in the absence of cytoplasmic $[Ca^{2+}]$ (~1 nM). The solid curves are Hill fits to the data. Half-inhibiting Mg^{2+} concentrations, $K_i(Mg^{2+})$, and numbers of experiments are summarized in Table II.

TABLE II

The Effect of Cytoplasmic Cs⁺ and Ca²⁺ and Luminal Ca²⁺ on Mg²⁺ Inhibition of Ryanodine-modified RyRs (+40 mV)

	Conditions			Experimental Data				Model Predictions		
	$[Cs^+]$	$[Ca^{2+}]_l$	$[Ca^{2+}]_c$	P_o control	n_i	$K_i(Mg^{2+})$		$K_i(Mg^{2+})$		
	mM	*mM*	*pCa*			*µM*	*(N, n)*	*Model 1*	*Model 2*	*Model 3*
1	50	0	9	0.67 ± 0.05	1.0 ± 1.0	90 ± 40	(5, 52)	97	121	79
2	50	0	7	0.71 ± 0.08	1.0 ± 1.0	2000 ± 900	(4, 5)	1197	518	1139
3	50	1	9	0.91 ± 0.07	1.4 ± 1.0	400 ± 100	(3, 21)	373	358	470
4	50	1	7	0.71 ± 0.10	1.4 ± 0.4	1420 ± 140	(6, 15)	1474	1534	1529
5	250	0	9	0.96 ± 0.05	1.7 ± 1.0	1050 ± 150	(7, 56)	1047	1167	1423
6	250	0	7	–	–	–	–	2147	1564	2482
7	250	1	9	0.94 ± 0.02	1.3 ± 0.5	3900 ± 900	(10, 38)	4400	3455	1813
8	250	1	7	0.98 ± 0.02	1.2 ± 1.0	8000 ± 3000	(4, 23)	5501	4631	2873
						Residual of least squares fits		0.022	0.048	0.067

The data was obtained from Hill fits (Eq. 8) to Mg²⁺ dose–responses of P_o. P_o control is the open probability in the absence of Mg²⁺, n_i is the Hill coefficient and $K_i(Mg^{2+})$ is the half-inhibitory [Mg²⁺]. Nominal zero luminal Ca²⁺ was obtained by using solutions with no added Ca²⁺ (~10 µM impurity Ca²⁺) or by chelating Ca²⁺ impurities with 2 mM BAPTA (~1 nM free Ca²⁺). *N* is the number of bilayers and *n* is the number of channels studied. The predictions of three models in the right three columns are compared with the experimental values of $K_i(Mg^{2+})$. Models 1 and 2 assume that cytoplasmic Ca²⁺ and Cs⁺ compete with Mg²⁺ at a common site and that luminal Ca²⁺ modulates the binding affinity of Mg²⁺ (Model 2) or both cytoplasmic Ca²⁺ and Mg²⁺ (Model 1) by a noncompetitive, allosteric action. Model 3 assumes that cytoplasmic Ca²⁺, Cs⁺, Mg²⁺, and luminal Ca²⁺ all compete at a common site. The quality of fit parameter is the residuals from model fits to the logarithms of $K_i(Mg^{2+})$. Ion binding affinities derived from Model 1 are listed in Table IV.

suggesting that Mg²⁺ is inhibiting at the Ca²⁺ activation site (*A*-site). The $K_{app}(Ca^{2+})_c$ is very much smaller than expected for the *I*-sites, which have Ca²⁺/Mg²⁺ affinities of ~1 mM. Interestingly, analysis of the data for the ryanodine-modified channels gives $K_{app}(Ca^{2+})_c \sim$ 100 nM (Table I), suggesting that ryanodine modification increases the Ca²⁺ sensitivity of the *A*-sites by ~10-fold. The increased Ca²⁺ sensitivity is not sufficient to account for the high P_o ($P_o \sim 1$) of ryanodine-modified RyRs at 1–10 nM cytoplasmic Ca²⁺. Rather, it is most likely due to a Ca²⁺-independent activation of RyRs by ryanodine, akin to that produced by DIDS and ATP (Fig. 1).

Effect of Monovalent Ions on Mg²⁺ Inhibition

In native RyRs, monovalent cations are known to compete with Ca²⁺ for the activation site to prevent channel activation (Meissner et al., 1997). Therefore we compared the Mg²⁺ inhibition of RyRs at high (250 mM) and low (50 mM) Cs⁺ concentrations in the absence of cytoplasmic Ca²⁺. Both in the presence and absence of luminal Ca²⁺, raising [Cs⁺] by fivefold increased $K_i(Mg^{2+})$ by 3- and 10-fold for ATP and ryanodine-modified RyRs, respectively (Fig. 5 and Table II, compare entries 1 and 5, 3 and 7; Table III, compare entries 1 and 3, 2 and 4). This is consistent with competition be-

TABLE III

The Effect of Cytoplasmic Cs⁺ and Ca²⁺ and Luminal Ca²⁺ on Mg²⁺ Inhibition of RyRs in the Presence of 2 mM ATP

	Conditions			Experimental Data				Model Predictions		
	$[Cs^+]$	$[Ca^{2+}]_l$	$[Ca^{2+}]_c$	P_o control	n_i	$K_i(Mg^{2+})$		$K_i(Mg^{2+})$		
	mM	*mM*	*pCa*			*µM*	*(N, n)*	*Model 1*	*Model 2*	*Model 3*
1	50	0.1	9	0.21 ± 0.16	–	8 ± 2	(2, 2)	7	9	7
2	50	1	9	0.45 ± 0.20	–	20 ± 5	(3, 5)	22	18	39
3	250	0.1	9	0.25 ± 0.07	1.6 ± 1.0	20 ± 4	(7, 21)	21	27	24
4	250	1	9	0.58 ± 0.06	1.1 ± 0.7	72 ± 18	(11, 26)	67	55	56
5	250	3	9	0.63 ± 0.07	1.0 ± 0.6	153 ± 40	(12, 43)	170	119	128
6	250	1	7	0.51 ± 0.06	1.8 ± 1.3	62 ± 12	(6, 20)	74	65	63
7	250	1	6	0.54 ± 0.12	1.7 ± 0.7	157 ± 18	(5, 13)	137	155	128
8	250	0.1	5	0.77 ± 0.04	1.4 ± 0.6	507 ± 60	(7, 30)	712	445	741
9	250	1	5	0.74 ± 0.06	1.6 ± 0.4	980 ± 100	(4, 31)	764	1050	777
						Residual of least squares fits		0.034	0.020	0.044

N is the number of bilayers and *n* is the number of channels studied. The analysis is described in the caption to Table II, and the ion binding affinities derived from Model 1 are listed in Table IV.
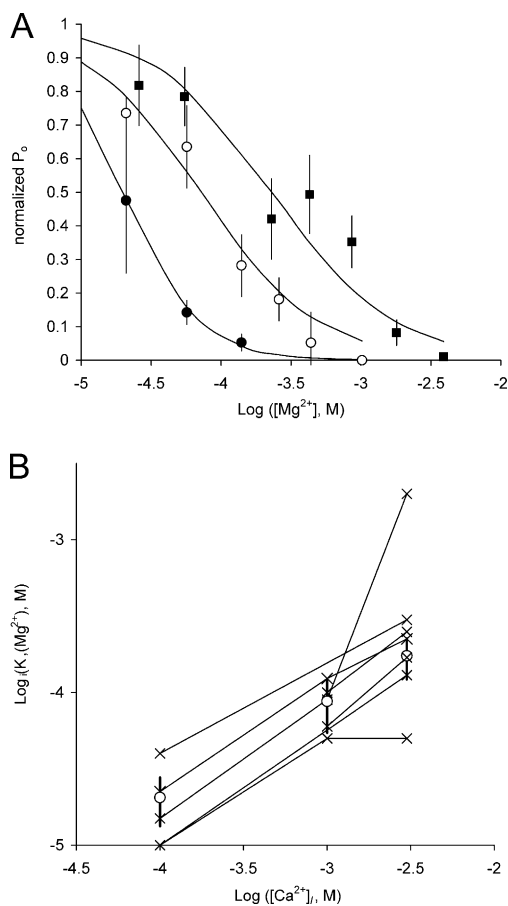
FIGURE 6. The effect of luminal $[Ca^{2+}]$ on inhibition of RyRs by cytoplasmic Mg^{2+}. Mg^{2+} inhibition of RyRs was measured in the presence of 1 nM cytoplasmic $[Ca^{2+}]$, 2mM ATP, and various luminal $[Ca^{2+}]$. (A) The mean dose–responses to Mg^{2+} were measured with luminal $[Ca^{2+}]$ of 0.1 mM (●, $N = 7$, $n = 21$), 1 mM (○, $N = 11$, $n = 26$), and 3 mM (■, $N = 13$, $n = 43$). The data points represent means of 3–13 measurements. The solid curves are Hill fits to the data. (B) x, half-inhibiting Mg^{2+} concentrations, $K_i(Mg^{2+})$, obtained from seven individual RyRs at two or more $[Ca^{2+}]_l$; ○, the mean from all experiments. N and n values listed in Table III.

tween Mg^{2+} and Cs^+ for common sites. The relatively strong Cs^+ dependence of Mg^{2+} inhibition in the case of ryanodine-modified channels suggests that at least two Cs^+ can bind at a Mg^{2+} inhibition site.

We considered the possibility that monovalent cations can substitute for Ca^{2+} as an agonist of RyRs that are modified by ATP, DIDS, and ryanodine and that Mg^{2+} inhibits RyRs by preventing the binding of cytoplasmic Cs^+ (Cs^+ is the major monovalent cation in our solutions). In ATP-activated RyRs in the absence of Ca^{2+} and Mg^{2+}, Cs^+ (50–250 mM) had no effect on channel activation. However, Cs^+ did appear to activate ryanodine-modified RyRs because decreasing $[Cs^+]$ in the cytoplasm from 250 to 50 mM decreased P_o. In ryanodine-modified RyRs, increasing $[Ca^{2+}]_c$ from 1 nM to sub-mM levels could not reverse the effect of decreasing

cytoplasmic $[Cs^+]$ from 250 to 50 mM (see Table V). Thus it appears that Cs^+ does not activate RyRs by binding at their A-sites. Although Cs^+ and Mg^{2+} do compete for the A-sites, it is unlikely that Mg^{2+} inhibits RyRs by preventing Cs^+ binding to the A-sites.

Effect of Luminal $[Ca^{2+}]$ on Mg^{2+} Inhibition

As mentioned above, increasing luminal $[Ca^{2+}]$ from ~0 to 1 mM both increased P_o in the absence of cytoplasmic Mg^{2+} and decreased the sensitivity of RyRs to Mg^{2+} inhibition (e.g., see Fig. 2). The Mg^{2+} dose–response curves for ATP-modified RyRs in the absence of cytoplasmic Ca^{2+} were obtained for three luminal Ca^{2+} loads (0.1, 1, and 3 mM) (Fig. 6 A). Mean values of $K_i(Mg^{2+})$ are shown in Fig. 6 B (circles), along with individual values for the 7 out of 12 experiments (crosses) where $K_i(Mg^{2+})$ was obtained from the same RyRs at two or more $[Ca^{2+}]_l$. These results show that, in spite of some channel-to-channel variations in $K_i(Mg^{2+})$, the effect of $[Ca^{2+}]_l$ was consistently observed. The mean $K_i(Mg^{2+})$ increased from 20 μM in 0.1 mM luminal Ca^{2+} to 72 μM at 1 mM Ca^{2+}_l and 153 μM in 3 mM Ca^{2+}_l (Table III, entries 3–5). A similar phenomenon was also observed in ryanodine-modified RyRs (Fig. 7; Table II, compare entries 1 and 3, 5 and 7). In ryanodine-modified RyRs, increasing $[Ca^{2+}]_l$ from ~0 to 1 mM markedly reduced the sensitivity of RyRs to Mg^{2+} inhibition. It caused a fourfold increase in $K_i(Mg^{2+})$ in the presence of both 50 and 250 mM Cs^+ (Fig. 5).

Ion Competition Models for Mg^{2+} Inhibition

To further understand the ionic mechanism underlying Ca^{2+} activation and Mg^{2+} inhibition of RyRs, we compared our data to the predictions of several models for ion binding at the RyR A-site (Tables II and III). In Table II, we fitted models to the $K_i(Mg^{2+})$ for ryanodine-modified channels in the presence of various combinations of high and low $[Ca^{2+}]_c$, $[Cs^+]$, and $[Ca^{2+}]_l$ using a least-squares criterion. In these models, the channel is open in its unbound form or when bound to Ca^{2+} or Cs^+, and the binding of Mg^{2+} closes the channel. We considered the possibility that cytoplasmic Ca^{2+}, Cs^+, Mg^{2+}, and luminal Ca^{2+} all compete at the A-site (Table II, Model 3). This provided relatively poor fit to the data and was unable to account for $K_i(Mg^{2+})$ at high $[Cs^+]$ and $[Ca^{2+}]_l$ (Table II, entry 7) and the effect of $[Ca^{2+}]_l$ on $K_i(Mg^{2+})$ of ATP-activated RyRs (Table III, entries 8 and 9). Several models were investigated that were based on noncompetitive binding between Ca^{2+}_l and cytosolic ions and also noncompetitive binding between the cytosolic ions. The only models that gave an improved fit to the data were those in which cytoplasmic Ca^{2+} and Cs^+ compete with Mg^{2+} at the A-site while luminal Ca^{2+} inhibits their binding via
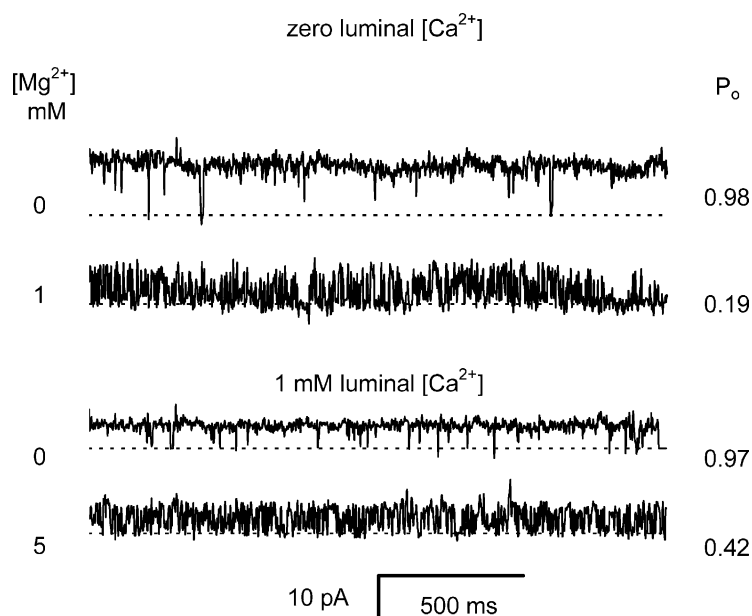
FIGURE 7. Recordings from a single, ryanodine-modified RyR showing the effect of luminal $[Ca^{2+}]$ on channel sensitivity to Mg^{2+} inhibition. In zero luminal Ca^{2+} ($[Ca^{2+}] < 10 \ \mu M$), 1 mM cytoplasmic Mg^{2+} reduced channel activation by $\sim$80%. Increasing luminal $[Ca^{2+}]$ to 1 mM markedly reduced the effect of Mg^{2+}, such that 5 mM Mg^{2+} was needed to produce $\sim$50% inhibition. Membrane potential was held at +40 mV. The current baselines are shown by dashed lines.

an allosteric interaction (Models 1 and 2). In Model 1, luminal Ca^{2+} prevents binding of both cytoplasmic Ca^{2+} and Mg^{2+}, while in Model 2 it only affects binding of cytoplasmic Mg^{2+}. Both models fit well, but Model 1 fit better with ryanodine-modified RyRs, and Model 2 fit better with ATP-activated RyRs. The predicted ion binding affinities for ryanodine-modified and ATP-activated RyRs were quite different and they are compared Table IV. Note that the affinities in Table IV represent different channel properties to the apparent affinities for the A-sites in the presence of Cs^+ in I. Ryanodine increases the A-site affinity for Ca^{2+} by 40-fold, Cs^+ by threefold, and decreases its affinity for Mg^{2+} by sevenfold and luminal Ca^{2+} by twofold.

Voltage Dependencies

The voltage dependencies of RyR regulation by Ca^{2+} and Mg^{2+} have been used to investigate the mechanism(s) of RyR activation by luminal Ca^{2+}. The rationale is that at −40 mV, the bilayer potential favors the flow of Ca^{2+} from the luminal to cytosolic sides of the RyR while positive potential inhibits such Ca^{2+} feedthrough. The voltage dependence of RyR activation by luminal Ca^{2+} obtained here (in cytoplasmic 2 mM ATP and 1 nM Ca^{2+}) is similar to that seen in previous studies (Sitsapesan and Williams, 1995; Tripathy and Meissner, 1996). At +40 mV, the RyR activated with a $K_a(Ca^{2+})_l \sim 1$ mM, while at −40 mV, $K_a(Ca^{2+})_l \sim 0.1$ mM.

One might expect that the voltage dependence of Ca^{2+} flow through the RyR could produce a voltage dependence in their Mg^{2+} inhibition, because at negative potentials, luminal Ca^{2+} flowing through the channel should compete with cytoplasmic Mg^{2+} for the A-sites. In marked contrast with this proposition, we found that

$K_i(Mg^{2+})$ for single RyRs in a bilayer was insensitive to voltage at all luminal $[Ca^{2+}]$ tested. In the case of 1 mM luminal Ca^{2+} (cytoplasmic 2 mM ATP and 1 nM Ca^{2+}) the difference in $K_i(Mg^{2+})$ between −40 mV (100 ± 40 μM; $N = 5$) and +40 mV ($130 \pm 40 \ \mu M$; $N = 12$) was not significant and considerably less than changes in $K_i(Mg^{2+})$ caused by varying luminal $[Ca^{2+}]$ (this is not the case for coupled groups of RyRs, see below). This is particularly important because it indicates that the effects of luminal Ca^{2+} on Mg^{2+} inhibition of single RyRs are not due to Ca^{2+} feedthrough.

Gating Kinetics of Single RyRs

More detailed information about the mechanisms of channel regulation by cytoplasmic and luminal ligands can be obtained from the rates of channel gating. For example, it has been argued that luminal Ca^{2+} cannot affect RyR closed dwell times by acting at cytoplasmic sites since they are inaccessible to luminal Ca^{2+} when the channel is closed (Xu and Meissner, 1998). We begin with an analysis of mean open and closed dwell times (τ_o and τ_c, respectively) of single channels and

TABLE IV
Ion Binding Affinities of the A-site

K_m	ATP (2 mM)	Ryanodine
Ca^{2+}_c	166 nM	4.1 nM
Ca^{2+}_l	729 μM	310 μM
Mg^{2+}	7.0 μM	46 μM
Cs^+	164 mM	54 mM

The ion binding affinities for the A-site of RyRs that are activated by ATP, predicted by Model 2 or ryanodine predicted by Model 1 (see MATERIALS AND METHODS) from the data in Tables II and III.

TABLE V

Ion-dependent Activation of Ryanodine-modified RyRs

$[Ca^{2+}]_c$	1 nM		10 μM–1 mM	
$[Cs^+]$, mM		(N, n)		(N, n)
50	0.50 ± 0.05	(7, 52)	0.64 ± 0.10	(7, 22)
250	0.90 ± 0.04	(9, 56)	0.96 ± 0.01	(5, 54)

The effects of cytoplasmic Cs^+ and Ca^{2+} on the open probability of ryanodine-modified RyRs in the near absence of luminal Ca^{2+} (i.e., either 1 nM obtained by buffering free Ca^{2+} with 2 mM BAPTA or 10 mM obtained with non-Ca^{2+}–buffered solutions and no added Ca^{2+}). N is the number of bilayers and n is the number of channels studied.

proceed to analyze the analogous closing and opening rates of RyR from multichannel recordings. The general pattern of RyR activation by Ca^{2+} (both luminal and cytosolic), Mg^{2+}, and ATP seen here closely follows that previously reported for the adenine nucleotides (see Fig. 7 in Laver et al., 2001). When P_o is $<\sim 0.2$, channel activation and inhibition was associated primarily with changes in τ_c, while at higher P_o, changes in channel activity were associated primarily with changes in τ_o (unpublished data). The dependencies of mean open and closed dwell times on luminal $[Ca^{2+}]$ and cytoplasmic $[Mg^{2+}]$ are summarized in Table VI. We find that luminal Ca^{2+} can modulate channel activity and Mg^{2+} inhibition via changes in both τ_o and τ_c, which implies a mode of action involving luminal sites on the RyR protein.

Gating Kinetics of RyRs in Coupled Groups

On average, 20% of fusion events incorporated groups of four to eight RyRs into the bilayer. Under the right experimental conditions (e.g., −40 mV and the pres-

FIGURE 8. Recordings from an experiment with ∼4 RyRs in the bilayer showing coupled gating at −40 mV. At +40 mV, the channels appeared to gate independently (see text). The baths contained symmetric 250 mM Cs^+ solutions with $[Ca^{2+}]_c = 1$ nM and $[Ca^{2+}]_l = 1$ mM. The current baselines are shown by dashed lines and the dashed lines (O1–O4) indicate current levels associated with increasing numbers of open channels.

TABLE VI

Effect of Mg^{2+} and Luminal Ca^{2+} on Gating Parameters

Conditions	P_o	τ_o, ms	τ_c, ms	N
$[Mg^{2+}]$ ($[Ca^{2+}]_l = 1$ mM)				
0	0.54 ± 0.1	5.5 ± 1.8	4.0 ± 0.7	12
55 μM	0.38 ± 0.07	5.4 ± 1.7	7.6 ± 1.4	11
230 μM	0.24 ± 0.05	3.4 ± 1	10 ± 2.5	8
430 μM	0.08 ± 0.03	1.8 ± 0.6	45 ± 25	5
$[Ca^{2+}]_l$ ($[Mg^{2+}] = 0$)				
10 μM	0.1 ± 0.04	0.86 ± 0.2	11 ± 4	4
1 mM	0.54 ± 0.1	5.5 ± 1.8	4.0 ± 0.7	12
3 mM	0.8 ± 0.1	35 ± 23	5 ± 3	5
$[Ca^{2+}]_l$ ($[Mg^{2+}] = 55$ μM)				
10 μM	0.058 ± 0.02	1.1 ± 0.3	22 ± 8	6
1 mM	0.38 ± 0.07	5.4 ± 1.7	7.6 ± 1.4	11
3 mM	0.67 ± 0.2	30 ± 21	4.3 ± 0.3	3

RyR open probability, P_o, Mean open (τ_o) and closed times (τ_c) obtained from bilayer recordings of single RyRs at 40 mV in cytoplasmic solutions containing 2 mM ATP and 1 nM Ca^{2+}.
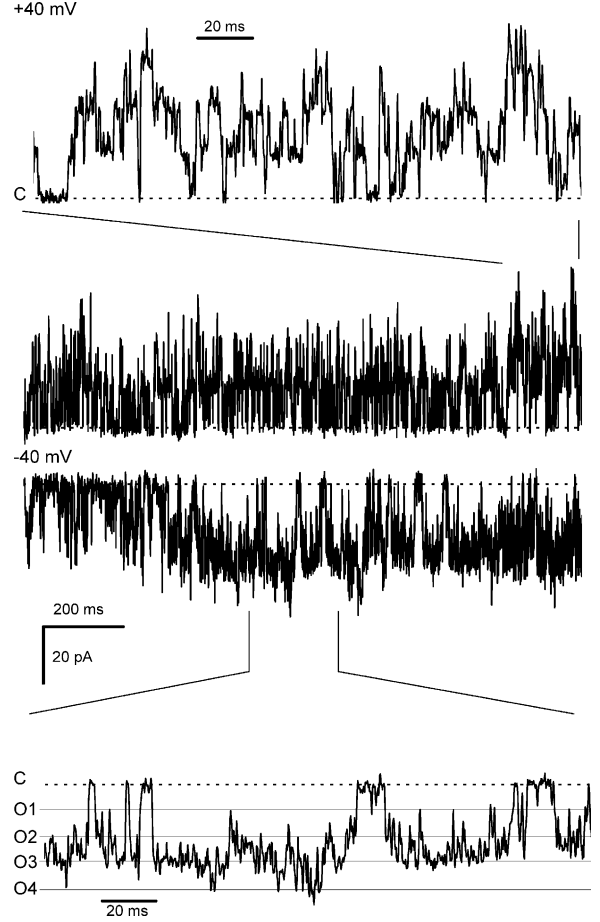
ence of ATP), the opening of one RyR in a group tended to promote the opening of other RyRs. Fig. 8 shows the activity of four, ATP-activated RyRs at positive and negative bilayer potentials. The current trace shows transitions between the current baseline (labeled C) and four equally spaced levels (O1–O4) corresponding to one to four open channels. At positive potentials, the weighting of each current level followed a binomial distribution expected from the gating in independent channels in the bilayer (unpublished data). At negative potentials, downward current steps frequently "bypassed" some of the current levels, indicating that several channels were opening in near synchrony. The weighting of current levels in these records markedly deviated from a binomial distribution. HMM analysis of these records (see MATERIALS AND METHODS) shows that the mean RyR opening rate associated with transitions between the current baseline and level O1, k_0^+,

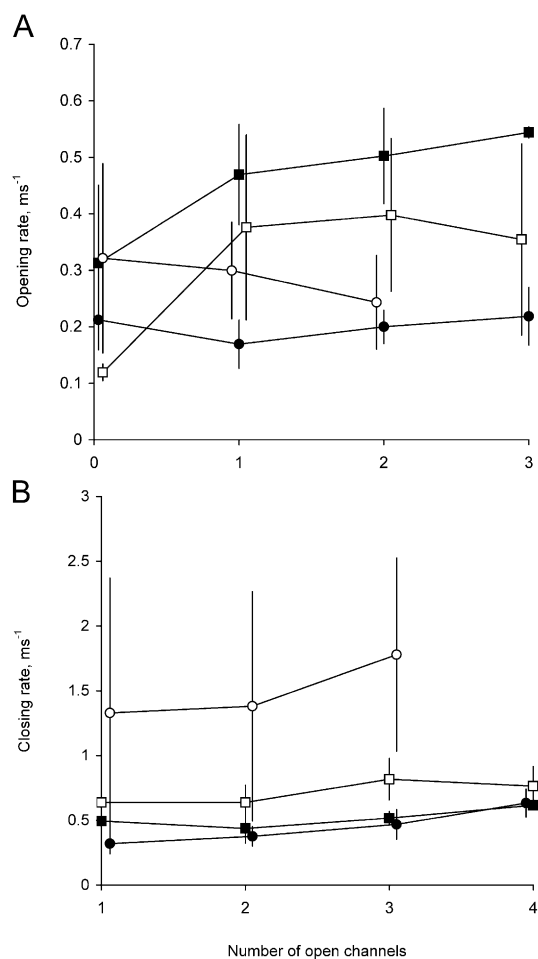750 *Luminal Ca^{2+} Modulates Mg^{2+} Inhibition of RyRs*

FIGURE 9. The dependence of mean channel opening rate (A) and closing rate (B) on the number of channels open in the bilayer under various experimental conditions at -40 mV. Cytoplasmic and luminal baths contained 250 mM Cs$^+$ solutions plus (cytoplasmic/luminal): ($\blacksquare$, $N = 7$) 2 mM ATP, 1 nM Ca^{2+}/1 mM Ca^{2+}; ($\bullet$, $N = 6$) 2 mM ATP, 1 nM Ca^{2+}/0.1 mM Ca^{2+} ($\square$, $N = 7$) 60–230 μM Mg^{2+}, 2 mM ATP, 1 nM Ca^{2+}/1 mM Ca^{2+}; ($\bigcirc$, $N = 4$) 100 μM Ca^{2+}/1 mM Ca^{2+}. The channel opening rate could be increased by the opening of other channels in the bilayer when ATP was present and when luminal [Ca^{2+}]$_l$ = 1 mM. Closing rates did not significantly depend on the presence of other open channels in the bilayer.
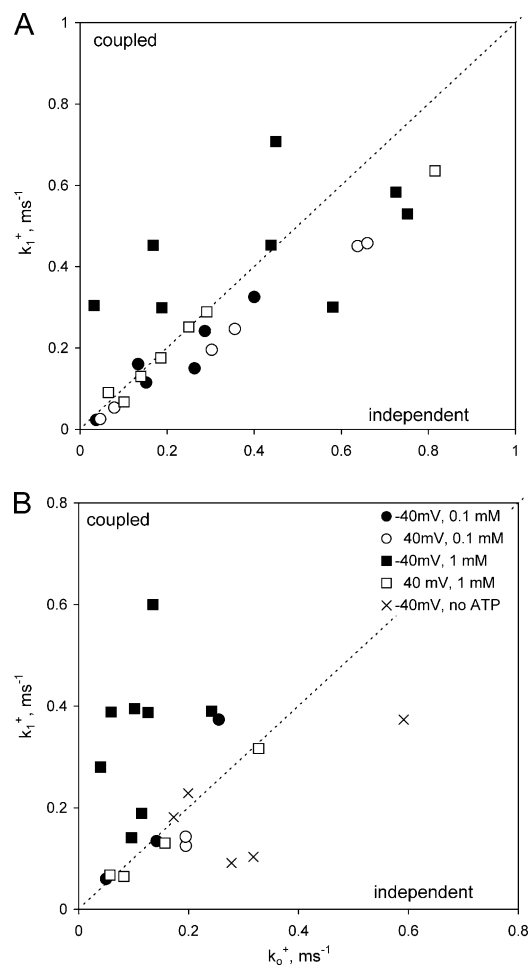
FIGURE 10. The dependence of opening rate on the presence of open channels in the bilayer obtained from individual experiments. For each experiment, the opening rates, k_0^+, in the absence of open channels (opening to level O1, see Fig. 8) are shown on the abscissa while opening rates in the presence of one open channel, k_1^+, (opening to level O2) is on the ordinate. The dashed line indicates independent gating of uniform channels. Datum points above this line indicate coupled channel openings while points below the line can arise independent gating of a heterogeneous group of channels. The legend shows the bilayer potential and luminal [Ca^{2+}] in the absence of cytoplasmic Mg^{2+} (A) or in the presence of 60–230 μM Mg^{2+} (B). Coupling between channels was promoted by luminal [Ca^{2+}] and cytoplasmic Mg^{2+} and was abolished by positive bilayer potentials or removing cytoplasmic ATP and using 100 μM Ca^{2+} as the primary channel activator.

was significantly slower than opening rates associated with transitions between levels O1–O4, k_1^+–k_4^+ (Fig. 9 A, $\square$; P = 0.003, paired t test), while there was no significant difference in the rates associated with transitions between levels O1–O4. Channel closing rates, k, did not depend on the number of open RyRs (Fig. 9 B). Thus, the coupling between RyRs was primarily due to the opening rate, which for each channel depended on whether or not one other channel was open in the bilayer.

One measure for the degree of RyR coupling is the difference between k_0^+ and k_1^+. The data from individual experiments are summarized in Fig. 10 by compar-

ing k_0^+ (abscissa) with k_1^+ (ordinate). Identical RyRs that gate independently would produce data points that lie on the diagonal dashed line, while data from coupled RyRs would lie above the line. Points that lie below the diagonal line can result from channel-to-channel differences in activity, which tends to bias the gating of the low activity RyRs to transitions between the upper current levels. In a large proportion of experiments where [Ca^{2+}]$_l$ = 1 mM and bilayer potentials were negative, RyR groups showed coupled gating. The

scatter in the data reflects genuine differences between gating kinetics in different experiments rather than statistical uncertainties in the HMM analysis (relative error <25%, see MATERIALS AND METHODS). In the absence of Mg^{2+} (Fig. 10 A, ■) three out of eight experiments exhibited significant coupling effects. This increased to seven out of seven in the same experiments performed in the presence of Mg^{2+} (Fig. 10 B, ■). Fig. 10 shows that RyR coupling is decreased by reducing $[Ca^{2+}]_l$ from 1 to 0.1 mM and abolished by positive bilayer potentials that oppose Ca^{2+} flow from the luminal to cytoplasmic baths (Fig. 10, A and B, empty symbols). Coupling is reversibly abolished by the absence of cytoplasmic ATP when the channels are activated by cytoplasmic Ca^{2+} (100 μM; Fig. 10 B, crosses).

The degree of coupling is markedly increased by the presence of cytoplasmic Mg^{2+} (Fig. 9 A and Fig. 10 B), which appears to selectively reduce k_0^+ (Fig. 11). Fig. 11 shows the Mg^{2+} dependencies of k_0^+ (●) and k_1^+ (■) in ATP-activated RyRs in the presence of 1 mM $[Ca^{2+}]_l$. At negative potentials (Fig. 11 A), Mg^{2+} has a much stronger effect on k_0^+ than k_1^+, while at positive potentials (Fig. 11 B), the effects of Mg^{2+} are similar. For comparison, we show the opening rates of single RyRs calculated from their mean closed time (○) which is similar to k_0^+.

DISCUSSION

Effects of Ion Binding at the A-site

The first main finding of this study is that Mg^{2+} is an RyR antagonist. Until now it was not clear whether Mg^{2+} was a competitive nonagonist that inhibited RyRs by preventing the agonist (Ca^{2+}) from binding, but itself could not open the channel. We find that Mg^{2+} closes RyRs that are activated by ATP, DIDS, and ryanodine in the absence of Ca^{2+} on both sides of the channel. Measurements of Ca^{2+} release from SR vesicles have previously demonstrated that Mg^{2+} inhibits RyRs in the absence of externally applied Ca^{2+} (Meissner et al., 1986). However, those experiments could not rule out the possibility that Mg^{2+} inhibited these RyRs by competing with Ca^{2+} that had been released from the vesicles. We ruled out the possibility that ATP, DIDS, and ryanodine had somehow modified the channel to allow its activation by monovalent ions and that Mg^{2+} inhibition occurred by occlusion of monovalent ions from the A-sites. Although Cs^+ did increase the activity of ryanodine-modified RyRs, it did so by a mechanism different to Ca^{2+} activation at the A-sites. However, our data indicates that Cs^+ did affect Mg^{2+} inhibition by competing with Mg^{2+} and Ca^{2+} at the A-sites (Tables II and III). Meissner et al. (1997) had come to a similar conclusion regarding competition between Ca^{2+} and monovalent cations for the A-sites based on [³H]ryanodine assays of
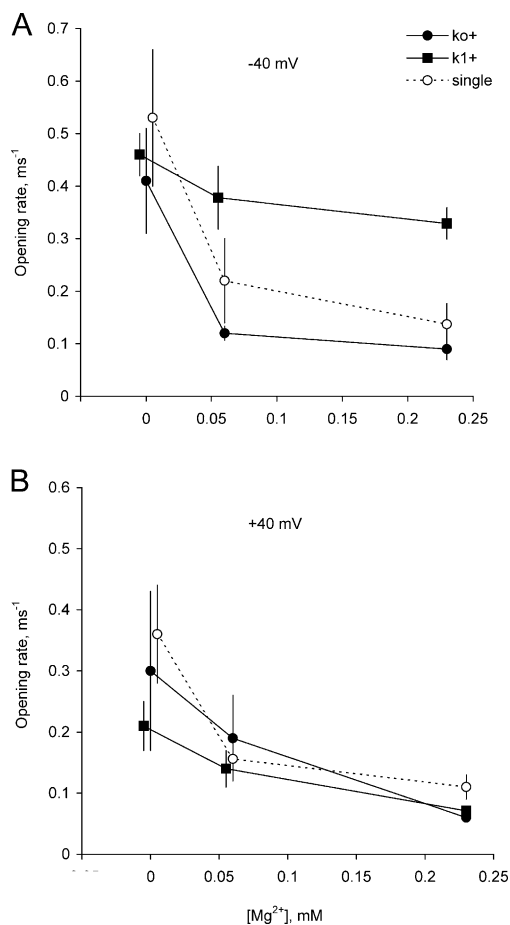

FIGURE 11. The effect of cytoplasmic Mg^{2+} on the channel opening rate of single RyRs (labeled single) and coupled groups of RyRs (labeled k_0^+ and k_1^+) at bilayer potentials of −40 mV (A) and +40 mV (B). The mean rate of the first channel opening k_0^+ (●) is similar to that seen for single channels (○, $N = 5$ at −40 mV and $N = 14$ at +40 mV). The inhibitory effect of cytoplasmic Mg^{2+} is seen here as a decrease in the channel opening rate. However, the opening rates of RyRs in the presence of an open channel, k_1^+, were less sensitive to cytoplasmic Mg^{2+} at negative bilayer potentials than at positive potentials. The data (●, ■) were compiled from experiments paired at +40 and −40 mV at 1 mM luminal Ca^{2+} ($N = 4$).

Ca^{2+} activation of RyRs. They found that Cs^+ and other alkali cations could inhibit RyRs by preventing Ca^{2+} from binding to the A-sites and activating the channel. In this regard, the action of Cs^+ is like that previously envisaged for Mg^{2+}, i.e., that it is a competitive nonagonist. In summary then, Ca^{2+} at the A-sites is an agonist, Cs^+ is a nonagonist and Mg^{2+} is an antagonist.

Luminal Ca^{2+} Alleviates Mg^{2+} Inhibition by an Allosteric Mechanism

Another major finding of this study is that the sensitivity of RyRs to Mg^{2+} inhibition is modulated by the level of luminal Ca^{2+}. Increasing $[Ca^{2+}]_l$ from 0.1 to 1 mM produced a fourfold decrease in the Mg^{2+} affinity of the

A-sites regardless of whether RyRs were in the presence of high or low cytoplasmic [Cs$^+$] and [Ca^{2+}], or whether they were stimulated by ATP or modified by ryanodine (Tables II and III). A number of our findings indicate that this effect is not due to competition at the *A*-sites between cytoplasmic Mg^{2+} and luminal Ca^{2+} that permeates the channel, but rather due to an allosteric mechanism. First, K_i(Mg^{2+}) for single RyRs was independent of the bilayer potential and hence Ca^{2+} feedthrough. Second, increased luminal Ca^{2+} alleviated Mg^{2+} inhibition via changing both the mean open and closed durations of the channel (Table VI). The effect of luminal Ca^{2+} on channel closures suggests that it has access to its site of action when the channel is impermeable to Ca^{2+}. Finally, the effect of luminal Ca^{2+} on Mg^{2+} inhibition is noncompetitive (Tables II and III). For example, competitive kinetics could not account for the combined effect of luminal Ca^{2+} and cytoplasmic Cs$^+$ on K_i(Mg^{2+}) (Table II, entries 5 and 7) or the significant effect of luminal Ca^{2+} on K_i(Mg^{2+}) in the presence 10 μM cytoplasmic Ca^{2+} (Table III, entries 8 and 9).

Comparison with Cardiac RyRs

Evidence for allosteric modulation of cardiac RyRs by luminal Ca^{2+} has been reported previously (Gyorke and Gyorke, 1998). However, the effect of luminal Ca^{2+} on cardiac RyRs (RyR2 isoform) is quite different from that found here for skeletal RyRs (RyR1). With RyR2, luminal Ca^{2+} modulates channel function by sensitizing the Ca^{2+} activation site (*A*-sites) and by reducing inhibition at the low affinity inhibition sites (*I*-sites) (Gyorke and Gyorke, 1998) and a reduction in maximal level of Ca^{2+} activation (P_{max}) (Xu and Meissner, 1998). In contrast, with ATP-activated RyR1, luminal Ca^{2+} has no evident effect on the Ca^{2+} sensitivity of either the *A*- or *I*-sites nor does it alter P_{max} (Table I). In addition, at low (<1 μM) cytoplasmic Ca^{2+}, luminal Ca^{2+} causes substantially more activation of RyR1 than of RyR2.

In single RyR1 channels, we did not detect competition between luminal Ca^{2+} and cytoplasmic Mg^{2+} for the A-sites, either by measuring the voltage dependence (i.e., Ca^{2+} flux dependence) of K_i(Mg^{2+}) or its dependence on Cs$^+$ competition. However, evidence of trans-channel competition has been reported in RyR2 by Xu and Meissner (1998) who noted that cytoplasmic Mg^{2+} induced a voltage dependence in the effect of luminal Ca^{2+}, which can be interpreted as a voltage-dependent Mg^{2+} inhibition.

Ca^{2+} Feedthrough Couples RyRs in Lipid Bilayers

The coupled opening of RyRs that we observe is very similar to the phenomenon reported by Copello et al. (2003) and Porta et al. (2004). They proposed that coupled gating of RyRs occurred in bilayers when Ca^{2+} flow (luminal to cytoplasm) through one channel raised the
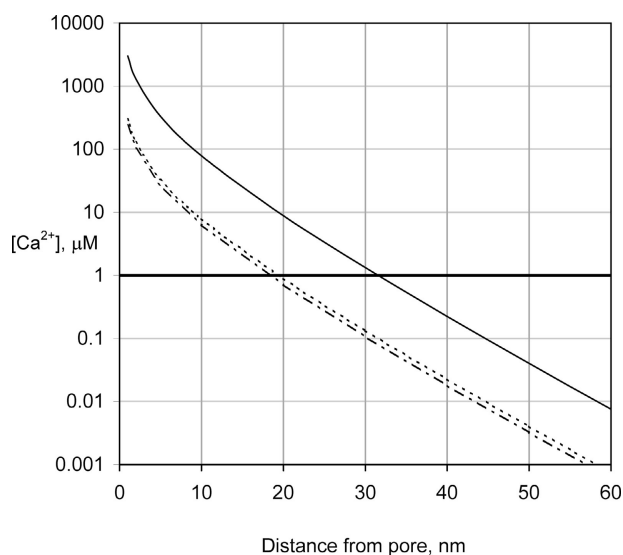

FIGURE 12. Theoretical cytoplasmic [Ca^{2+}] profiles generated by luminal Ca^{2+} fluxes emanating from an RyR pore into a medium containing 4.5 mM BAPTA. Ca^{2+} fluxes were calculated using the rate model developed by Tinker et al. (1992) and the [Ca^{2+}] profiles were calculated using Eq. 13 from Stern (1992) with ion diffusion constants given by Xu and Meissner (1998). Solid curve, [Ca^{2+}]$_l$ = 1 mM, −40 mV; long dashes, [Ca^{2+}]$_l$ = 0.1 mM, −40 mV; short dashes, [Ca^{2+}]$_l$ = 1 mM, +40 mV. [Ca^{2+}] > 1 μM (horizontal line) significantly activates RyRs and increases K_i(Mg^{2+}) (see Figs. 1 and 4).

local [Ca^{2+}]$_c$ sufficiently to activate neighboring RyRs. Several of our findings support their conclusion. First, the coupling of channel openings was promoted under conditions which favored the flow of luminal Ca^{2+} through the channel (i.e., negative potential and high [Ca^{2+}]$_l$). Second, coupling did not occur in the presence of high (100 μM) cytoplasmic Ca^{2+} (Fig. 10). At these concentrations, the *A*-sites are virtually saturated and the feedthrough of luminal Ca^{2+} would have no significant activating effect. Finally, the opening of one channel in a group of RyRs caused an approximately fivefold reduction in Mg^{2+} inhibition of the other RyRs (Fig. 11), suggesting that local [Ca^{2+}]$_c$ is indeed increased and is competing with Mg^{2+} for the *A*-sites. From the effects of cytoplasmic [Ca^{2+}] on P_o and K_i(Mg^{2+}) (Figs. 1 and 4), one can estimate [Ca^{2+}]$_c$ to be ∼10 μM at RyRs neighboring an open RyR. The separation of these RyRs in the bilayer may be estimated from theoretical concentration profiles of [Ca^{2+}] in the vicinity of a pore (Stern, 1992) (Fig. 12). The [Ca^{2+}] near an open channel falls off sharply with distance because Ca^{2+} emerging from the pore is rapidly chelated by BAPTA (4.5 mM) in the bath. Fig. 12 shows that at −40 mV, 1 mM luminal Ca^{2+} generates 1 μM [Ca^{2+}] in the cytoplasmic bath at 30 nm from an open channel. Reversing the polarity of the potential or reducing [Ca^{2+}]$_l$ to 0.1 mM decreases this distance to ∼20 nm.

Freeze fracture electron micrographs show that RyRs within the triad junction are organized into square, two-dimensional arrays with the pores of nearest and second nearest neighbors being separated by 31 and 43 nm, respectively (Protasi et al., 1997). The *A*-sites could be as close as 20 nm from the pore of another channel. Separations in the range 20–30 nm (Fig. 12) would nicely explain the coupling we observe at -40 mV and 1 mM $[Ca^{2+}]_l$ and the loss of coupling at 0.1 mM $[Ca^{2+}]_l$ and $+40$ mV. This suggests that during isolation and reconstitution, the RyR arrays in muscle are not completely disrupted and that rafts containing 3–10 RyRs remain stable in lipid bilayers.

The close packing of RyRs in the membrane makes it seem possible that RyRs could interact physically. In fact, a coupling phenomenon has been identified in CHAPS-purified RyRs (RyR1 and RyR2; Marx et al., 1998, 2001) that is likely to stem from a physical interaction between RyRs. Those authors found that protein fractions enriched in RyR multimers produced synchronously gated channels in $\sim 10\%$ of instances when reconstituted with lipid bilayers. Functional coupling required FKSO6 binding protein, FKBP 12 and FKBP 12.6, but was independent of luminal Ca^{2+}. Our experimental conditions were not designed to optimize observation of this type of coupling since our membrane preparations were not enriched in RyR multimers. We found such behavior in RyRs to be quite rare, observing it in only two instances out of many hundreds of recordings when using cardiac SR vesicles (Honen, B., personal communication). Therefore, RyR coupling by luminal Ca^{2+} is the dominant mechanism operating in our experiments.

Coupled and Single RyRs are Regulated Differently by Luminal Ca^{2+}

Our data shows that Mg^{2+} inhibition is highly dependent on the situation of the RyR. Mg^{2+} inhibition of single RyRs, measured by open probability and opening rate, was not affected by bilayer potential. However, in clusters, RyRs responded quite differently to Mg^{2+}. Although the opening of the first RyR in a group was similar to the single channel situation (Fig. 11, compare ● and ○), Mg^{2+} had a markedly reduced effect on subsequent openings, which we explained in terms of the raised local $[Ca^{2+}]_c$ reducing $K_i(Mg^{2+})$ of the neighboring closed RyRs (see above). However, the fact that Ca^{2+} feedthrough does not also decrease $K_i(Mg^{2+})$ for the first channel opening means that Mg^{2+} inhibition for any particular channel is somehow unaffected by the raised $[Ca^{2+}]$ originating from its own pore. This is surprising since the local $[Ca^{2+}]$ near the open channel will be much higher than near its closed neighbors.

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 an RyR in a raft, even when the channel is closed, the *A*-sites can still have access to luminal Ca^{2+} via adjacent open channels. With single RyRs, it is then possible 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 an RyR in a raft. Therefore there is scope for luminal Ca^{2+} to have a bigger effect on RyRs in rafts than individually. This difference should become quite significant when RyR $P_o <$ 0.2 where changes in P_o are mediated almost entirely through changes in closed dwell time. The differing accessibility of *A*-sites to luminal Ca^{2+} in single and coupled RyRs could also influence the binding of luminal Ca^{2+} and cytoplasmic Mg^{2+} at the *A*-sites. The unbinding rate of Mg^{2+} might not be fast enough to allow luminal Ca^{2+} and cytoplasmic Mg^{2+} to reach equilibrium at the *A*-sites during the open time of a single RyR (Zahradnikova et al., 2003). This could be the reason why luminal Ca^{2+} and cytoplasmic Mg^{2+} do not exhibit competitive binding kinetics with single RyRs. The situation with coupled RyRs would be quite different. When an RyR is exposed to luminal Ca^{2+} via neighboring channels, there is more time for Ca^{2+} and Mg^{2+} to attain equilibrium at the *A*-site before channel opening.

In any case, *A*-site–mediated Mg^{2+} inhibition of an RyR is effectively "immune" to the Ca^{2+} emanating from its own pore. This can be seen, albeit to a lesser degree, for luminal Ca^{2+} activation in the absence of Mg^{2+}. The first channel opening in a cluster frequently has a slower rate than the second opening (compare k_0^+ and k_1^+ in Fig. 10 A). Therefore, RyRs can be more strongly activated by the Ca^{2+} from neighboring channels than by the Ca^{2+} from their own pore.

Mechanism of Luminal Ca^{2+} Activation

In this study, we found evidence supporting both the feedthrough and the true luminal regulation hypotheses for luminal Ca^{2+} regulation of RyRs. We observed coupled gating of RyRs that is due to feedthrough of luminal Ca^{2+} to the cytoplasmic Ca^{2+} sites on the RyR. We also find that luminal Ca^{2+} alters Mg^{2+} inhibition by a noncompetitive mechanism, indicating the presence of luminal Ca^{2+} sites that regulate RyR activity. Both mechanisms are probably important for the stimulation of Ca^{2+} release in muscle by increased store load (see below). Though there is strong evidence that the Ca^{2+} feedthrough activates RyR rafts, the question of how luminal Ca^{2+} activates single RyRs is not totally resolved since it remains possible that RyRs are "immune" to their own Ca^{2+} flux.

The fact that RyR activation by luminal Ca^{2+} requires the presence of specific secondary activators of the channel such as ATP, suramin (Sitsapesan and Williams, 1994), or caffeine (Xu and Meissner, 1998) has been taken as evidence against the Ca^{2+} feedthrough mechanism (Sitsapesan and Williams, 1997). However, our finding that ATP is required for Ca^{2+} coupling of RyRs leads to an alternative interpretation of this phenomenon. In the absence of secondary activators, any opening of RyRs would be associated with Ca^{2+} binding at the A-sites. Thus when luminal Ca^{2+} gains access to the A-sites through the open channel, Ca^{2+} will not augment channel opening because the A-sites are already occupied. However, when ATP is present, the RyRs can open even when the A-sites are unoccupied, leaving scope for luminal Ca^{2+} to enhance RyR activation.

The nature of the luminal Ca^{2+}–sensing sites is still not determined. It is known that the luminal proteins calsequestrin, triadin, and junction are associated with RyRs and modulate their activity (Beard et al., 2004). These proteins can confer on RyRs a means of sensing luminal $[Ca^{2+}]$ by either Ca^{2+}-dependent dissociation from the RyR complex or by regulating RyRs in a Ca^{2+}-dependent manner, as is the case for calmodulin. It is unlikely that calsequestrin dissociation underlies the effects of luminal Ca^{2+} in Mg^{2+} inhibition because luminal Ca^{2+} was kept in the range that stabilizes calsequestrin binding to the RyR complex.

Ryanodine Modification of RyRs

The effects of ryanodine modification of RyRs were broadly similar to those seen in previous studies. Ryanodine markedly stabilized channel openings to a lower than normal conductance, channel activity was relatively insensitive to cytoplasmic Ca^{2+} (Fig. 1). There are two interpretations for the loss of Ca^{2+} sensitivity. One is that the Ca^{2+} activation dependence is shifted to such low $[Ca^{2+}]$ that RyRs are fully activated over the experimentally attainable $[Ca^{2+}]$ range. The alternative is that ryanodine-modified RyRs do not require cytoplasmic Ca^{2+} to open. The decisive experiment is to find Ca^{2+} levels sufficiently low to deactivate ryanodine-modified RyRs. Unfortunately, on this key experiment, reports are divided. Experiments on purified, recombinant cardiac RyRs show that ryanodine-modified RyRs deactivate ($P_o \sim 0.2$) in the presence of 1 mM EGTA and no added Ca^{2+} (Du et al., 2001; Masumiya et al., 2001) while ryanodine-modified sheep cardiac RyRs remained fully active in the presence of Ca^{2+} buffers and no added Ca^{2+} (Rousseau et al., 1987; Laver et al., 1995) (the actual $[Ca^{2+}]_{free}$ in these studies is uncertain because impurity Ca^{2+} levels were not specified).

In the present study, we did not see reproducible deactivation of ryanodine-modified RyRs at low $[Ca^{2+}]$. In only a few instances did these RyRs show a clear decrease in activity at 0.1–1 nM Ca^{2+}. The reason why reduced activity at low $[Ca^{2+}]$ was frequently seen in studies on recombinant RyR2 is not clear. It could be due to differences between native RyRs and those purified from nonmuscle cells or it could be due to the different bathing solutions used for single channel recording (KCl vs. $CsCH_3O_3S$). However, in spite of these differences, sensitivity of RyRs to Mg^{2+} inhibition seen here is consistent with the study of Masumiya et al. (2001), which obtained a $K_i(Mg^{2+}) \sim 2$ mM in the presence of 100 nM cytoplasmic Ca^{2+}, zero luminal $[Ca^{2+}]$, and symmetric 250 mM KCl (our model predicts $K_i(Mg^{2+}) =$ 2.0 mM). Du et al. (2001) also found that Mg^{2+} inhibition was independent of membrane potential over the range $\pm$ 30 mV.

The ion selectivity of the A-site is strongly and reproducibly altered by ryanodine. In the presence of 250 mM Cs^+, the apparent affinity of the A-sites for Ca^{2+} is 100 nM (see $K_{app}(Ca^{2+})_c$ in Table I), which means that RyRs should always deactivate at cytoplasmic $[Ca^{2+}] <$ 100 nM if Ca^{2+} at the A-sites is required for channel activation. This was not the case in most RyRs studied here. The decrease in activity seen by others at nM Ca^{2+}, and occasionally seen here, probably does not involve the same mechanism as Ca^{2+} activation in native RyRs.

Control of Calcium Release by Luminal Calcium and Cytoplasmic Mg^{2+}

The results of this study give new insight into how SR luminal Ca^{2+} and cytoplasmic Mg^{2+} influence Ca^{2+} release from the SR under physiological conditions in muscle fibers. Mg^{2+} exerts inhibitory effects on RyR1 by acting at the I-site as well as the A-site (see INTRODUCTION). We have extended the dual-inhibition model to include the effects of luminal Ca^{2+} on the ion binding properties of the A-sites. The inhibitory effect of Mg^{2+} at the I-sites, which is evidently not affected by luminal $[Ca^{2+}]$ (note similar Ca^{2+} affinity of the I-sites at 0.01 and 1 mM luminal Ca^{2+} in Table I), tempers the effects of luminal Ca^{2+} on the overall Mg^{2+} inhibition. This is because the overall $K_i(Mg^{2+})$ for both Mg^{2+} inhibition mechanisms is always less than the $K_i(Mg^{2+})$ for either A- and I-sites alone (Laver et al., 1997). For the I-sites, $K_i(Mg^{2+})$ is $\sim$250 μM at physiological ionic strength ($\sim$100 mM) in the presence of ATP (Table I). At the normal resting cytoplasmic $[Ca^{2+}]$ ($\sim$100 nM), the $K_i(Mg^{2+})$ of the A-sites and overall Mg^{2+} inhibition are similar, ranging from $\sim$20 μM at very low luminal Ca^{2+} (0.1 mM) to $\sim$100 μM at quite high luminal Ca^{2+} (3 mM) (i.e., in this situation, the overall inhibition is largely set by the A-site properties). Thus, even though the ATP present in the cytoplasm has a strong stimulatory action on RyR1 (Fig. 1), the presence of physiolog-

ical free Mg^{2+} (1 mM) will keep the channel P_o very low even if the SR is very loaded with Ca^{2+} (Mg^{2+} is present at four times the $K_i(Mg^{2+})$ of I-site and 10 to 50 times the $K_i(Mg^{2+})$ of the A-site). Moreover, as the SR becomes progressively more depleted of Ca^{2+}, increased Mg^{2+} inhibition would cause a large reduction (>25-fold) in channel activity and Ca^{2+} release. This is much larger than the two to fivefold decrease in activity that one would expect in the absence of Mg^{2+} (Tables III and VI). Therefore, modulation of Mg^{2+} inhibition by luminal Ca^{2+} is a significant regulator of Ca^{2+} release from the SR.

It is also apparent that heavy loading of the SR with Ca^{2+} will make RyRs more prone to activation by cytoplasmic Ca^{2+} and/or caffeine. If the cytoplasmic $[Ca^{2+}]$ rises above the resting level, the $K_i(Mg^{2+})$ at the A-site will increase substantially. For the case of high SR luminal $[Ca^{2+}]$ (3 mM), $K_i(Mg^{2+})$ for the A-sites will increase from ∼100 μM at 100 nM cytoplasmic Ca^{2+} to 180 μM at 1 μM Ca^{2+}, 340 μM at 3 μM Ca^{2+}, and 900 μM at 10 μM Ca^{2+}. As a result, the overall $K_i(Mg^{2+})$ will increase from ∼80 μM at 100 nM cytoplasmic Ca^{2+} to 110 μM at 1 μM $Ca^{2+}{}_c$, 150 μM at 3 μM $Ca^{2+}{}_c$, and 200 μM at 10 μM $Ca^{2+}{}_c$. Consequently, if the cytoplasmic $[Ca^{2+}]$ is raised above the resting level, or if caffeine is applied (caffeine increases the sensitivity of the A-site for Ca^{2+} relative to Mg^{2+}; Balog et al. 2001), the total inhibitory effect of Mg^{2+} will decrease and the channel will activate to some extent. Nevertheless, peak activation will still be ultimately limited by the prevailing Mg^{2+} inhibition at the I-site.

Thus, the above interplay of I-site and A-site properties would seem adequate to account for the findings that (a) appreciable calcium-induced calcium release (CICR) cannot be induced at physiological $[Mg^{2+}]$ if the SR is loaded at <∼25% of its maximum capacity ($[Ca^{2+}]_l$ ∼ 1 mM, Endo, 1985; Saiki and Ikemoto, 1999), (b) caffeine-induced release and CICR and are greatly potentiated by heavily loading the SR with Ca^{2+} and attenuated by raising the free $[Mg^{2+}]$ above 1 mM (Endo, 1985; Nelson and Nelson, 1990; Lamb et al., 2001), (c) the peak rate of caffeine-induced Ca^{2+} release in muscle fibers is very much lower than that produced by action potential stimulation (Lamb et al., 2001; Posterino and Lamb, 2003), and (d) the SR can be depleted of some but not all of its Ca^{2+} if the cytoplasmic $[Mg^{2+}]$ is lowered to 50 μM, and fully depleted by lowering $[Mg^{2+}]$ or by applying caffeine in the presence of low $[Mg^{2+}]$ (Lamb and Stephenson, 1994; Fryer and Stephenson, 1996).

Voltage Sensor Control of Calcium Release in Skeletal Muscle

The above considerations show how the levels of luminal Ca^{2+} and cytoplasmic Mg^{2+} can affect the sensitivity of skeletal muscle fibers to CICR and caffeine-induced

Ca^{2+} release. However, these are not the normal mechanisms triggering Ca^{2+} release in vertebrate skeletal muscle. Instead, Ca^{2+} release is controlled largely by the DHPRs in the T-tubules (see INTRODUCTION). At rest, CICR is greatly down-regulated by strong Mg^{2+} inhibition exerted at physiological $[Mg^{2+}]$ (Meissner et al., 1986; Laver et al., 1997). DHPRs must be able to overcome this inhibition because RyRs are near maximally activated (i.e., P_o ∼ 1) during action potential stimulation (Posterino and Lamb, 2003, and references therein). The DHPRs do not appear to bypass Mg^{2+} inhibition of RyRs, because raising the $[Mg^{2+}]$ to >3 mM considerably inhibits the ability of the DHPRs to trigger Ca^{2+} release (Lamb and Stephenson, 1991, 1994; Westerblad and Allen, 1992; Anderson and Meissner, 1995; Blazev and Lamb, 1999). Furthermore, the DHPRs are not able to activate the RyRs unless the RyRs are also stimulated by ATP in the cytosol (see above references). The preceding two observations show that DHPR activation is insufficient to activate RyRs independently of cytoplasmic factors acting on the RyR. This raises the possibility that DHPRs activate RyRs by raising the overall $K_i(Mg^{2+})$, thereby reducing their inhibition by Mg^{2+} and allowing cytosolic ATP to activate the channel, with the released Ca^{2+} then able to reinforce this activation (Lamb and Stephenson, 1991, 1994).

Not all published data can be easily explained solely by the removal of RyR Mg^{2+} inhibition. O'Brien et al. (2002) investigated the ability of the DHPRs to activate mutated RyR1s (E4032A) in which Ca^{2+} and ATP activation was virtually abolished. They found that DHPR activation of these RyRs could still produce ∼20% of the Ca^{2+} release of wild-type RyRs, which suggests that Ca^{2+} and ATP activation was not absolutely necessary for DHPR controlled Ca^{2+} release. However, Ca^{2+} and ATP activation of the E4032A mutant RyR was determined in bilayer experiments where luminal Ca^{2+} was absent. Therefore, it is possible that in the presence of physiological luminal $[Ca^{2+}]$ (1 mM), the mutant RyRs show substantially more activation by ATP as is known to be the case for the wild-type RyRs.

Exactly how DHPRs modulate the dual mechanism of Mg^{2+} inhibition is not clear. At the very least, the DHPRs must overcome the Mg^{2+} inhibition exerted at the I-site (Laver et al., 1997). Importantly, the results here also show that reducing the I-site inhibition would not be enough by itself to open the RyRs because, unless the SR was very loaded with Ca^{2+}, RyR1 would still be inhibited by Mg^{2+} on the A-sites. However, if the DHPRs could reduce the inhibitory effect of Mg^{2+} at the A-sites as well as at the I-sites, the RyR would be potently activated irrespective of the level of SR Ca^{2+} loading and the resting cytoplasmic $[Ca^{2+}]$. This immediately raises the intriguing prospect that the DHPRs are able to

commandeer the mechanism by which SR luminal Ca^{2+} reduces Mg^{2+} inhibition at the *A*-sites. In fact this seems quite plausible, as this mechanism must involve an allosteric interaction mediated from the luminal side to the cytoplasmic side of the RyR, and it is apparent that DHPR activation must also involve long-range allosteric effects within the RyR. Moreover, this "commandeering" would readily explain why DHPRs can potently activate RyRs irrespective of the level of Ca^{2+} loading in the SR and thereby, unlike applied caffeine or cytoplasmic Ca^{2+}, completely empty the SR of Ca^{2+} in the presence of physiological $[Mg^{2+}]$ (Kurebayashi and Ogawa, 2001; Posterino and Lamb, 2003).

In conclusion, cytoplasmic Mg^{2+} is an important regulator of RyRs in muscle, which is very sensitive to both the cytoplasmic and luminal milieu. The 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. Finally, measurements of single RyRs in artificial bilayers have had a major impact on our understanding of the mechanisms of Ca^{2+} release. In addition, this study demonstrates that RyR arrays like those found in muscle can be regulated by mechanisms that are different to those identified in single channels.

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