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Magnesium Inhibition of Ryanodine-Receptor Calcium Channels: Evidence for Two Independent Mechanisms

D.R. Laver, T.M. Baynes, A.F. Dulhunty

Muscle Research Group, Division of Neuroscience, John Curtin School of Medical Research, Australian National University, GPO Box 334, Canberra, ACT 2601, Australia

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Abstract. The gating of ryanodine receptor calcium release channels (RyRs) depends on myoplasmic Ca^{2+} and Mg^{2+} concentrations. RyRs from skeletal and cardiac muscle are activated by μM Ca^{2+} and inhibited by mM Ca^{2+} and Mg^{2+}. $^{45}Ca^{2+}$ release from skeletal SR vesicles suggests two mechanisms for Mg^{2+}-inhibition (Meissner, Darling & Eveleth, 1986, *Biochemistry* **25:**236–244). The present study investigates the nature of these mechanisms using measurements of single-channel activity from cardiac- and skeletal RyRs incorporated into planar lipid bilayers.

Our measurements of Mg^{2+}- and Ca^{2+}-dependent gating kinetics confirm that there are two mechanisms for Mg^{2+} inhibition (Type I and II inhibition) in skeletal and cardiac RyRs. The mechanisms operate concurrently, are independent and are associated with different parts of the channel protein. Mg^{2+} reduces P_o by competing with Ca^{2+} for the activation site (Type-I) or binding to more than one, and probably two low affinity inhibition sites which do not discriminate between Ca^{2+} and Mg^{2+} (Type-II). The relative contributions of the two inhibition mechanisms to the total Mg^{2+} effect depend on cytoplasmic [Ca^{2+}] in such a way that Mg^{2+} inhibition has the properties of Types-I and II inhibition at low and high [Ca^{2+}] respectively. Both mechanisms are equally important when [Ca^{2+}] = 10 μM in cardiac RyRs or 1 μM in skeletal RyRs. We show that Type-I inhibition is not the sole mechanism responsible for Mg^{2+} inhibition, as is often assumed, and we discuss the physiological implications of this finding.

Key words: Magnesium inhibition — Calcium inhibition — Sarcoplasmic reticulum — Cardiac muscle — Skeletal muscle — Ryanodine receptor — Artificial BLM

Correspondence to: D.R. Laver

Introduction

The sarcoplasmic reticulum (SR) in striated muscle stores Ca^{2+} which is released to generate muscle contraction. Upon depolarization of the T-tubule membrane, dihydropyridine receptors (DHPR-L-type calcium channels) are activated and trigger Ca^{2+} release from the SR via the ryanodine receptor (RyR-calcium channels in the terminal cisternae membrane). In cardiac muscle the influx of Ca^{2+} through the DHPR is though to open the RyR, whereas in skeletal muscle, Ca^{2+} influx is not a prerequisite for RyR activation. In both skeletal and cardiac isoforms of the RyR, channel openings are activated by μM [Ca^{2+}] and inhibited by mM [Ca^{2+}] (Kawano & Coronado, 1991; Sitsapesan, Boraso & Williams, 1991; Laver et al., 1995) and Mg^{2+} (Meissner, 1994; Coronado et al., 1994). Mg^{2+} inhibition is believed to play an important role in regulating the opening of skeletal RyRs in muscle fibers. For example, in toad skeletal muscle it has been shown that Mg^{2+} prevents opening of RyR channels in the resting muscle (Lamb & Stephenson, 1991). The same authors also proposed that during excitation-contraction (EC) coupling DHPRs in skeletal muscle open RyRs by removing their Mg^{2+} inhibition (Lamb & Stephenson, 1992). In agreement, Ritucci and Corbett (1995) have demonstrated modulation of Mg^{2+}-inhibition in skeletal RyRs by DHPRs, which are functionally coupled to RyRs in triad vesicles.

Measurements of $^{45}Ca^{2+}$ release by SR vesicles from skeletal muscle (Meissner et al., 1986) suggests that there are at least two mechanisms for Mg^{2+} inhibition. In μM Ca^{2+}, Mg^{2+} prevented Ca^{2+} activation of RyRs. In contrast, Mg^{2+} reduced $^{45}Ca^{2+}$ efflux by an unknown

mechanism in mM Ca^{2+}. Meissner et al. (1986), Meissner (1994) and Lamb (1993) argued that the latter process was the same as Ca^{2+} inhibition. However, measurements of Ca^{2+} release from vesicles could not be used to positively establish a link between Ca^{2+} and Mg^{2+} inhibition (Meissner et al., 1986; Coronado et al., 1994). Millimolar cytoplasmic concentrations of Ca^{2+} can also reduce Ca^{2+} efflux through RyRs by reducing the electrochemical driving force on Ca^{2+} (Coronado et al., 1994) and both Mg^{2+} and Ca^{2+} can reduce channel conductance (Smith et al., 1988; Tinker et al., 1992). It is not surprising that Meissner et al. (1986) left characterization of the Mg^{2+} inhibition mechanisms to future studies of single channels.

Single-channel studies have not identified two types of Mg^{2+} inhibition. Rather, they present a confusing picture of the Mg^{2+} effect and the possibility of more than one Mg^{2+}-inhibition mechanism is often overlooked (e.g., Coronado et al., 1994). Two studies linked Mg^{2+} inhibition with Ca^{2+} activation in skeletal (e.g., Smith et al., 1986) and cardiac RyRs (Ashley & Williams, 1990) because Mg^{2+} appeared to compete with Ca^{2+} for the high affinity Ca^{2+}-activation site. However, these observations are at odds with more recent observations from the same laboratories which imply there is no link between Ca^{2+} activation and Mg^{2+} inhibition. Boraso and Williams (1994) showed that 5 mM H_2O_2, which removed the Ca^{2+} requirement for channel activation, had no effect on Mg^{2+} inhibition. Smith et al. (1988) showed that CHAPS solubilization of skeletal RyRs removed Mg^{2+} inhibition yet had no effect on Ca^{2+} activation. Two other studies suggest that Mg^{2+} inhibition is not linked with Ca^{2+} activation, but it is associated with Ca^{2+} inhibition. Mack et al. (1994) demonstrated that 5 μM bastadin added to skeletal RyRs alleviated both Ca^{2+} and Mg^{2+} inhibition but had no effect on Ca^{2+} activation. Mickelson et al. (1990) found that Ca^{2+} and Mg^{2+} inhibition were attenuated in malignant hyperthermia susceptible (MHS) porcine muscle, but Ca^{2+} activation was normal.

Because previous single channel measurements have not explored the possibility that there are two Mg^{2+}-inhibition mechanisms we have carried out a detailed study of the Mg^{2+} and Ca^{2+} dependence of RyRs in lipid bilayers. We investigated the relationship between Mg^{2+} inhibition and Ca^{2+} regulation of RyRs by comparing the effects of Mg^{2+} on three groups of RyRs which had distinctly different Ca^{2+} regulation properties, namely, skeletal RyRs, normal cardiac RyRs and cardiac RyRs in which the Ca^{2+}-inhibition mechanism was either absent or had markedly reduced Ca^{2+} sensitivity. To reduce the number of regulation mechanisms operating in RyRs, and simplify our investigation, we studied Mg^{2+} inhibition under circumstances where Ca^{2+} was the sole activating ligand. Our measurements confirm that there are two mechanisms which operate concurrently in RyRs and which determine Mg^{2+} inhibition over different ranges of cytoplasmic [Ca^{2+}]. We also show that Mg^{2+} and Ca^{2+} at mM concentrations share a common inhibitory mechanism. We use simple kinetic schemes to describe Mg^{2+} inhibition and elucidate the role of each inhibition mechanism in skeletal and cardiac RyRs in vivo.

In testing the Mg^{2+}-inhibition model we have focused on cardiac RyRs since they provide a simpler system for studying Ca^{2+} and Mg^{2+} regulation of RyRs in general. In cardiac RyRs the Ca^{2+} activation and inhibition occur over widely separated, nonintersecting [Ca^{2+}] ranges (Chu et al., 1993; Laver et al., 1995) so that Ca^{2+} activation and inhibition can be studied individually. This is not true for skeletal RyRs where the Ca^{2+} dependence of activation and inhibition overlap so that channel gating is significantly affected by both processes at mid-range [Ca^{2+}] (~10–100 μM). The same principle was found to apply to Mg^{2+} regulation in that cardiac RyRs are less sensitive to inhibition by Mg^{2+} at high [Ca^{2+}] than are skeletal RyRs (Meissner et al., 1986, 1987). Only in cardiac RyRs, were the Ca^{2+} dependence of the Mg^{2+}-inhibition mechanisms sufficiently well separated to allow us to directly measure the kinetics of each individual mechanism. The kinetic model for cardiac RyRs was then adapted to model the properties of Mg^{2+} inhibition in skeletal RyRs.

Materials and Methods

Materials

Full details of the materials and methods are given elsewhere (Laver et al., 1995). Skeletal SR vesicles were prepared from the back and leg muscles of New Zealand rabbits and cardiac vesicles were prepared from sheep hearts. Bilayers separating two aqueous baths (*cis* and *trans*) were formed from a mixture of PE, PS and PC (5:3:2, by weight) in n-decane using the film drainage technique of Mueller et al. (1962). Incorporation of ion channels with the bilayers was achieved by fusion with SR vesicles as described by Miller and Racker (1976). SR vesicles added to the *cis* bath and the cytoplasmic side of the SR membrane faced the *cis* solution when fused with the bilayer.

The luminal (*trans*) bath contained (in mM) 50 CsCl and either 0.1 or 1 $CaCl_2$. During initial experiments it was believed that the higher *trans* [$CaCl_2$] may have promoted vesicle fusion. More recent measurements were carried out in the presence of the lower [$CaCl_2$] since it had no discernible effect on vesicle fusion and the RyR conductance was significantly higher. The cytoplasmic (*cis*) bath contained either 250 or 500 CsCl and various free [Ca^{2+}] and [Mg^{2+}]. Prior to vesicle fusion the *cis* [Ca^{2+}] was either 0.1 or 1 mM. During initial experiments raising *cis* [Ca^{2+}] or adding [Mg^{2+}] was done by adding stock solutions of the chloride salts to the bath while stirring vigorously. Otherwise, exchange of solutions was achieved by perfusing the whole bath with 4–15 volumes (6–20 ml) of [Ca^{2+}]-buffered solution depending on the degree of solution exchange required. The bathing solutions were buffered to pH 7.4 using 10 mM TES and CsOH and to a range of pCa using $CaCl_2$ and either 2 mM BAPTA (used for free [Ca^{2+}] less than 1 μM)

or 2 mM dibromo BAPTA (used for free [Ca^{2+}] between 3 and 10 μM). Free [Ca^{2+}] was measured using an ion meter (Radiometer ION83).

In later experiments solution exchange at the *cis* face of the bilayer was achieved by flowing solutions from a vinyl tube directly onto the bilayer surface for the duration of the recording. This local perfusion technique is the same as that used by Laver and Curtis (1996) with a modification which allowed for a choice of up to 16 perfusion solutions. Solution flow rates of only 1–2 μl/sec which could be sustained for extended periods without rupturing the bilayer, produced complete solution exchanges within 5 sec. The gating of RyRs in response to solution changes appeared to reach steady state within 5 to 10 sec from the onset of solution exchange. Using this technique, RyRs were perfused with many solutions for periods lasting 30 sec to 2 min during each experiment. We found that the main advantages of this technique were that solution exchange was rapid in comparison to the whole-chamber perfusion system and that only small quantities of solution were needed. Hence, the local perfusion method is well suited for solutions containing relatively expensive buffers such as dibromo BAPTA.

Recording and Analysis of Single-Channel Data

Fusion of vesicles containing RyRs with the bilayer occurred every 5 to 10 minutes when the *cis* bath contained 250 mM Cs and 1 mM $CaCl_2$. Due to the relatively slow fusion rate it was not important to carry out special precautions to prevent the further fusion of vesicles during an experiment. Perfusion of the *cis* bath by low-[Ca^{2+}] buffers in the normal course of most experiments eliminated subsequent vesicle fusion. Electrical potentials are given with respect to the *trans* chamber as ground and positive current is directed from the *cis* to *trans* bath. Unless otherwise stated channel recordings were made at a bilayer potential of +40 mV which is near the reversal potential for Cl^-. During the experiments the bilayer current and potential were recorded at a bandwidth of 5 kHz on videotape using pulse code modulation. For measurements of unitary current and open probability, P_o, the current signal was replayed through a 1 kHz, low pass, 8-pole Bessel filter and sampled at 2 kHz. Unitary current was determined from inspection of the current records.

Open probability was calculated from the time-average current divided by the unitary current and the number of channels (up to 3 RyRs were sometimes present during P_o measurements). The number of RyRs in each bilayer was determined from the number of current levels seen under conditions where the RyRs had relatively high open probability. On occasion RyRs would go into long closed periods lasting several tens of seconds to minutes. Using a threshold duration of 1 to 3 sec these closures were excluded from our determinations of P_o. This threshold was based on the closed time frequency distributions which showed that for cytoplasmic [Ca^{2+}] > 1 μM nearly all the closed durations were associated with exponential decay constants shorter than 0.1 sec. The ramifications of this type of analysis are given in the Discussion. P_o was calculated from sections of record lasting 10 to 60 sec (excluding long closures) and containing between 10^3 and 10^5 events.

Unitary current and time-averaged currents were measured using an in-house program (Channel2, developed by Professor P. W. Gage and Mr. M. Smith). Unless otherwise stated the scatter in the data is given by the standard deviation (SD).

The gating kinetics were quantified by the probability distributions of open and closed durations obtained from single-channel recordings with steady baseline and apparently stationary gating kinetics (i.e., no gating mode changes were apparent). Frequency distributions were compiled from channel records varying in duration from 10 to 60 sec and containing between 10^3 and 10^5 events. Event durations were extracted from idealized, two-level signals (namely, open and closed), modelled on the current records by the Hidden Markov Model algorithm (HMM, the details of this program are given by Chung et al., 1990). HMM assumes that the signal is sampled at *discrete time intervals* and is composed of a two-state, Markov signal buried in *uncorrelated*, Gaussian noise. To satisfy the HMM assumptions the current signal was slightly undersampled (replayed at 5 kHz and digitized at 10 kHz) to eliminate correlations between adjacent data points. Unlike other channel analysis algorithms the accuracy of HMM is not diminished by under sampling since it ''knows'' that the data is sampled at discrete time intervals. A detailed justification of this sampling rate is given by Laver et al. (1995). Probability distributions of open and closed durations were calculated by normalizing their frequency distributions. The data are presented using variable bin widths with equal separation on a log scale as described by Sigworth and Sine (1987). The time constants and the relative areas of these components were inferred from least-squares fits of multi-exponential functions to the ''Sigworth & Sine'' plots. The probability distributions were fitted by the sum of up to three exponentials since fitting larger numbers of exponentials to the data provided only a marginal improvement in the quality of the fit parameter (the root-mean-square of the residuals).

The Double Gate Model

Ca^{2+} and Mg^{2+} Regulation of RyRs: Development of a Model

Here we present a model for Mg^{2+} inhibition of RyRs which provides the framework for our experimental approach as well as a means for understanding how two Mg^{2+}-inhibition mechanisms contribute to the overall Mg^{2+} inhibition of RyRs. In this model RyR gating is controlled by two Mg^{2+}-/Ca^{2+}-dependent gates (see Fig. 1); one is responsible for Ca^{2+} activation and one form of Mg^{2+} inhibition and the other is responsible for Ca^{2+} inhibition and a second form of Mg^{2+} inhibition. The gates are assumed to operate independently and such that the channel conducts only when both gates are open. Evidence for independent gating has been obtained elsewhere from the response of RyRs to rapid plunges in [Ca^{2+}] from inhibiting levels to sub activating levels (Laver & Curtis, 1996). The model predictions are relatively insensitive to the assumption of independence (*not shown*).

The regulation of the two putative gates by Mg^{2+} and Ca^{2+} is described here in terms of simplified kinetics schemes which account for the open probability of RyRs and their mean open and closed durations. The model does *not* attempt to account for the detailed shape of the probability distributions for open and closed events shown in this paper. Comprehensive kinetic models describing the Ca^{2+} and Mg^{2+} dependence of RyR channel gating have not been devised, here or elsewhere, because the gating kinetics are complex. Even the details of the gating kinetics associated with the Ca^{2+}-activation mechanism are still to be resolved (*see below*). For the sake of brevity the two, putative Mg^{2+}-inhibition mechanisms are labeled Type-I and Type-II. We show later that according to this model Mg^{2+} inhibition at low [Ca^{2+}] is largely determined by Type-I inhibition and at high [Ca^{2+}] by Type-II inhibition.

Type-I: Mg^{2+} inhibition of the Ca^{2+} activation site. Measurements of Ca^{2+}-induced Ca^{2+} release from SR vesicles (Meissner et al., 1986) and some earlier single channel studies (Chu et al., 1993 and references cited therein) have obtained Hill coefficients for Ca^{2+} activation of ~1 suggesting that the binding of a single Ca^{2+} can open RyRs. However, a recent single channel study (Sitsapesan & Williams, 1994*a*) obtained a much higher Hill coefficient indicating cooperativity

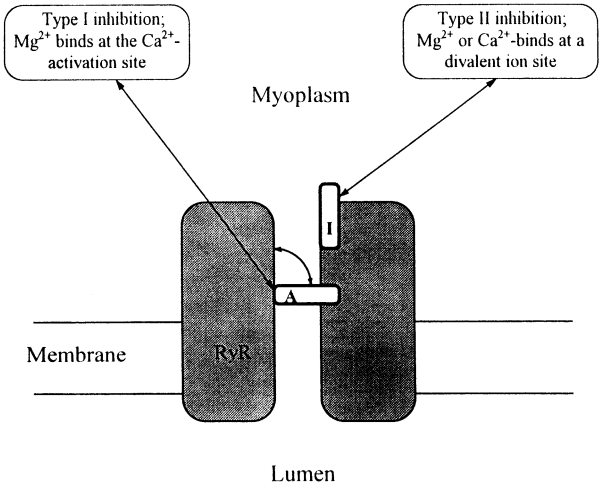

Fig. 1. A schematic diagram of a RyR which illustrates the main aspects of the model for Mg^{2+} inhibition of cardiac and skeletal RyRs. Ion gates within the channel for Ca^{2+} activation and Ca^{2+} inhibition of the RyR are labeled (A) and (I) respectively. These gates are assumed to operate independently and such that both gates must be open for the channel to conduct. In Type-I inhibition Mg^{2+} prevents opening of the activation gate by competing with Ca^{2+} for the activation site. However, unlike Ca^{2+} binding (~1 μM affinity) the binding of Mg^{2+} at this site (~1 mM affinity) does not open the channel (*see* Scheme 2). In Type-II inhibition the binding of Mg^{2+} of Ca^{2+} (~mM affinity) at a common set of sites (probably 2 sites) closes the inhibition gate.

between Ca^{2+} binding at several sites on the RyR[1]. Previous models for Mg^{2+} inhibition at the Ca^{2+}-activation site assume that Mg^{2+} reduces P_o primarily by competing with Ca^{2+} for the activation site on the closed channel but unlike Ca^{2+}, the binding of Mg^{2+} at this site does not open the channel (Scheme 1; Meissner et al., 1986; Ashley & Williams 1990).

$$\begin{array}{ccc} C & \leftrightarrow & O.\,(Ca^{2+}) \\ \updownarrow & & \\ C.\,(Mg^{2+}) & & \end{array} \qquad \text{SCHEME 1}$$

According to this model the $[Mg^{2+}]$ required to induce a 50% reduction in P_A, K_A, is given by Eq. 1.

$$K_A = K_{MA} \cdot \frac{K_{CA} + [Ca^{2+}]}{K_{CA}} \tag{1}$$

where K_{CA} and K_{MA} are the binding affinities for Ca^{2+} and Mg^{2+} at the channel activation site. However, Scheme 1 does not explain the Mg^{2+} sensitivity of RyRs at $[Ca^{2+}]$ levels less than 1 μM (at low $[Ca^{2+}]$, Eq. 1 reduces to $K_A \cong K_{Mg}$). K_{CA} is constrained to be ~1 μM to account for the Ca^{2+}-activation properties of RyRs. In that case K_A is relatively insensitive to $[Ca^{2+}]$ lower than ~1 μM (*see* Fig. 2, thin dashed line) whereas those of Smith et al. (1986) and data in this paper, show that K_A should be nearly proportional to $[Ca^{2+}]$ down to 300 nM or less than 10 nM respectively.

To account for the K_{Mg} values obtained here at 0.3 μM Ca^{2+} we adopted Scheme 2 to describe both Ca^{2+}-activation and Type-I Mg^{2+}-inhibition of RyRs. According to Scheme 2.

$$\begin{array}{ccccc} C & \leftrightarrow & C.\,(Ca^{2+}) & \leftrightarrow & O.\,(2Ca^{2+}) \\ \updownarrow & & \updownarrow & & \\ C.\,(Mg^{2+}) & & C.\,(Ca^{2+} + Mg^{2+}) & & \end{array} \qquad \text{SCHEME 2}$$

K_A then is given by Eq. 2.

$$K_A = \frac{1 + (K_{CA1}K_{CA2}/[Ca^{2+}]^2) + (K_{CA2}/[Ca^{2+}])}{(K_{CA2}/K_{MA2}[Ca^{2+}]) + (K_{CA1}K_{CA2}/K_{MA1}[Ca^{2+}]^2)} \tag{2}$$

where K_{CA1} and K_{CA2} are the binding constants for Ca^{2+} at the empty- and Ca^{2+}-occupied closed states respectively and, K_{MA1} and K_{MA2} are those for Mg^{2+}. Scheme 2, like Scheme 1, describes how Mg^{2+} prevents channel opening by binding to the Ca^{2+}-activation sites. In addition, Scheme 2 describes how RyRs could be activated by the binding of two Ca^{2+} as expected from the value of the Hill coefficient for channel activation obtained by Sitsapesan and Williams (1994*a*, *see above*). Predictions of Scheme 2 are consistent with the values of K_{Mg} we measured in low $[Ca^{2+}]$ (*see* Fig. 2, dashed line labeled Type-I inhibition). The model predicted Ca^{2+}-activation characteristics for RyRs consistent with those reported here when K_{CA1} ~ 1 nM and K_{CA2} ~ 1 μM.

Type-II: Mg^{2+} inhibition at the Ca^{2+}-inhibition site. The Hill coefficients for Ca^{2+} inhibition indicate that it is mediated by the binding of more than one, and possibly two ions. The fact that Ca^{2+} inhibition is associated with a decrease in channel mean open time and an increase in mean closed time indicates that Ca^{2+} can inhibit the channel from both the open and closed conformations. In the model Ca^{2+} and Mg^{2+} share a common Type-II inhibition mechanism and bind to divalent ion sites with the same affinity, K_{XI}. Therefore, we describe Type-II inhibition by the condensed model, Scheme 3.

$$\begin{array}{ccc} O & \leftrightarrow & Io.\,(2X^{2+}) \\ \updownarrow & & \updownarrow \\ C & \leftrightarrow & Ic.\,(2X^{2+}) \end{array} \qquad \text{SCHEME 3}$$

States *Io* and *Ic* represent the nonconducting, inhibited states of the channel. The $[Mg^{2+}]$ required to induce a 50% reduction in P_I , K_I, depends on $[Ca^{2+}]$ and the ion binding affinities according to Eq. 3.

$$K_I = \sqrt{2 \cdot [Ca^{2+}]^2 + K_{X1}^2} - [Ca^{2+}] \tag{3}$$

Combined effects of Type I and Type II mechanisms. The gates for Types I and II inhibition are assumed to operate independently and such that the channel conducts only when both gates are open. Thus the probability of an open channel is equal to the probability that both gates are open which, in turn, is equal to the product of the open probabilities of each gate, $P_A \times P_I$. The exact solution for 50% reduction of P_o by Mg^{2+} (K_{Mg}) is not given for brevity. Numerical solutions for K_A, K_I and K_{Mg} are shown in Fig. 2 using model parameters which account for the Mg^{2+} inhibition we observed in both cardiac and skeletal RyRs.

In Fig. 2 it can be seen that sensitivity of the overall inhibition to Mg^{2+} has a biphasic dependence on $[Ca^{2+}]$ such that at low $[Ca^{2+}]$ it is similar to Type-I inhibition and at high $[Ca^{2+}]$ it is like Type-II inhi-

[1] It has been pointed out by Laver et al. (1995) that these differences may result from the way the data are averaged. The average $[Ca^{2+}]$ response of P_o in a population of variable RyRs, as occurs in vesicle studies, would smear the $[Ca^{2+}]$ response so that the apparent Hill coefficients are reduced. Though variability in RyR properties is frequently observed in bilayer studies, it has not yet been established in SR vesicles or in vivo.

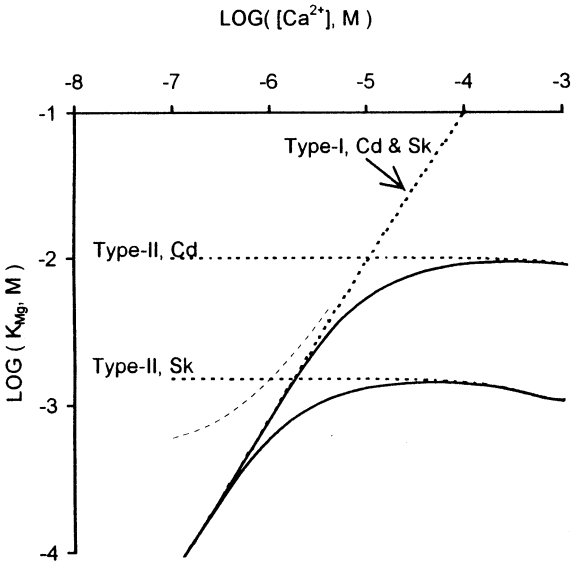

Fig. 2. Model predictions for K_{Mg} showing the relative contributions of the Type-I and Type-II mechanisms to Mg^{2+} inhibition of cardiac (Cd) and skeletal (Sk) RyRs. The solid lines are two of the model fits to the data in Fig. 4 which use the parameter values: K_{CA1} = 1 nM, K_{CA2} = 1 μM, K_{MA1} = 0.7 μM K_{MA2} = 1 mM. The only difference between the two fits are the values of K_{XI} which are: 1.5 mM for skeletal RyRs, 10 mM for cardiac RyRs. The thick dashed lines show the Ca^{2+} dependence of the $[Mg^{2+}]$ required to halve the open probability of the gates associated with Type-I and Type-II mechanisms, K_A and K_I respectively (Eqs. 2 and 3). The thin dashed line shows the predicted values of K_A using a 3-state model for Type-I inhibition which has been used in previous studies (*see* Scheme 1). It can be seen that for the cardiac RyRs (upper solid line) K_{Mg} at high and low $[Ca^{2+}]$ extremes is similar to that expected solely from Type-II and Type-I mechanisms respectively.

bition. The emergence of Types-I and -II inhibition at opposite $[Ca^{2+}]$ extremes can be predicted from relative values of K_A and K_I given by Eqs. 2 and 3. When $K_A << K_I$ then Mg^{2+} inhibition will be similar to that expected from Type-II inhibition alone whereas when $K_I << K_A$ then Type-I inhibition will dominate. The relative importance of the two processes in determining K_{Mg}, crosses over at $[Ca^{2+}]$ where $K_I = K_A$. Under experimental conditions $[Ca^{2+}] << K_{XI}$ and $K_{AC} << K_{XI}$ so that the cross over occurs at $[Ca^{2+}]_{co}$ given by:

$$[Ca^{2+}]co = \frac{K_{AC}K_{XI}}{K_{AM}} \cong 10^{-3} \cdot K_I \qquad (4)$$

Near $[Ca^{2+}]_{co}$ Mg^{2+} inhibition is determined by the kinetic properties of both Types-I and -II mechanisms.

ABBREVIATIONS

ATP	adenosine-5′-triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-NNN′N′-tetra-acetic acid
Dibromo-BAPTA	5,5′-dibromo 1,2-bis(2-aminophenoxy)ethane-NNN′N′-tetraacetic acid, tetrapotassium salt
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate
PC	palmitoyl-oleoyl-phosphatidylcholine
PE	palmitoyl-oleoyl-phosphatidylethanolamine
PS	palmitoyl-oleoyl-phosphatidylserine
TES	N-tri[Hydroxymethyl] methyl-2-aminoethanesulfonic acid

Results

Fusion of cardiac SR vesicles incorporated both Cl^- channels and RyRs into bilayers. RyRs had properties previously described by Laver et al. (1995). Millimolar concentrations of Mg^{2+} and Ca^{2+} in either the *cis* or *trans* baths reduced the Cs^+ conductance of RyRs in a similar way to that found in previous studies (e.g., Tinker et al., 1992). Increasing *trans* $[Ca^{2+}]$ from 0.1 to 1 mM had no effect on channel gating. This agrees with the findings of Sitsapesan and Williams (1994*b*) where the gating of sheep cardiac RyRs, activated solely by Ca^{2+}, was independent of *trans* $[Ca^{2+}]$. The gating properties of RyRs were examined over a wide range of *cis* $[Ca^{2+}]$ and $[Mg^{2+}]$. Ca^{2+} caused activation of RyRs within the range of 0.1 to 10 μM and inhibited RyRs at concentrations in excess of 1 mM. The maximum open probabilities within bursts for cardiac RyRs were 0.63 ± 0.27 (mean ± SD, n = 21) and for skeletal RyRs P_o = 0.43 ± 0.25 (n = 12).

The inhibition of RyRs by divalent ions X^{2+} (either Mg^{2+} or Ca^{2+}) was characterized by fitting a Hill equation to the relationship between P_o and $[X^{2+}]$, i.e.,

$$\frac{P_o([X^{2+}])}{P_o(0)} = \frac{1}{1 + ([X^{2+}]/K_X)^{H_x}} \qquad (5)$$

When X≡Mg then P_o (0) is the channel open probability in the absence of Mg^{2+} and when X≡Ca then P_o (0) is the open probability of the fully activated channel at $[Ca^{2+}]$ between 10 and 100 μM. K_X is the $[X^{2+}]$ which produces 50% reduction on P_o and H_X is the Hill coefficient which represents the number of ions which bind cooperatively to produce channel inhibition.

Different Mg^{2+}-inhibition Kinetics are Associated with Ca^{2+} Activation and Inhibition

As expected, RyR channel openings were inhibited by addition of mM Mg^{2+} to the *cis* bath (Fig. 3). However, the kinetics of the Mg^{2+} effect shown in Fig. 3 were different in the presence of μM and mM *cis* Ca^{2+}. At 1 μM Ca^{2+} the channel openings appear to be long in the presence and absence of Mg^{2+} but after the addition of Mg^{2+} there are fewer openings. In contrast with 1 μM Ca^{2+}, the addition of Mg^{2+} in 1 mM Ca^{2+} caused RyR gating to be more flickery and this effect was similar to that seen when RyRs are inhibited by Ca^{2+} alone. The

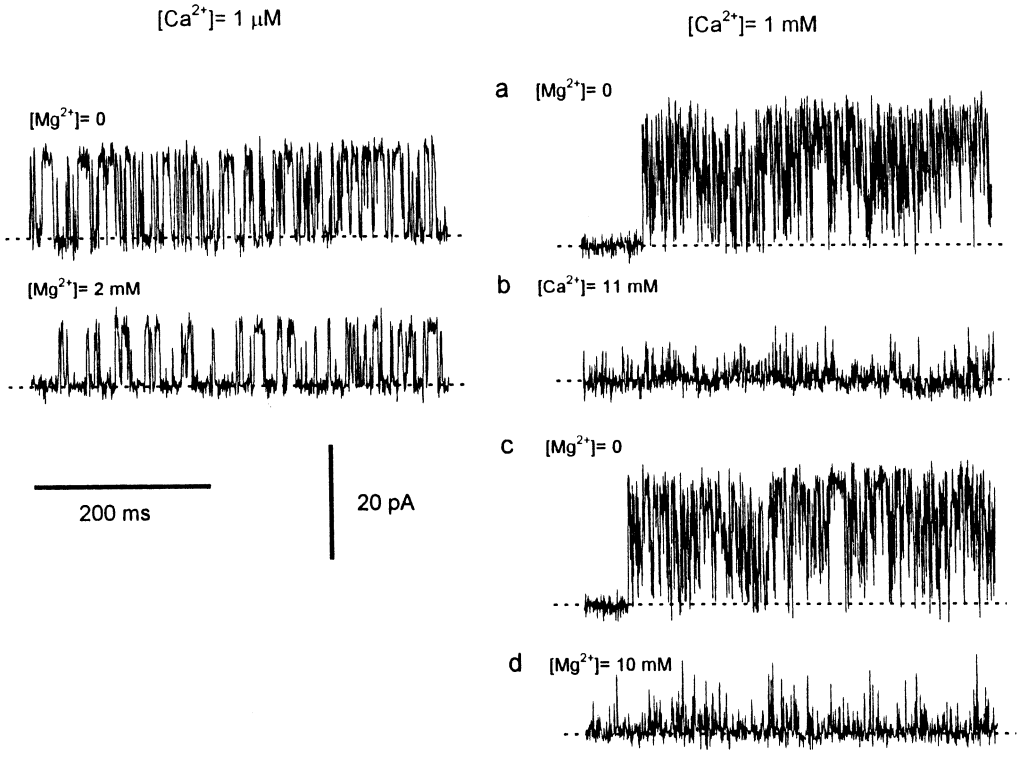

Fig. 3. Single-channel recordings of cardiac RyRs in lipid bilayers showing the effects of Mg^{2+} inhibition on channel gating. The signals are displayed here with a bandwidth of 1 kHz. The *cis* bath contained 250 mM CsCl. The bilayer voltage was +40 mV and the current baseline is at the bottom of each trace (dashed lines). The left panel (experiment D10231; *trans* $[Ca^{2+}] = 1$ mM) shows that Mg^{2+} inhibition in 1 μM Ca^{2+} increases the duration of channel closures. In the right panel (experiment D15152; *trans* $[Ca^{2+}] = 0.1$ mM) 10 mM Ca^{2+} was added to a RyR initially in 1 mM Ca^{2+} and this inhibits the channel. The *cis* bath was flushed with solutions containing 1 mM Ca^{2+} and maximal channel activity was restored. Then 10 mM Mg^{2+} was added to the *cis* bath. Gating pattern of the RyR inhibited by 10 mM Mg^{2+} + 1 mM Ca^{2+} appeared to be the same as that inhibited by 11 mM Ca^{2+} alone. Mg^{2+} inhibition in 1 mM Ca^{2+} also appeared to induce a more flickery gating pattern than in 1 μM Ca^{2+}. The smaller unitary currents in the left panel show the effect of elevated *trans* $[Ca^{2+}]$.

gating kinetics are explored more fully in the later section dealing with open and closed probability distributions. We found K_{Mg} increased with increasing $[Ca^{2+}]$, in general agreement with other studies of RyRs from cardiac- (Ashley & Williams, 1990) and skeletal muscle (Smith et al., 1986).

To investigate the possible links between Mg^{2+} inhibition and Ca^{2+} regulation in RyRs we compared the effects of Mg^{2+} on normal RyRs with those seen in RyRs in which the Ca^{2+}-inhibition mechanism was either absent or had more than 10-fold reduced Ca^{2+} sensitivity. Cardiac RyRs with attenuated or absent Ca^{2+}-inhibition mechanisms were obtained in three ways. (i) Approximately 20% of the RyRs which were incorporated into bilayers in the presence of 250 mM CsCl were not inhibited by high $[Ca^{2+}]$ (Laver et al., 1995). (ii) Solubilizing cardiac RyRs with CHAPS prior to their fusion with the bilayer or (iii) Exposing the cytoplasmic side of RyRs to 500 mM CsCl for ~1 min (Laver et al., 1995). The $[Ca^{2+}]$ dependencies of K_{Mg} were the same in all cardiac RyRs with attenuated Ca^{2+} inhibition (Fig. 4, filled circles). In these channels K_{Mg} increased proportionally with $[Ca^{2+}]$ up to ~100 μM[2].

We also observed three cardiac RyRs in which the gating activity did not depend on *cis* or *trans* $[Ca^{2+}]$. In those channels neither Ca^{2+} activation, inhibition nor Mg^{2+} inhibition were detected.

The $[Ca^{2+}]$ dependence of Mg^{2+} inhibition depended on the presence or absence of Ca^{2+} inhibition in individual RyRs (Fig. 4). In cardiac RyRs that showed Ca^{2+} inhibition (open circles) the Ca^{2+} dependence of K_{Mg} was biphasic; K_{Mg} increased with increasing $[Ca^{2+}]$ and reached a plateau when $[Ca^{2+}]$ exceeded 10 μM. In skeletal RyRs (squares) K_{Mg} was relatively insensitive to

[2] The mean value of K_{Mg} for channels with attenuated Ca^{2+} inhibition at 1 mM Ca^{2+} shown in Fig. 2 underestimates the true K_{Mg} since the mean did not include several experiments where K_{Mg} was above the experimental range of $[Mg^{2+}]$.

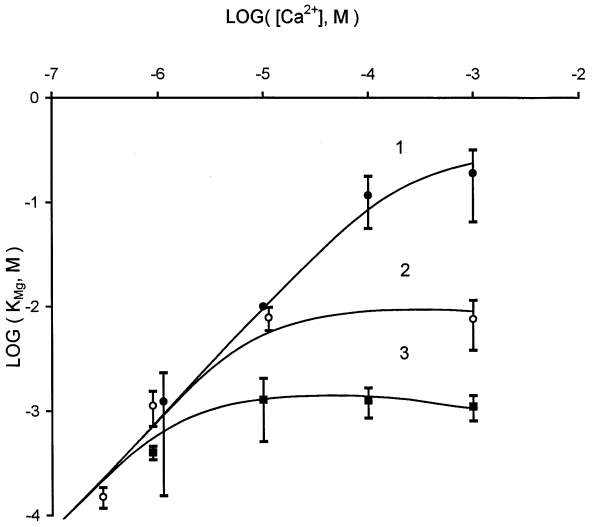

Fig. 4. The mean (±SEM) *cis* $[Mg^{2+}]$ causing 50% inhibition of RyRs, K_{Mg}, plotted against *cis* $[Ca^{2+}]$ for three distinct groups of RyR, namely. (1, ●)- Cardiac RyRs which were insensitive to inhibition by mM *cis* Ca^{2+} (23 measurements of K_{Mg} from 18 RyRs in either *cis* 250 or 500 mM CsCl). (2, ○)- Cardiac RyRs in *cis* 250 mM CsCl which could be inhibited by mM *cis* Ca^{2+} (21 measurements of K_{Mg} from 17 RyRs). (3, ■)- Skeletal RyRs (20 measurements of K_{Mg} from 19 RyRs). RyRs were classified as not showing Ca^{2+} inhibition by either direct measurement of inhibition ($n = 4$; *cis* $[Cs^+] = 250$ mM) or by the assumptions that 500 mM *cis* CsCl ($n = 13$) and CHAPS solubilization ($n = 1$) remove or severely attenuate Ca^{2+} inhibition. Three model predictions for the Ca^{2+} dependence of K_{Mg} are compared with the data. In each case the binding constants used in Scheme 2 (Type-I inhibition) were the same where $K_{CA1} = 1$ nM, $K_{CA2} = 1$ μM, $K_{MA1} = 0.7$ μM and $K_{MA2} = 1$ mM. The parameter values of K_{XI} in Scheme 3 were adjusted to simulate the three RyR groups; (i) $K_{XI} = 300$ mM, (ii) $K_{XI} = 10$ mM, (iii) $K_{XI} = 1.5$ mM. Differences between each group of RyRs appeared to result entirely from differences in Type-II inhibition.

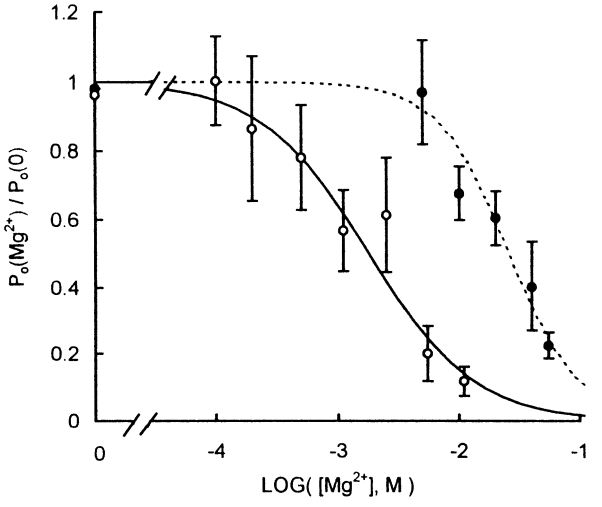

Fig. 5. The $[Mg^{2+}]$ dependence of cardiac RyR P_o in *cis* 250 mM CsCl and $[Ca^{2+}]$ of either 1 μM (○; average of 7 experiments with individual K_{Mg} ranging from 0.4 to 2 mM) or 1 mM (●; average of 6 experiments where K_{Mg} ranged from 14 to 35 mM) where RyRs were found to be inhibited by $[Ca^{2+}]$ (*data not shown*). Of the total of 11 experiments carried out in 1 mM Ca^{2+} K_{Mg} ranged from 2 mM to 35 mM. Five of these experiments were excluded from the averaging process to restrict the range of K_{Mg} and so more accurately reflect the different slopes of the Mg^{2+} dependencies in high and low $[Ca^{2+}]$. The lines show fits of the data with Eq. 5 with the following parameters: (solid line)- $H_{Mg} = 1$, $K_{Mg} = 1.8$ mM. (dashed line)- $H_{Mg} = 1.5$, $K_{Mg} = 26$ mM. Fits to individual experiments give a higher Hill coefficient for Mg^{2+} inhibition in 1 mM Ca^{2+} ($H_{Mg} \sim 2$, *see* text).

$[Ca^{2+}]$ between 1 μM and 1 mM. A biphasic $[Ca^{2+}]$ dependence has also been seen in the Mg^{2+} inhibition of CICR from SR vesicles from skeletal muscle (Meissner et al., 1986). This was interpreted as indicative of two Mg^{2+}-inhibition processes; the one which is $[Ca^{2+}]$ dependent dominates Mg^{2+} inhibition at low $[Ca^{2+}]$ and the other which appears to be $[Ca^{2+}]$ independent produces the plateau at high $[Ca^{2+}]$. The properties of these processes in cardiac RyRs are studied separately in the following section by analyzing Mg^{2+} inhibition at 1 μM Ca^{2+} (representing the low $[Ca^{2+}]$ extreme, *see* the Double Gate Model) and 1 mM Ca^{2+} (representing the high $[Ca^{2+}]$ extreme). Since the plateau appears to be shifted to high $[Ca^{2+}]$ in channels that lack sensitivity to Ca^{2+} inhibition (Fig. 4) it appears that mechanism for Mg^{2+} inhibition of RyRs in high $[Ca^{2+}]$ is linked with the Ca^{2+} inhibition mechanism.

Figure 5 shows the $[Mg^{2+}]$ dependence of inhibition at 1 μM and 1 mM $[Ca^{2+}]$ in cardiac RyRs which could be inhibited by Ca^{2+}. It can be seen that the concentration-dependence of Mg^{2+} inhibition is steeper in the presence of 1 mM Ca^{2+} (filled circles) than in 1 μM Ca^{2+} (open circles). On average we found that Hill coefficients for Mg^{2+} inhibition, H_{Mg}, of single RyRs in 0.1–1 mM Ca^{2+} were 2.0 ± 0.4 (±SD, $n = 15$) whereas with $[Ca^{2+}]$ in the range 1–10 μM Ca^{2+}, H_{Mg} were 1.3 ± 0.5 ($n = 11$). We were unable to obtain reliable estimates of H_{Mg} in 1 mM Ca^{2+} from RyRs with attenuated Ca^{2+} inhibition since they did not show a sufficient degree of Mg^{2+} inhibition within the experimental range of $[Mg^{2+}]$. For skeletal RyRs, an H_{Mg} of 2.0 ± 0.8 ($n = 5$) in 1 mM Ca^{2+} was similar to that found for cardiac RyRs. However, in 1–3 μM Ca^{2+} an H_{Mg} of 3.0 ± 0.8 ($n = 3$) was higher than that for cardiac RyRs.

We also found that the sensitivity of skeletal RyRs to inhibition by Mg^{2+} and Ca^{2+} was reversibly dependent on [CsCl]. Increasing [CsCl] in the *cis* bath from 250 mM to 500 mM raised K_{Mg} from 1.1 ± 0.2 mM to 5.2 ± 1.0 mM and raised K_{Ca} from 0.7 ± 0.2 mM to 2.0 ± 0.5 mM for skeletal RyRs. These effects are consistent with the results of $[H^3]$-ryanodine binding studies (Shomer et al., 1993) which showed that sensitivity of pig skeletal RyRs to Ca^{2+} inhibition is reduced in proportion with the

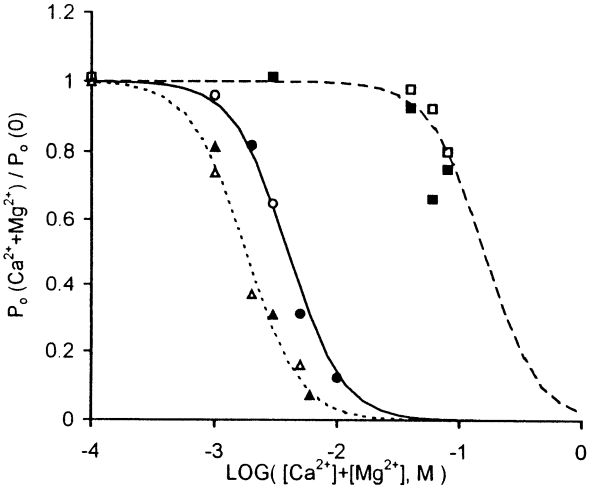

Fig. 6. Inhibition of 3 cardiac RyRs by *cis* Ca^{2+} and Mg^{2+} in the presence of 1 mM Ca^{2+} plotted against the total divalent ion concentration ([Ca^{2+}] + [Mg^{2+}]). In these experiments Mg^{2+} inhibition (filled symbols) was produced by adding $MgCl_2$ to the *cis* bath which also contained 0.1 mM Ca^{2+}. Subsequently, the bath was perfused with Mg^{2+}-free solution containing 0.1 mM Ca^{2+} and then $CaCl_2$ was added to the bath to inhibit the same channel (open symbols). The data are fitted with Eq. 5 using Hill coefficients of 2 and the following values of K_{Mg}: (triangles; experiment D15223)- 1.8 mM, (circles; experiment D15217)- 4 mM, (squares; experiment D14135)- 160 mM. The inhibition of RyRs appears to depend on the total divalent ion concentration rather than specifically on [Ca^{2+}] or [Mg^{2+}].

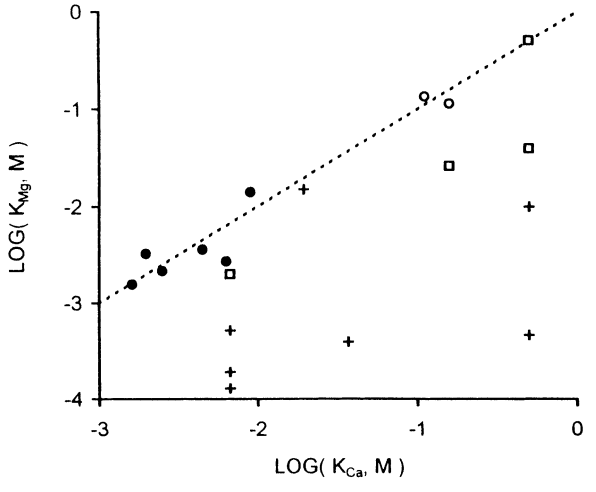

Fig. 7. Correlation between the sensitivity to inhibition by Ca^{2+} and Mg^{2+} in individual cardiac RyRs in *cis* 250 mM CsCl. Half inhibition by Mg^{2+}, K_{Mg}, and by Ca^{2+}, K_{Ca}, was determined on the same RyRs as described in the legend to Fig. 6 except that Mg^{2+} inhibition was measured in the presence of various *cis* [Ca^{2+}]. The correlation between K_{Ca} and K_{Mg} is shown in several cases; (●)- Mg^{2+} inhibition in the presence of 1 mM Ca^{2+} where RyRs showed normal Ca^{2+} inhibition, (○)-Mg^{2+} inhibition in the presence of 1 mM Ca^{2+} where the RyRs showed reduced Ca^{2+} inhibition because they had been exposed to *cis* 500 mM CsCl for one minute prior to measurements of divalent ion inhibition, (□)-Mg^{2+} inhibition in the presence of 100 μM Ca^{2+}, (+)-Mg^{2+} inhibition in the presence of 10 μM Ca^{2+}. Estimates of K_{Mg} and K_{Ca} which exceeded the experimental range of [Ca^{2+}] or [Mg^{2+}] are not accurate. These data points have been set to K_{Ca} and/or K_{Mg} of 500 mM and are not included in the statistical analysis. There is a close correlation between K_{Mg} and K_{Ca} when Mg^{2+} inhibition is measured in the presence of 1 mM Ca^{2+} which is not as apparent at lower [Ca^{2+}].

square of the ionic strength (i.e., ~100-fold reduction by increasing [NaCl] from 100 to 1,000 mM).

Equivalence of Ca^{2+} Inhibition and Mg^{2+} Inhibition of Cardiac RyRs in 1 mM Ca^{2+}

In this study the kinetics of Ca^{2+} inhibition and Mg^{2+} inhibition of cardiac RyRs in 1 mM Ca^{2+} appeared to be identical. In RyRs which could be inhibited by Ca^{2+} the mean values of H_{Mg} and K_{Mg} for Mg^{2+} inhibition (H_{Mg} = 2.0 ± 0.4 and K_{Mg} = 14 ± 13 mM, n = 12) was not significantly different from those for Ca^{2+} inhibition (H_{Ca} = 2.3 ± 0.5 and K_{Ca} = 13 ± 13 mM; n = 18). The large standard deviations in the distribution of K_{Ca} and K_{Mg} reflects the considerable variation in the sensitivity of RyRs to inhibition by Ca^{2+} and Mg^{2+}. Variations in Ca^{2+} sensitivity have been observed in previous studies (Laver et al., 1995). In Figs. 6 to 8 we show correlations between Ca^{2+} inhibition and Mg^{2+} inhibition in the variable RyR population by comparing both forms of inhibition in the same RyR. Figure 6 shows the dependence of P_o on divalent ion concentration in three cardiac RyRs (squares, circles and triangles) which showed very different X^{2+} sensitivities, spanning the range observed in this study. The results show that the concentration-dependence of inhibition was the same in each channel, whether X^{2+} was Ca^{2+} alone (open symbols) or Mg^{2+} plus 1 mM Ca^{2+} (filled symbols). Therefore, the P_o of individual RyRs during inhibition depends on the total divalent ion concentration [X^{2+}] rather than the relative contributions of [Ca^{2+}] and [Mg^{2+}].

The generality of the equivalence of Ca^{2+} and Mg^{2+} (in the presence of 1 mM Ca^{2+}) throughout our experiments is shown in Fig. 7 where values of K_{Ca} and K_{Mg} obtained from the same channels are compared. Measurements of K_{Ca} for 6 cardiac RyRs (filled circles) shown in Fig. 7 are spread throughout the range 2 to 10 mM and for another 2 RyRs (Cs^+ treated; open circles) K_{Ca} were ~ 100 mM. There is a tight correlation (correlation coefficient, r = 0.95 or coefficient of determination, r^2 = 0.9) and equality between values of K_{Mg} (in 1 mM Ca^{2+}) and K_{Ca} obtained from these channels. In contrast to this were much poorer correlations (r = 0.17) between K_{Ca} and K_{Mg} from another 11 cardiac RyRs in the presence of 10 and 100 μM Ca^{2+} (crosses & squares in Fig. 7). This clearly shows that the Mg^{2+}/Ca^{2+} equivalence does not apply under low [Ca^{2+}] conditions.

Close association of the Ca^{2+}- and Mg^{2+}-inhibition mechanisms is shown again in Fig. 8. In this experiment

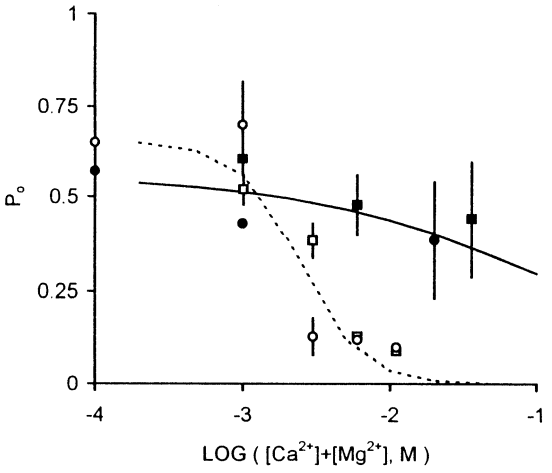

Fig. 8. Measurement of P_o *vs.* total divalent ion concentration. The figure shows Ca^{2+} and Mg^{2+} inhibition in a cardiac RyR in *cis* 250 mM CsCl, both before and after exposing of the *cis* face of the RyR to 500 mM CsCl (Experiment D15152). The inhibiting effects of Ca^{2+} (○) and Mg^{2+} (□; in the presence of 1 mM Ca^{2+}) were measured shortly after it was incorporated into the bilayer. [Cs^+] in the *cis* chamber was raised to 500 mM by adding an aliquot of 2M CsCl. After one minute the *cis* bath was perfused with 250 mM CsCl and the effects of Ca^{2+} (●) and Mg^{2+} (■; in the presence of 1 mM Ca^{2+}) were measured again. High [Cs^+] has the same, irreversible effect on Ca^{2+} and Mg^{2+} inhibition.

cardiac RyRs showing Ca^{2+} inhibition had been incorporated with bilayers in the presence of 250 mM CsCl. Both Mg^{2+} and Ca^{2+} inhibition showed similar concentration dependencies (open symbols). [Cs^+] in the *cis* bath was then raised to ~500 mM for 1 min and then returned to 250 mM. This treatment drastically reduced the sensitivity of RyR inhibition to both Ca^{2+} and Mg^{2+} (filled symbols).

The Effect of Ca^{2+} and Mg^{2+} Inhibition on RyRs Open and Closed Durations

Probability distributions of ''log-binned'' event durations, produced by normalizing the event frequency histograms, show the gating kinetics of Mg^{2+} inhibition in detail. The Mg^{2+} inhibition of cardiac RyRs (those RyRs which showed Ca^{2+} inhibition) produced markedly different gating kinetics in the presence of <3 μM and 1 mM [Ca^{2+}]. Specific examples of these differences are shown in Figs. 9 and 10 which were derived from data of which segments are shown in Fig. 3. Figure 9 shows that 2 mM Mg^{2+} with 1 μM Ca^{2+}, which reduced P_o to 50% of the control value also significantly increased the probability of long closed durations (Fig. 9*A*) but produced no significant change in the open time distribution (Fig. 9*B*). In contrast to the Mg^{2+} effect in 1 μM Ca^{2+}, Fig. 10 shows that 10 mM Mg^{2+} in 1 mM Ca^{2+}, which reduced P_o by 50% both increased the probability of long closed durations (Fig. 10*A*) and increased the probability of

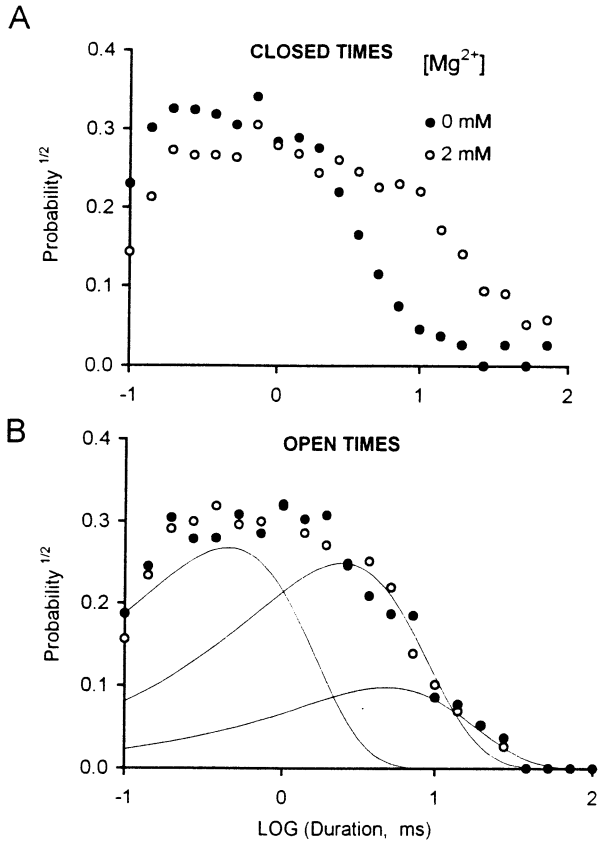

Fig. 9. The effect of Mg^{2+} on the probability distributions of channel closed (*A*) and open (*B*) durations of cardiac RyRs in the presence of 1 μM *cis* [Ca^{2+}]. The probabilities were calculated from number of events/bin divided by the total number of events (~1500 events in each record). The histograms were extracted from single-channel recordings of ~15 second duration in experiment D10231 as outlined in Materials and Methods (part of this record is shown in Fig. 3). The event durations are ''log-binned'' and displayed using the approach of Sigworth and Sine (1987). The distributions of open and closed times could be fit by the sum of three exponentials shown separately by the three curves in part B. 50% inhibition of the RyR by the addition of 2 mM Mg^{2+} had no significant effect on the open time distribution but it did significantly increase the probability of long channel closures.

short open durations (Fig. 10*B*) of cardiac RyRs. Figure 10 also shows that adding 10 mM Mg^{2+} or Ca^{2+} to the *cis* bath initially containing 1 mM Ca^{2+} had the same effect on channel gating. The open and closed event probability distributions in 10 mM Mg^{2+} plus 1 mM Ca^{2+} and 11 mM Ca^{2+} are identical, indicating again that the RyR inhibition mechanism depends only on the total divalent ion concentration and not the relative amounts of Ca^{2+} and Mg^{2+}.

The different effects of Mg^{2+} inhibition on the gating kinetics in μM and mM Ca^{2+} are highlighted by the exponential analysis of the probability distributions. In Figs. 11 to 13 the effects of Ca^{2+} and Mg^{2+} on the shape of all the open and closed probability distributions are summarized in terms of the individual exponential com-

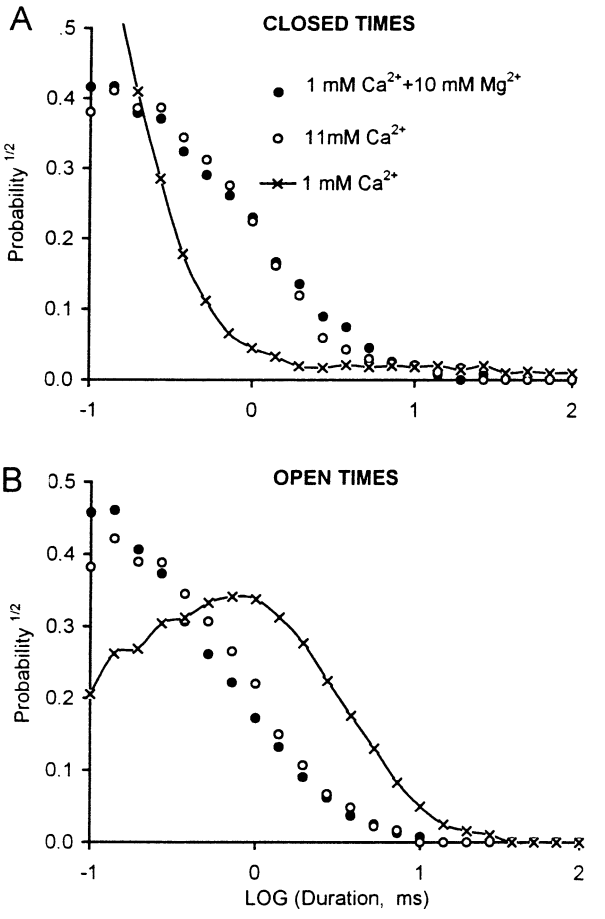

Fig. 10. The effect of adding 10 mM Mg^{2+} or 10 mM Ca^{2+} to cardiac RyRs initially in the presence of 1 mM [Ca^{2+}]. The probability distributions are generated from 20–40 second records containing ~20,000 events and are displayed in the same way as in Fig. 9. These are the results of one for two experiments (D15152 shown here and D16332) where the gating of a single cardiac RyR was initially measured when the *cis* bath contained 250 mM CsCl + 1 mM $CaCl_2$ (—x—). The measurement was repeated after 10 mM $CaCl_2$ was added to the bath (open circles). The *cis* bath was then flushed with solution containing 1 mM $CaCl_2$. The *cis* [Mg^{2+}] was increased to 10 mM before the final measurement was made (closed circles). In both experiments a 50% inhibition of the RyR, by the addition of either 10 mM Ca^{2+} or Mg^{2+}, had identical effects on channel gating.

ponents comprising these distributions. The open and closed probability distributions were complex; having at least two or three resolvable exponential components (e.g., Fig. 9*B*, lines). The variations in K_{Mg} at different [Ca^{2+}] and also between individual RyRs under the same conditions made it difficult either to measure gating kinetics for a given P_o or to obtain a meaningful comparison of RyR gating at a given [Mg^{2+}]. To overcome these problems in Figs. 11 to 13 the areas, A_i, and time constants T_i, of short (circles), medium (squares) and long (triangles) exponential components are plotted against the relative inhibition of the channel by [Ca^{2+}] or [Mg^{2+}] rather than against [Ca^{2+}] or [Mg^{2+}] directly. Figure 11 shows the effects of Mg^{2+} on the gating of cardiac RyRs ($n = 7$) in the presence of 1 μM Ca^{2+}. The Mg^{2+}-dependent increase in mean closed time under these conditions is mediated by an increase in all the exponential closed time constants with no consistent change in relative area. Mg^{2+} had no significant effect on any of the open duration parameters. In the presence of 1 mM Ca^{2+}, Mg^{2+} inhibition ($n = 7$) had a distinctly different effect on the exponential components to that seen in 1 μM Ca^{2+} (Fig. 12, filled symbols). In this case Mg^{2+} inhibition increased mean closed times by increasing the relative area and time constant of the second exponential component (squares). Mg^{2+} inhibition decreased the mean open times by reducing the relative area of the second exponential component (squares) while leaving the time constants unaffected. Figure 12 also shows that the gating kinetics of Mg^{2+} inhibition (closed symbols) are identical those of Ca^{2+} inhibition (open symbols).

Mg^{2+} and Ca^{2+} had very similar actions on the gating of cardiac and skeletal RyRs. Figure 13 summarizes the effects of Ca^{2+}, and Mg^{2+} inhibition in 0.1 to 1 mM Ca^{2+}, on the gating kinetics of skeletal RyRs. Ca^{2+} (open symbols) and Mg^{2+} (closed symbols) had identical effects on A_i and τ_i (open) and τ_i (closed). Though Ca^{2+} and Mg^{2+} both produced similar changes in A_i, absolute differences in A_i were apparent. These differences appeared to originate from differences in gating between the uninhibited RyRs used for each group of experiments and are not necessarily due to specific effects of Ca^{2+} and Mg^{2+}. We also found that the Mg^{2+} dependencies of A_i and τ_i skeletal RyRs for [Ca^{2+}] down to 1 μM (*not shown*) were similar to those shown in Figure 13 and similar to those seen in cardiac RyRs only in high (e.g., 1 mM) [Ca^{2+}] (*c.f.* Figs. 11 and 12).

The kinetics of Mg^{2+} inhibition of cardiac RyRs in mid-range [Ca^{2+}] (~10 μM) appeared to be the amalgamation of those seen in high and low [Ca^{2+}]. In 10 μM Ca^{2+} up to 80% inhibition of RyRs by Mg^{2+} was mediated solely by an increase in the mean closed duration, a property of Mg^{2+} inhibition in low [Ca^{2+}]. Mg^{2+} inhibition above 80% was due to both a decrease in mean open duration and a decrease in mean closed duration, a property of Mg^{2+} inhibition in high [Ca^{2+}].

The analysis of the effects of Mg^{2+} inhibition on the open and closed durations of cardiac RyRs shown here is consistent with the trends reported by Ashley and Williams (1990) who also used sheep cardiac RyRs. They also found that Mg^{2+} inhibition in the presence of high (100 μM) Ca^{2+} reduced mean open times by altering the relative areas of the exponential components without changing the time constants (*cf.* Fig. 12). Mg^{2+} also increased their observed mean closed durations. However, the trends we observe for closed times are too complex to directly compare with their results.

The kinetics of Ca^{2+} inhibition reported here and previously by Laver et al. (1995) are substantially dif-

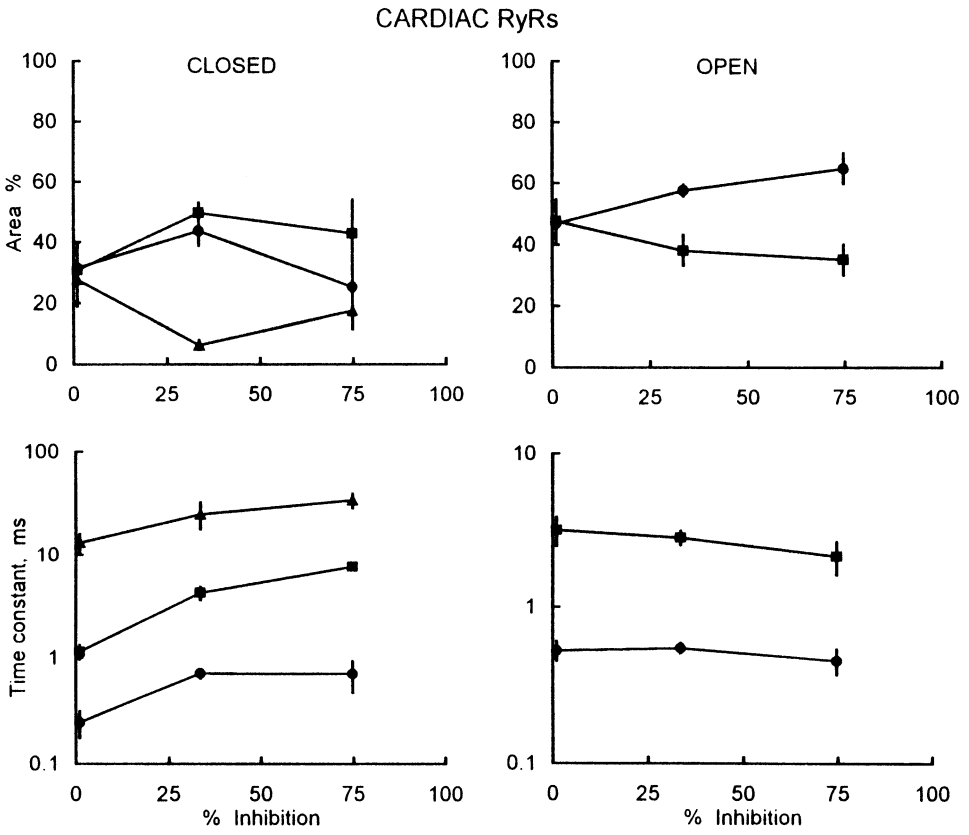

Fig. 11. The effect of Mg^{2+} inhibition in 1 μM *cis* Ca^{2+} on the channel lifetime parameters of 5 cardiac RyRs. The parameters were obtained from least-squares fits of double or triple exponentials to 22 pairs of open and closed probability distributions (*see* Materials and Methods). The degree of Mg^{2+} inhibition was determined from channel open probabilities in the presence and absence of Mg^{2+} using the formula:

$$\% \text{ Inhibition} = 100 \times [1 - P_o(Mg^{2+})/P_o(0)].$$

Parameters associated with exponentials having short, medium and long time constants are represented by ●, ■ and ▲ respectively and the error bars represent standard errors on the mean. Data were grouped for averaging over ranges of P_o less than 0.2. Mg^{2+} inhibition here is due to an increase in the closed time constants. Mg^{2+} has no significant effect on the open time frequency distributions.

ferent from the gating kinetics associated with a decline in P_o of sheep cardiac RyRs described in an abstract by Sitsapesan, Boraso and Williams (1991). In their study Ca^{2+} inhibition had no significant effect on mean open times and half inhibition occurred at only 0.5 to 3 mM. In their experiments RyRs, activated by Ca^{2+} alone, have a much lower P_o ~0.2 than we generally measured for complete records, even allowing for the intraburst gaps. As yet we have no explanation for the different gating of Ca^{2+} activated sheep cardiac RyRs between the two studies.

Discussion

Open Probability within Bursts as a Measure of RyR Inhibition

We chose to measure the RyR activity using the open probability, P_o, calculated from channel activity *within bursts* in order to circumvent the statistical scatter introduced by including very variable and relatively infrequent, long, channel closures. Excluding long closures from calculations of P_o probably accounts for the higher maximum values of P_o that we obtain (P_o = 0.63 for cardiac RyRs and 0.43 for skeletal RyRs) compared to P_o in some other reports. Maximum values of P_o for cardiac RyRs were found elsewhere to be 0.44 (Chu et al., 1993) and for skeletal RyRs reported values are 0.4 (Smith, Coronado & Meissner, 1986), 0.35 (Shomer et al., 1993) and 0.13 (Chu et al., 1993). However, our maximum P_o values are much lower than those seen by Rousseau et al. (1986, P_o ~1) where Ca^{2+} was the permeant ion. The exclusion of long RyR closures from our analysis is unlikely to compromise our measurements of Mg^{2+} inhibition since the predominant changes in the gating kinetics associated with Mg^{2+} inhibition occurred on the millisecond time scale. Though this study presents evidence for two Mg^{2+}-inhibition mechanisms

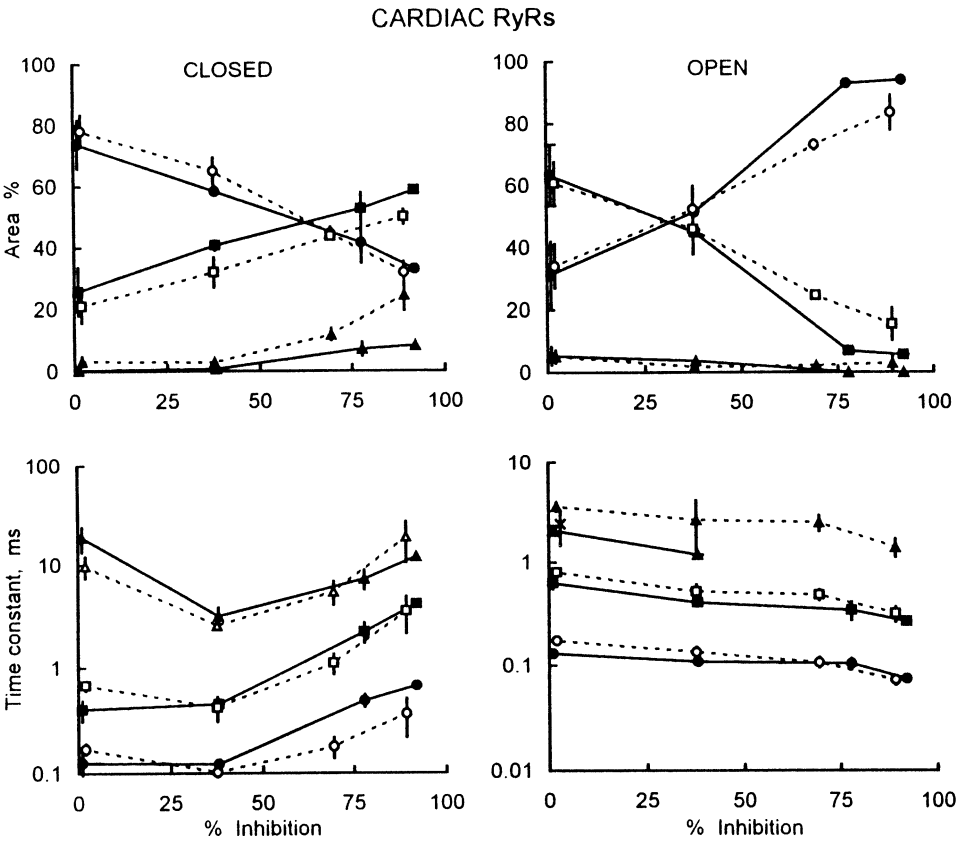

Fig. 12. A comparison of the effects of Mg^{2+} inhibition in 1 mM *cis* Ca^{2+} (full symbols, 5 RyRs, 14 pairs open and closed distributions) and Ca^{2+} inhibition (open symbols, 5 RyRs, 21 pairs of distributions) on cardiac RyR lifetime parameters. The symbol meanings and presentation are described in the legend to Fig. 11. Both Ca^{2+} and Mg^{2+} inhibition here is due to a change in the relative areas of exponential components of the open and closed probability distributions. The closed time constants showed similar complex dependencies on $[Mg^{2+}]$ or $[Ca^{2+}]$ whereas the open time constants showed no dependence on either $[Mg^{2+}]$ or $[Ca^{2+}]$. These effects of Mg^{2+} here are quite distinct from those produced by Mg^{2+} in 1 μM Ca^{2+} shown in Fig. 11.

in cardiac and skeletal RyRs we cannot exclude the possibility that other, unidentified, Mg^{2+}-dependent mechanisms regulate RyRs via their burst kinetics. So far we have not observed any evidence for such a mechanism.

Two Mg^{2+}-Inhibition Mechanisms

We present measurements of Mg^{2+} inhibition in single cardiac RyRs which show that there are at least two Mg^{2+}-inhibition mechanisms. Our results support Meissner's et al. (1986) interpretation of the Mg^{2+} inhibition of $^{45}Ca^{2+}$ efflux from SR vesicles. There are three broad lines of evidence in the present study for the existence of at least two mechanisms which contribute to the nature of Mg^{2+} inhibition depending on the cytoplasmic $[Ca^{2+}]$: (i) the biphasic $[Ca^{2+}]$ dependence of the $[Mg^{2+}]$ required for half inhibition, K_{Mg}, (*see* Fig. 4); (ii) the marked differences in the kinetics of Mg^{2+} inhibition at low and high $[Ca^{2+}]$ (Fig. 5 and *cf.* Figs. 9 and 10, 11 and 12); (iii) modification of RyRs by CHAPS solubilization or exposure of RyRs to 500 mM Cs^{+} irreversibly attenuated or abolished Mg^{2+} inhibition at high $[Ca^{2+}]$ without affecting Mg^{2+} inhibition at low $[Ca^{2+}]$ (Figs. 4 and 7) or the actions of ATP and caffeine (Laver et al., 1995). The last group of results also indicate that the two Mg^{2+} inhibition processes are probably associated with different amino acid sequences on the RyR protein.

The mechanism which mediates Mg^{2+} inhibition at high $[Ca^{2+}]$ appears to be the same gating mechanism responsible for Ca^{2+} inhibition since: (i) channels which lose Ca^{2+} inhibition by exposure to CHAPS or high [CsCl] also lose their Mg^{2+} inhibition at high $[Ca^{2+}]$ (Figs. 4 and 8); (ii) in spite of significant variations in the sensitivity of RyRs to Ca^{2+} inhibition, Ca^{2+} and Mg^{2+} always bound with similar affinity and Hill coefficient (Figs. 6 and 7); (iii) the ion concentration-dependence and gating kinetics of Ca^{2+} inhibition and Mg^{2+} inhibition at high $[Ca^{2+}]$ are indistinguishable (Figs. 6 and 10–13).

The mechanism which mediates Mg^{2+} inhibition at low $[Ca^{2+}]$ is probably associated with the Ca^{2+}-activation mechanism since activating Ca^{2+} and inhibiting Mg^{2+} display competitive binding at low $[Ca^{2+}]$ (*see*

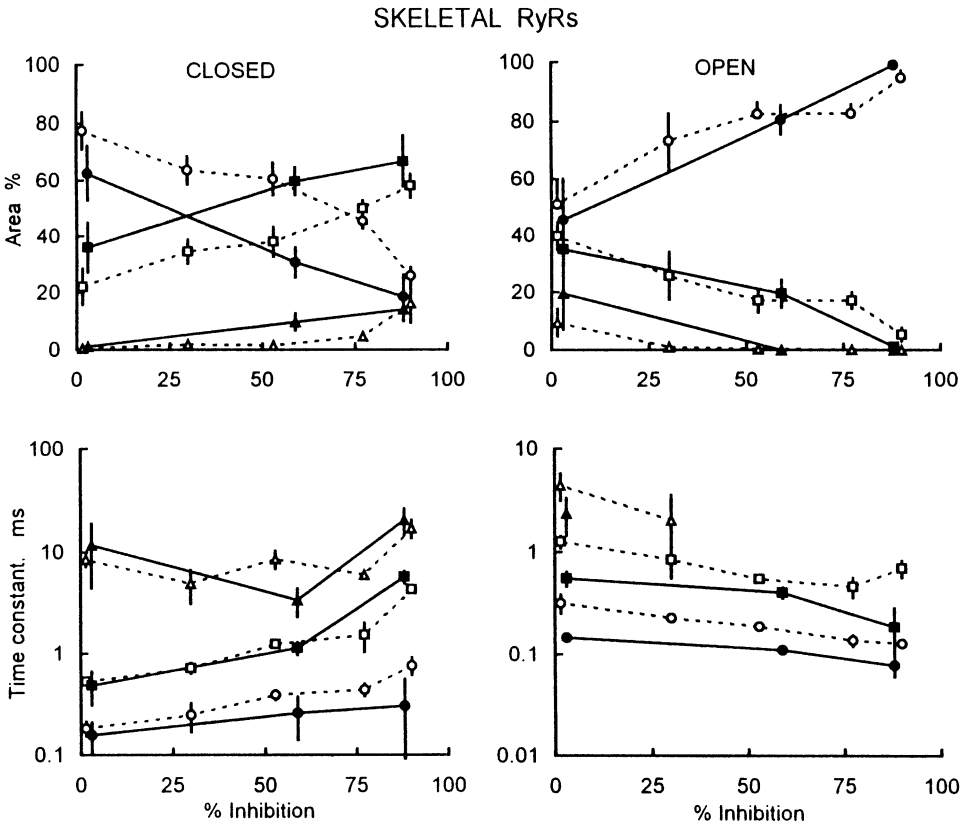

Fig. 13. A comparison of the effects of Mg^{2+} inhibition in 0.1–1 mM *cis* Ca^{2+} (full symbols, 4 RyRs, 14 pairs open and closed distributions) and Ca^{2+} inhibition (open symbols, 7 RyRs, 31 pairs of distributions) on skeletal RyR lifetime parameters. The symbol meanings and data presentation are described in the legend to Figs. 11 and 12. Like that seen for cardiac RyRs in Fig. 12 both Ca^{2+} and Mg^{2+} inhibition is due to a change in the relative contributions of components with long and short time constants to the open and closed probability distributions.

below). This mechanism for Mg^{2+} inhibition would account for the loss of Mg^{2+} inhibition at low $[Ca^{2+}]$ only in the three RyRs that had lost their Ca^{2+} activation (i.e., RyRs were fully activated independently of $[Ca^{2+}]$). However, we can not rule out the possibility that RyRs in this study which have lost sensitivity to both μM and mM Ca^{2+} have also suffered nonspecific damage that has abolished their sensitivity to all ligands.

Although we have not focused on skeletal RyRs our results indicate that Mg^{2+} inhibition, over the entire experimental range of *cis* $[Ca^{2+}]$ ($[Ca^{2+}] > 1$ μM), is mainly due to the mechanism responsible for Ca^{2+} inhibition in these channels. This is based on our findings that the kinetics of Mg^{2+} inhibition in skeletal RyRs (i) are similar to that of Ca^{2+} inhibition; (ii) are also similar to Mg^{2+} inhibition in cardiac RyRs at high $[Ca^{2+}]$ which is closely correlated with Ca^{2+} inhibition; (iii) are quite different to the kinetics of Mg^{2+} inhibition of cardiac RyRs at low $[Ca^{2+}]$ which is associated with Ca^{2+} activation. The range of *cis* $[Ca^{2+}]$ over which we see the correlation between Ca^{2+}- and Mg^{2+} inhibition is similar to that reported by Meissner et al. (1986, when *cis* $[Ca^{2+}] > 5$ μM divalent ion inhibition showed no specificity between Ca^{2+} and Mg^{2+}). Though we have not resolved two Mg^{2+}-inhibition mechanisms in skeletal RyRs, Meissner et al. (1986) detected Mg^{2+} inhibition in skeletal RyRs which is associated with Ca^{2+} activation at 1 nM cytoplasmic Ca^{2+}. Our findings contradict a commonly held hypothesis which suggests that Mg^{2+} binding at the Ca^{2+}-activation site is the sole contributor to inhibition of RyRs in artificial bilayers (e.g., Coronado et al., 1994).

The Suitability of Normal and Modified RyRs for Studying Mg^{2+} Inhibition

RyRs which were incorporated into bilayers from native SR vesicles are functionally normal since they are activated by cytosolic μM Ca^{2+}, mM ATP and caffeine, inhibited by mM Ca^{2+} and μM ruthenium red and they are ''locked'' into a substate by 30 μM ryanodine (Laver et al., 1995). Modification of cardiac RyRs by CHAPS solubilization or exposure to high $[Cs^+]$ abolished divalent cation-inhibition without affecting either channel conductance, substate activity or normal regulation of RyRs by the above mentioned ligands (Laver et al., 1995). Thus the modifying effects of CHAPS and Cs^+

on cardiac RyRs in this study appears to be specific for the divalent cation-inhibition mechanism.

The properties of our CHAPS solubilized RyRs appear to be the same as those of purified sheep cardiac RyRs prepared by others (Sitsapesan and Williams, 1994*a*), but their study did not comment on the effects of CHAPS on Ca^{2+} inhibition. Smith et al. (1988) did not address Ca^{2+} inhibition either, though they did observe a loss of Mg^{2+} inhibition after CHAPS solubilization of skeletal RyRs which was not accompanied by a loss of Ca^{2+} activation. Since we see the same effect in solubilized cardiac RyRs it is likely that their RyRs had also lost Ca^{2+} inhibition after CHAPS solubilization.

Though specific effects of Cs^+ on cardiac RyRs have not been reported by other laboratories, chaotropic ions are known to affect RyR function. For example, cytosolic, ClO_4^-, SCN^- and I^- stimulate activity of cardiac and skeletal RyRs in μM Ca^{2+} and mM Mg^{2+} (Ma et al., 1993). It is believed that these ions act by altering the interactions between the modulatory FK506 binding protein and the RyR (Ma, Bhat & Zhao, 1995). We have no evidence for the presence or absence of accessory proteins after exposure of cardiac RyRs to high $[Cs^+]$.

A number of other channel treatments have produced quite specific modifications to RyR function. Boraso and Williams (1994) found that 5 mM H_2O_2 in the cytosolic bath activated sheep cardiac RyRs at subactivating $[Ca^{2+}]$ without modifying channel conductance or gating in response to the normal regulating ligands (*see above*). In that study Mg^{2+} inhibition was measured in the presence of 10 μM Ca^{2+} where the inhibition is mediated primarily by the inhibition gate (Type-II inhibition at Gate I, *see* Fig. 1). Thus it appears that H_2O_2 opens the activation gate (Gate A in Fig. 1) and not the inhibition gate. The opposite specificity was observed for bastadin which alleviated Ca^{2+}- and Mg^{2+} inhibition (this was measured in the presence 30 μM Ca^{2+} so that Mg^{2+} inhibition was presumably mediated by Type-II inhibition) and left Ca^{2+} activation unaffected.

We believe the specific action of the above modifiers of RyR function make them useful probes for the regulation mechanisms operating in these channels. The different effects of these agents on the activation and inhibition gates further supports our contention that these gates operate independently and are associated with different parts of the RyR protein. Since the structural changes occurring as a result of CHAPS and Cs^+ treatments are poorly understood we could not attempt much structural interpretation from our data and further studies are required to conclusively establish the structural identity and independence of the two Ca^{2+}/Mg^{2+} gating mechanisms. We wish to stress that the main conclusions of this paper do not rely on results obtained from modified RyRs (*see previous section*). However, the effects of Ca^{2+} and Mg^{2+} on modified and normal RyRs do form a consistent picture of how the two gating mechanisms contribute to the overall Ca^{2+}- and Mg^{2+} regulation of these channels.

Evaluation of the Model

Three numerical predictions for the Ca^{2+} dependence of K_{Mg} are compared with our experimental values obtained from the three groups of RyRs in Fig. 4. In each case the binding constants used in Scheme 2 (Type-I inhibition) were the same where $K_{CA1} = 1$ nM, $K_{CA2} = 1$ μM, $K_{MA1} = 0.7$ μM and $K_{MA2} = 1$ mM. Differences between each group of RyRs appeared to result entirely from differences in Type-II inhibition. The parameter values of K_{XI} in Scheme 3 were adjusted to simulate: (i) RyRs which fail to show significant Ca^{2+} inhibition; $K_{XI} = 300$ mM; (ii) cardiac RyRs which show Ca^{2+} inhibition; $K_{XI} = 10$ mM; (iii) skeletal RyRs which show Ca^{2+} inhibition; $K_{XI} = 1.5$ mM. In each case the values of K_{XI} were able to accurately account for sensitivity of these RyR groups to Ca^{2+} inhibition.

Substitution of the model parameters into Eq. 5 shows that the Mg^{2+} sensitivity of Types-I and -II mechanisms crossover, on average at $[Ca^{2+}]_{co} \sim 10$ μM in cardiac RyRs and ~1 μM in skeletal RyRs (*see* Fig. 2). This explains why it was necessary to analyze Mg^{2+} inhibition at $[Ca^{2+}]$ extremes well separated from 10 μM to characterize each inhibition mechanism individually in cardiac RyRs and why it was more difficult to do this for skeletal RyRs where $[Ca^{2+}]_{co}$ lay at the lower end of the experimental $[Ca^{2+}]$ range. For cardiac RyRs 1 μM Ca^{2+} was generally low enough to ensure that the Type I mechanism could be studied without significant interference from the Type II mechanism. The Ca^{2+}-dependent Mg^{2+} sensitivity of the Type-I mechanism is such that when $[Ca^{2+}] < 10^{-3} \times K_I$, K_{Mg} will be largely determined by the Type-I mechanism. In our hands K_I for cardiac RyRs in 250 mM Cs^+ varied over the range 2 mM to 20 mM with a mean of 8 mM. In 37 measurements of Ca^{2+} inhibition, 19% of channels had K_I values less than 5 mM. Characteristics of the Type I mechanism in this study were derived from data collected from six experiments where the $[Ca^{2+}]$ was thought to be considerably less than $10^{-3} \times K_I$. In four experiments this was verified by direct measurement of K_I.

Type-I inhibition (Scheme 2) explains: (i) the increase in K_{Mg} as $[Ca^{2+}]$ increases from 0.3 to 10 μM (Fig. 4); (ii) our observation that channels which have lost their Ca^{2+} requirement for activation don't show Type-I Mg^{2+} inhibition; (iii) why Mg^{2+} inhibition in low $[Ca^{2+}]$ is mediated mainly by an increase in mean closed times (Figs. 9 and 11). The value of $H_{Mg} = 1$ for Type-I inhibition is in agreement with previous studies ($H_{Mg} = 1.1$ in low $[Ca^{2+}]$; Meissner et al., 1986) and suggests that the binding of a single Mg^{2+} to RyRs is sufficient to prevent the channel from opening.

Type-II mechanism determines the plateau phase in the $[Ca^{2+}]$ dependence of K_{Mg} in Fig. 4. The nonspecificity of the Type-II mechanism explains why both Ca^{2+}, and Mg^{2+} in the presence of 1 mM Ca^{2+}, have the same binding kinetics and produce identical gating kinetics for an individual channel in spite a large variations between channels and the effects of channel modifiers.

The Dual Nature of Mg^{2+} Inhibition Resulting from Types-I and II Mechanisms

Mg^{2+} inhibition by Types-I and -II mechanisms appear to have quite different gating signatures. Type-I inhibition (at low $[Ca^{2+}]$) increases mean closed time but has no effect on mean open times whereas Type-II inhibition causes both a decrease in mean open time and an increase in mean closed time (*see* Figs. 9–12). The Mg^{2+} dependence of mean durations at mid-range $[Ca^{2+}]$ (10 μM) suggests that both Type-I and II mechanisms operate concurrently in RyRs. Under these conditions, the onset of Mg^{2+} inhibition had the signature of the Type-I mechanism whereas advanced inhibition behaved more like Type-II mechanism. The concurrency of both inhibition mechanisms may also explain the relatively high values of H_{Mg} measured for skeletal RyRs in 1 μM Ca^{2+}. The superposition of Types-I and II mechanisms, under circumstances where they have similar Mg^{2+} sensitivities, would produce a Hill coefficient equal to the sum of the Hill coefficients of both mechanisms.

The dual nature of Mg^{2+} inhibition exhibited by RyRs at low and high $[Ca^{2+}]$ explains the different characteristics of Mg^{2+} inhibition reported in the literature. Single-channel studies which found that Mg^{2+} prevented Ca^{2+} activation of skeletal (e.g., Smith et al., 1986) and cardiac RyRs (Ashley & Williams, 1990) were based on measurements made at $[Ca^{2+}] < 1$ μM. Studies which suggested a link between Ca^{2+}- and Mg^{2+} inhibition were carried out at Ca^{2+} concentrations well above $[Ca^{2+}]_{co}$ (Boraso & Williams, 1994 $[Ca^{2+}] = 10$ μM; Mack et al., 1994, $[Ca^{2+}] = 30$ μM; Mickelson et al., 1990, $[Ca^{2+}] = 6$ μM).

Shomer et al. (1995) found that the optimal $[Ca^{2+}]$ for RyR open probability is increased from 10 μM to 4 mM by the presence of 5 mM Mg^{2+} and concluded that Mg^{2+} inhibits RyRs solely by competing with Ca^{2+} binding at the Ca^{2+}-activation site; thus contradicting the dual Mg^{2+}-inhibition model. However, the dual Mg^{2+}-inhibition model also predicts an upward shift in the optimal $[Ca^{2+}]$ for RyR open probability in the presence of Mg^{2+}. Therefore a shift in optimal $[Ca^{2+}]$, in itself, does not rule out a dual Mg^{2+}-inhibition mechanism.

Shomer et al. (1995), in studying $[^3H]$-ryanodine binding and single channels in bilayers report no significant difference in the sensitivity of RyRs from normal and MHS muscle to Mg^{2+} inhibition whereas their Ca^{2+} sensitivities to inhibition are significantly different. They conclude that Mg^{2+} inhibition is not associated with Ca^{2+} inhibition. However, there findings are complicated by an unexplained contradiction in the $[^3H]$-ryanodine binding results with earlier, more detailed reports by same authors (Mickelson et al., 1990). Moreover, the $[Ca^{2+}]$ at which Mg^{2+} inhibition was determined was not stated by Shomer et al. (1995) so that a direct comparison of their data with predictions of the Double Gate Model is not possible. However, the results of Shomer et al. (1995) are at variance with the findings of a bilayer study by Laver et al. (1996) which showed that RyRs from MHS pig muscle are less inhibited by Mg^{2+} than RyRs from normal muscle.

A Comparison of Ca^{2+} and Mg^{2+} Regulation in Skeletal and Cardiac RyRs

Our determinations of K_{Mg} are consistent with other measurements from RyRs in lipid bilayers. In skeletal RyRs in the presence of 2 μM $[Ca^{2+}]$, Smith et al. (1986) measured a K_{Mg} of 1.5 mM which is similar to our result ($K_{Mg} \sim 1$ mM). A comparison of Mg^{2+}-inhibition measurements in skeletal and cardiac RyRs in 10 μM $[Ca^{2+}]$ (Rousseau et al., 1986; Meissner & Henderson, 1987) shows that skeletal RyRs are inhibited by lower $[Mg^{2+}]$ than cardiac RyRs which is also consistent with our data.

Several lines of evidence indicate that skeletal and cardiac RyRs possess similar Ca^{2+}- and Mg^{2+}-regulation mechanisms with the main difference between them being that in the cardiac isoform, Type-II inhibition has a 10-fold lower Ca^{2+} and Mg^{2+} sensitivity. (i) It is possible to account for the observed differences in Mg^{2+} and Ca^{2+} sensitivity of RyRs from skeletal and cardiac muscle solely by variations in Type-II inhibition (*c.f.* Figs. 2 and 4). (ii) Mg^{2+} inhibition in the plateau phase of its Ca^{2+} dependence in skeletal and cardiac RyRs have similar Hill coefficients. (iii) Mg^{2+} inhibition has similar effects on the open and closed lifetime parameters in both isoforms (*c.f.* Figs. 12 and 13). (iv) Both skeletal and cardiac RyRs show Type-II inhibition that does not discriminate between Ca^{2+} and Mg^{2+}. For cardiac RyRs the nonspecificity is demonstrated in Figs. 6 to 8 and for skeletal RyRs by Meissner et al. (1986).

Meissner et al. (1986) showed that inhibition of $^{45}Ca^{2+}$ release from skeletal SR vesicles, via RyRs, by Ca^{2+} and Mg^{2+} was the same provided $[Ca^{2+}]$ exceeded 5 μM. In this study the same equivalence of Ca^{2+} and Mg^{2+} occurred in cardiac RyRs provided $[Ca^{2+}]$ exceeded 100 μM. One substantial difference between the two studies is that inhibition of skeletal RyRs in Meissner et al. (1986) occurred at much lower divalent ion concentrations than observed here; $K_{Ca} = K_{Mg} \sim 1$ mM and $H_{Mg} \sim 2$ in this study, compared with $K_{Ca} = 150$ μM and $K_{Mg} = 100$ μM and $H_{Mg} = 1.6$ (Meissner et al., 1986). The order of magnitude difference between the values of K_{Ca} and K_{Mg} obtained here (using 250 mM

CsCl) and by Meissner et al. (1986, using 100 mM KCl) is to be expected from the differences in the ionic strength of the bath solutions (K_{Ca} and K_{Mg} depend on ionic strength, see below).

Mg^{2+} Inhibition in Intact Fibers

The kinetics of RyR activation by Ca^{2+} and inhibition by Ca^{2+} and Mg^{2+} are altered by physiological (mM) concentrations of ATP. Consequently the relative contributions of Types-I and -II inhibition under physiological conditions will differ from those determined in this study. For example, skeletal RyRs can be activated by mM ATP in the absence of Ca^{2+} (Smith et al., 1986; Meissner, 1994). Physiological [ATP] may open RyRs even if Mg^{2+} is bound to the Ca^{2+}-activation site. In that case Type-I inhibition would be bypassed in vivo and would not contribute to Mg^{2+} inhibition of RyRs in skeletal muscle. Other differences between our experimental conditions and those in muscle is the ionic strength of the aqueous phase and the chaotropic nature of Cs^+ compared with Na^+ and K^+. We find that K_I for Mg^{2+} and Ca^{2+} inhibition increases 3–4-fold when the ionic strength (CsCl) is increased from 250 mM to 500 mM. Shomer et al. (1993) reported a 100-fold increase in K_I between 100 mM and 1,000 mM NaCl determined from [H^3]-ryanodine binding assays. Thus, it is likely that with in vivo ionic strength (~150 mM) the $[Ca^{2+}]_{co}$ will occur at 3–4-fold lower [Ca^{2+}] than we report here. The irreversible, effects of Cs^+ on cardiac RyR were not apparent in the skeletal isoform so that the chaotropic nature of Cs^+ is probably not an important consideration when inferring RyR function in vivo from our single channel measurements.

Our results do suggest that it is possible for different Mg^{2+}-inhibition mechanisms to prevail in skeletal and cardiac muscle under physiological conditions; [Mg^{2+}] ~ 1 mM (*see* citations in Lamb & Stephenson, 1992) and [Ca^{2+}] varies from 100 nM at rest to 10 μM during contraction (Ruegg, 1986; Hirota et al., 1989). In cardiac muscle Mg^{2+} inhibition of RyRs may act mainly via the Type-I mechanism whereas in skeletal muscle the Type-II mechanism could be more significant.

A Possible Role for Type-II Mg^{2+} Inhibition in Skeletal Muscle

Our findings provide an answer to the question of why RyRs are inhibited by myoplasmic [Ca^{2+}] well in excess of that expected during muscle contraction. It seems likely that it is not the Ca^{2+} inhibition of RyRs that is physiologically relevant at all, but rather it is the Mg^{2+} inhibition with which Ca^{2+} shares a common mechanism (as suggested by Lamb, 1993). Furthermore, a direct role for Ca^{2+} inhibition in the presence of physiological [Mg^{2+}] (1 mM) is unlikely since the inhibition sites would be saturated by Mg^{2+} and a rise in [Ca^{2+}] as high as 1 mM would cause no significant increase in channel inhibition. Our findings also support Lamb's (1993) contention that deficiencies in Ca^{2+} release via RyRs in MHS muscle are due to the mutation induced changes in RyR inhibition by Mg^{2+} rather than in changes in Ca^{2+} inhibition.

It appears that Type-II inhibition is the most fragile and variable property of cardiac RyRs. Sensitivity of Type-II inhibition to divalent ions can be altered without affecting other channel properties by either changing ionic strength (Shomer et al., 1993), oxidation state of the RyR (Eager, Roden & Dulhunty, 1997), or exposing RyRs to H_2O_2 (Boraso & Williams, 1994), toxins (Mack et al., 1992), CHAPS or high [CsCl] (Laver et al., 1995). Therefore, the proposition that EC coupling in skeletal muscle may operate via a change in RyR sensitivity to Mg^{2+} (Lamb & Stephenson, 1992), is reasonable. Recent measurements of $^{45}Ca^{2+}$ release from skeletal triad vesicles (Ritucci & Corbett, 1995) clearly demonstrate that the Mg^{2+} sensitivity of RyRs is reduced 13-fold by depolarization of the transverse tubule. Though the particular Mg^{2+}-inhibition mechanism which is modulated by DHPRs is yet to be established, it is likely to involve the Type-II mechanism since removal of the Type-I mechanism can produce, at most, a 3-fold reduction in RyR Mg^{2+} sensitivity. In contrast, the lifting of Type-II inhibition during EC coupling may allow RyRs to be opened by resting [ATP] and [Ca^{2+}]. Relief of Type-I inhibition by rising myoplasmic [Ca^{2+}] during Ca^{2+} release from the SR (*see* Fig. 2) would provide a strong positive feedback mechanism for Ca^{2+} release. Reimposition of the Type-II inhibition upon DHPR depolarization would close RyRs even if myoplasmic [Ca^{2+}] was high. Thus modulation of the Type-II mechanism could explain the regulation of RyRs during EC coupling in skeletal muscle.

Conclusions

Cardiac and skeletal RyRs possess two Mg^{2+}-sensitive gates (Type-I and II) that produce Mg^{2+} inhibition via a reduction in the open probability of RyRs. In Type-I inhibition, Mg^{2+} competes with Ca^{2+} for the high affinity Ca^{2+}-activation site, thus preventing Ca^{2+} from opening the channel. Type-II inhibition results from the binding of Mg^{2+} to low affinity sites which do not discriminate between Ca^{2+} and Mg^{2+}. Calcium inhibition of RyRs also acts via the Type-II inhibition mechanism. Both Type-I and II mechanisms act independently, concurrently on the channel and such that the channel conducts only when both gates are open.

The relative contributions of Types I and II inhibition mechanisms to the total Mg^{2+} effect depend on cytoplasmic [Ca^{2+}] in such a way that the Type-II mechanism becomes a significant determinant of Mg^{2+} inhibition when cytoplasmic [Ca^{2+}] is equal to, or greater than 10 μM in cardiac muscle or 1 μM in skeletal muscle.

Type-I inhibition only determines the Mg^{2+} sensitivity of RyR inhibition at much lower [Ca^{2+}] and so is not the sole mechanism responsible for Mg^{2+} inhibition, as is often assumed.

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