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Differences in the regulation of RyR2 from human, sheep, and rat by Ca^{2+} and Mg^{2+} in the cytoplasm and in the lumen of the sarcoplasmic reticulum

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Regulation of the cardiac ryanodine receptor (RyR2) by intracellular Ca^{2+} and Mg^{2+} plays a key role in determining cardiac contraction and rhythmicity, but their role in regulating the human RyR2 remains poorly defined. The Ca^{2+}- and Mg^{2+}-dependent regulation of human RyR2 was recorded in artificial lipid bilayers in the presence of 2 mM ATP and compared with that in two commonly used animal models for RyR2 function (rat and sheep). Human RyR2 displayed cytoplasmic Ca^{2+} activation (K_a = 4 µM) and inhibition by cytoplasmic Mg^{2+} (K_i = 10 µM at 100 nM Ca^{2+}) that was similar to RyR2 from rat and sheep obtained under the same experimental conditions. However, in the presence of 0.1 mM Ca^{2+}, RyR2s from human were 3.5-fold less sensitive to cytoplasmic Mg^{2+} inhibition than those from sheep and rat. The K_a values for luminal Ca^{2+} activation were similar in the three species (35 µM for human, 12 µM for sheep, and 10 µM for rat). From the relationship between open probability and luminal [Ca^{2+}], the peak open probability for the human RyR2 was approximately the same as that for sheep, and both were ~10-fold greater than that for rat RyR2. Human RyR2 also showed the same sensitivity to luminal Mg^{2+} as that from sheep, whereas rat RyR2 was 10-fold more sensitive. In all species, modulation of RyR2 gating by luminal Ca^{2+} and Mg^{2+} only occurred when cytoplasmic [Ca^{2+}] was <3 µM. The activation response of RyR2 to luminal and cytoplasmic Ca^{2+} was strongly dependent on the Mg^{2+} concentration. Addition of physiological levels (1 mM) of Mg^{2+} raised the K_a for cytoplasmic Ca^{2+} to 30 µM (human and sheep) or 90 µM (rat) and raised the K_a for luminal Ca^{2+} to ~1 mM in all species. This is the first report of the regulation by Ca^{2+} and Mg^{2+} of native RyR2 receptor activity from healthy human hearts.

INTRODUCTION

The cardiac ryanodine receptor (RyR2), an intracellular Ca^{2+} release channel, plays a key role in excitation-contraction coupling in the heart. Depolarization of the sarcolemma opens voltage-dependent L-type Ca^{2+} channels (dihydropyridine receptors), which allows Ca^{2+} to enter the cell. The subsequent increase in cytoplasmic Ca^{2+} activates RyR2, which releases Ca^{2+} from the SR. Intracellular Ca^{2+}, Mg^{2+}, and ATP are allosteric regulators of RyR2 (Meissner and Henderson, 1987; Meissner, 1994; Laver and Honen, 2008) that play an important role in determining normal cardiac contraction and rhythmicity (Meissner, 1994; Bers, 2002), and their disruption can lead to sudden cardiac death (Blayney and Lai, 2009; Katz et al., 2009). Ca^{2+} in the SR lumen and cytoplasm activates RyR2, whereas Mg^{2+} (free concentration of ~1 mM in cytoplasm and lumen [Meissner, 1994]) is a channel inhibitor. During diastole, cytoplasmic and SR luminal Ca^{2+} concentrations are ~100 nM and ~1 mM, respectively (Ginsburg et al., 1998). During systole, clusters of RyR2s release Ca^{2+} into the confined region between the SR and sarcolemma/T-tubule membrane. Computer simulations of Ca^{2+} release estimate that cytoplasmic Ca^{2+} concentration near the RyR2 peaks at ~200 µM and that luminal Ca^{2+} declines to ~200 µM (Laver et al., 2013).

Single channel studies of RyR2 isolated from animal hearts (e.g. sheep, rat and dog) have provided valuable insights into the regulation of RyR2 by intracellular Ca^{2+}, Mg^{2+}, and ATP (Sitsapesan and Williams, 1997; Györke et al., 2002; Laver, 2005, 2007; Györke and Terentyev, 2008). These studies provide evidence for four different Ca^{2+}-dependent mechanisms, controlled by four Ca^{2+}/Mg^{2+} sites on each RyR2 subunit (Laver, 2010). RyR2 can

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be activated by Ca^{2+} binding to either the cytoplasmic side of the channel with ~2 μM affinity (A site [Smith et al., 1986; Hymel et al., 1988; Sitsapesan and Williams, 1994a]) or to the luminal side of the channel with ~0.1 mM affinity (L site [Sitsapesan and Williams, 1994b; Laver, 2007]). In addition, two mechanisms for cytoplasmic Ca^{2+} inhibition of RyR2 have been identified, with channels inhibited by millimolar concentrations of cytoplasmic Ca^{2+} at the I_1 site (Meissner, 1986; Laver et al., 1995) and partially inhibited by micromolar concentrations at the I_2 site (Laver, 2007). Mg^{2+} inhibits RyR2 by competing with Ca^{2+} at the A site (Smith et al., 1986) and the L site (Laver and Honen, 2008) where, unlike Ca^{2+}, Mg^{2+} binding does not cause channel opening. Mg^{2+} also inhibits RyR2 because it acts as a surrogate for Ca^{2+} at the I_1 site (Laver et al., 1997a).

Interest in the function of human RyR2 has been spurred by the recent understanding that heart dysfunction associated with heart failure is generally associated with aberrant Ca^{2+} fluxes across the sarcolemma and SR of cardiac cells (Bers et al., 2003). Although the regulation of human RyR2 by intracellular Ca^{2+} and Mg^{2+} is central to our understanding of SR Ca^{2+} fluxes, its regulation characteristics are still sparsely defined (Marx et al., 2000; Jiang et al., 2002). One study has examined cytoplasmic Ca^{2+} activation, and this was done using recombinant human RyR2 (Wehrens et al., 2004). Here we present the first analysis of RyR2 isolated from human heart and its regulation by intracellular Ca^{2+} and Mg^{2+}. We also compare these gating properties of human RyR2 with RyR2 from sheep and rat heart (two commonly used animal models for RyR2 function).

MATERIALS AND METHODS

Heart tissue

Human left ventricle tissue (n = 4) was obtained from healthy donor hearts (Table 1 summarizes the donor heart details). These hearts were collected at the site of organ donation by the St. Vincent's Hospital (Darlinghurst) surgical team. The hearts were flushed with ice-cold cardioplegia, packaged under sterile conditions, transported by the Australian Red Cross Blood Service and delivered to the Bosch Institute. Although these hearts showed no sign of disease, they were not required for orthotopic heart transplantation for a range of reasons, including tissue incompatibility. Trans-mural sections of left ventricle free wall (~1 g) from these hearts were snap frozen in liquid nitrogen (−196°C) in not more than 4 h, and usually within 3 h, of death. Human tissues were obtained with approval from the Human Research Ethics Committees of both the University of Newcastle (approval number H-2009-0369) and the University of Sydney (approval numbers #09-2009-12146 and #2012/2814).

Animal tissues were obtained with approval from the Animal Care and Ethics Committee of the University of Newcastle (approval number #A-2009-153). Sheep hearts were obtained from ewes anesthetized with 5% pentobarbitone (intravenously) followed by oxygen/halothane and killed by barbiturate overdose (pentobarbitone, 150 mg/kg intravenously) before the heart was removed. Rat heart tissue was obtained from male rats (Sprague-Dawley, 6–9 wk). Rats were anesthetized with isoflurane and then decapitated. Hearts were rapidly removed and perfused in a Langendorff apparatus. The blood was removed by flushing the hearts with ice-cold Krebs-Henseleit buffer (mM: 120 NaCl, 25 $NaHCO_3$, 10 glucose, 5 KCl, 2 $MgCl_2$, 1 NaH_2PO_4, and 2.5 $CaCl_2$) for 2 min. Hearts were then perfused with warmed (37°C) and oxygenated (95% O_2–5% CO_2) Krebs-Henseleit buffer for 5 min. After perfusion, the whole hearts were snap frozen in liquid N_2. All tissue samples were stored at −80°C.

Preparation of SR vesicles

Heart muscle was minced and homogenized in a Waring blender (four 15-s bursts at high speed) in homogenizing buffer containing 0.3 M sucrose, 10 mM imidazole, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine, 0.5 mM dithiothreitol, 3 mM NaN_3, and 20 mM NaF, pH 6.9, followed by 10 manual strokes of a loose glass/glass Dounce homogenizer. The homogenate was centrifuged at 8,000 *g* for 20 min using an Optima L-100XP ultracentrifuge (Beckman Coulter). The supernatant was then centrifuged at 170,000 *g* for 30 min. The resultant pellet was resuspended with homogenizing buffer containing 0.65 M KCl using a glass/glass Dounce homogenizer, incubated for 30 min on ice, and then centrifuged at 8,000 *g* for 15 min. The supernatant was centrifuged at 170,000 *g* for 1 h, and the resulting microsomes from the pellet were resuspended in storage buffer (homogenizing buffer + 0.65 M KCl), snap-frozen in liquid nitrogen, and stored at −80°C.

Single channel measurements

RyRs from human, rat, and sheep heart were incorporated into artificial lipid bilayers that were formed from phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt) in 50 mg/ml *n*-decane. For single channel recording, the cis (cytoplasmic) and trans (luminal) solutions contained 250 mM Cs^+ (230 mM cesium methanesulfonate and 20 mM cesium chloride). During recordings, the composition of trans solution was altered by means of aliquot additions of stock solutions, and the cis solution was exchanged for solutions with specified free Ca^{2+} and Mg^{2+}. Solution exchange was performed using continuous local perfusion via a tube placed in close proximity to the bilayer, which could produce solution change within 1 s.

TABLE 1

Characteristics of hearts (Human Heart Tissue Repository, University of Sydney)

Sample	Section	Sex	Age	Cause of death	P_o (n)
6.008	LV	M	40	Stroke	0.06 ± 0.02 (20)
6.028	LV	F	62	Hypoxic brain injury	0.10 ± 0.05 (18)
7.012	LV	M	19	Motor cycle accident	0.05 ± 0.03 (2)
6.048	LV	F	54	Subarachnoid hemorrhage	0.12 ± 0.06 (16)

Hearts were not required for organ transplantation. The open probability (mean ± SEM) was measured in the presence of 100 nM cytoplasmic Ca^{2+} and 0.1 mM luminal Ca^{2+} from n experiments. LV, left ventricle.

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All solutions were buffered using 10 mM TES (*N*-Tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid; ICN Biomedicals) and titrated to pH 7.4 using CsOH (ICN Biomedicals). A Ca^{2+} electrode (Radiometer) was used in our experiments to determine the purity of Ca^{2+} buffers and Ca^{2+} stock solutions as well as free [Ca^{2+}] when [Ca^{2+}] was >100 nM. Free Ca^{2+} was adjusted with $CaCl_2$ and buffered using either (a) 4.5 mM BAPTA (1,2-bis(o-aminophenoxy)ethane- *N,N,N′,N′*-tetraacetic acid; Invitrogen) for free [Ca^{2+}] < 1 µM, (b) dibromo BAPTA (up to 2 mM) for free [Ca^{2+}] between 1 and 10 µM, or (c) sodium citrate (up to 6 mM) for free [Ca^{2+}] between 10 and 50 µM (sodium citrate was only used as a buffer in the absence of Mg^{2+}). Because all solutions applied in the cis bath contained ATP (ATP chelates Ca^{2+} and Mg^{2+}), free levels of Mg^{2+} (added as $MgCl_2$) were calculated using estimates of ATP purity and effective Mg^{2+} binding constants that were determined previously under our experimental conditions (Laver et al., 2004). The cesium salts were obtained from Sigma-Aldrich; $CaCl_2$ and $MgCl_2$ were obtained from BDH Chemicals.

Acquisition and analysis of ion channel recordings

An Axopatch 200B amplifier (Molecular Devices) was used to control the bilayer potential and record unitary currents. Electrical potential differences were expressed as cytoplasmic potential relative to the luminal potential (at virtual ground). The channel currents were recorded during the experiments using a 50-kHz sampling rate and 5-kHz low pass filtering. Before analysis, the current signal was redigitized at 5 kHz and low pass filtered at 1 kHz with a Gaussian digital filter. Single channel open probability (P_o) was measured using a threshold discriminator at 50% of channel amplitude. These parameters were measured from single channel records using Channel3 software (Nicholas W. Laver). Unless otherwise stated, measurements were performed with the cis solution voltage clamped at −40 mV.

Statistics

Unless otherwise stated, data are presented as means ± SEM. Individual readings of open probability were derived from 30–200 s of RyR2 recording depending on experimental conditions. At

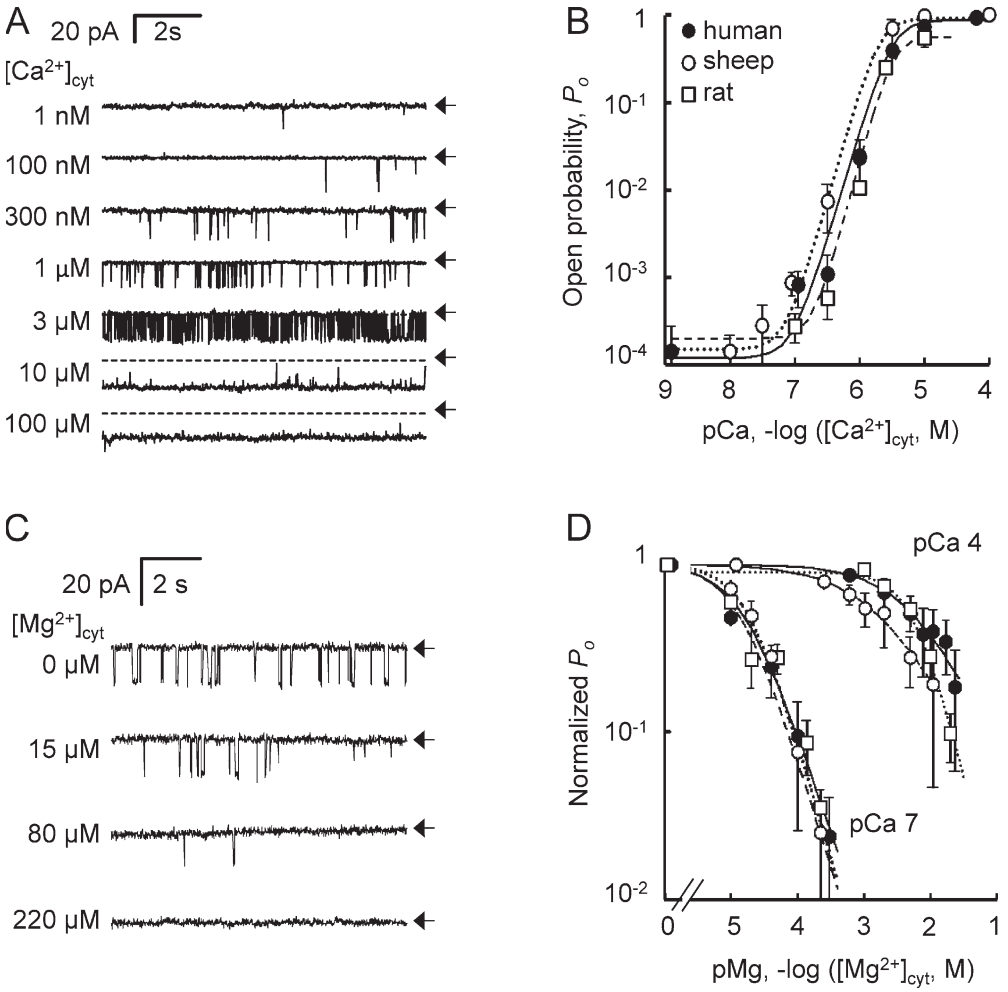

Figure 1. RyR2 regulation by cytoplasmic Ca^{2+} and Mg^{2+}. (A) Single channel recordings of RyR2 from healthy human heart with cytoplasmic [Ca^{2+}] indicated to the left of each trace. Channel recordings were taken in the virtual absence of luminal Ca^{2+} (1 nM) and in the presence of 2 mM ATP in the cytoplasm. Membrane potential is at −40 mV, and channel openings are downward current jumps from the baseline (arrows). (B) Cytoplasmic Ca^{2+} dependence of RyR2 P_o from human, sheep, and rat (as indicated in the legend, which also applies to D) under the same experimental conditions. (C) Single channel recordings of human RyR2 with cytoplasmic [Mg^{2+}] indicated to the left of each trace. Channel recordings were taken in the presence of cytoplasmic pCa 7 and 2 mM ATP (luminal pCa 4). (D) Inhibition of RyR2 by cytoplasmic [Mg^{2+}] in the presence of both cytoplasmic pCa 7 and pCa 4 and luminal pCa 4. The number of experiments and the Hill parameters are listed in Table 2. Data for rat at pCa 7 and sheep RyR2 in D were obtained from Li et al. (2013) and Laver and Honen (2008). Error bars indicate SEM.

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moderate to high levels of RyR2 activation, 10^3 to 10^4 opening events were recorded for each condition. At low levels of RyR activity, care was taken to ensure that the duration of recordings was sufficient to capture at least 30 channel openings. Hill equations were fitted to the dose–response data by the method of least squares, and errors on the Hill equation parameters were derived using the simplex method. The significance of the parameter differences between species was tested using Student's *t* test with a p-value <0.05 defining statistical significance.

RESULTS

Ionic conductance

Human RyR2 incorporated into lipid bilayers had a cesium ion conductance of 575 ± 5 pS ($n = 6$) in symmetric 250 mM CsCl ($[Ca^{2+}] < 1$ µM), which is close to values of 525 ± 10 pS ($n = 6$) for sheep RyRs and 460 ± 10 pS ($n = 6$) for rat RyR2s obtained under the same conditions. We compared the gating kinetics of cardiac RyR2s from four healthy human hearts (see Table 1 for source information). Myocytes from these four hearts were previously found to have contractility and Ca^{2+} handling properties that are consistent with healthy donor hearts (Wijnker et al., 2011; van Dijk et al., 2012; Hamdani et al., 2013; Mollova et al., 2013; Sequeira et al., 2013). We found no significant difference in open probability, P_o, among RyRs from these four hearts (Table 1). Therefore, in our analysis of the concentration dependencies of cytoplasmic and luminal Ca^{2+} and Mg^{2+} regulation of RyR2, the data were pooled from all heart samples. Regulation by intracellular Ca^{2+} and Mg^{2+} of human RyR2 was compared

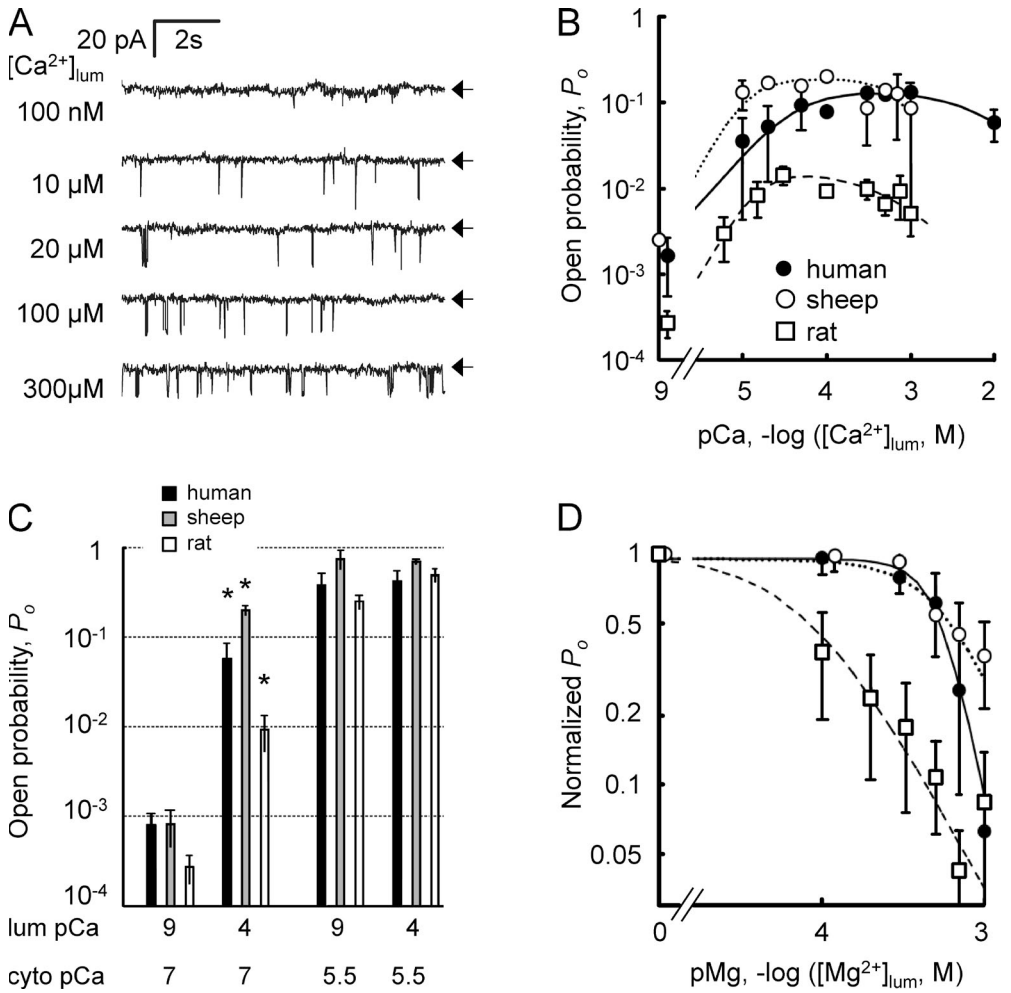

Figure 2. RyR2 regulation by luminal Ca^{2+} and Mg^{2+}. (A) Single channel recordings of RyR2 from healthy human heart with luminal $[Ca^{2+}]$ indicated to the left of each trace. Channel recordings were taken in the presence of 100 nM cytoplasmic Ca^{2+} (pCa 7) and 2 mM ATP in the cytoplasm at −40 mV. Channel openings are downward current jumps from the baseline (arrows). (B) Effect of luminal Ca^{2+} on RyR2 P_o from human, sheep, and rat as indicated in the legend (legend also applies to D). The luminal $[Ca^{2+}]$ activation response of RyR2 was obtained using cytoplasmic pCa 7 and 2 mM ATP. (C) P_o of RyR2 from human, sheep, and rat in the presence of various combinations of cytoplasmic and luminal $[Ca^{2+}]$. Asterisks indicate significant difference to luminal pCa 9 (*, $P < 0.05$). (D) Luminal $[Mg^{2+}]$ inhibition response of RyR2 with cytoplasmic pCa 7 (2 mM ATP) and luminal pCa 4. The solid and dashed curves show Hill fits to the data. The number of experiments and the Hill parameters for rat and sheep RyR2 are listed in Table 3. Data points show the mean ± SEM. Data for rat RyR2 in B and D were obtained from Li et al. (2013).

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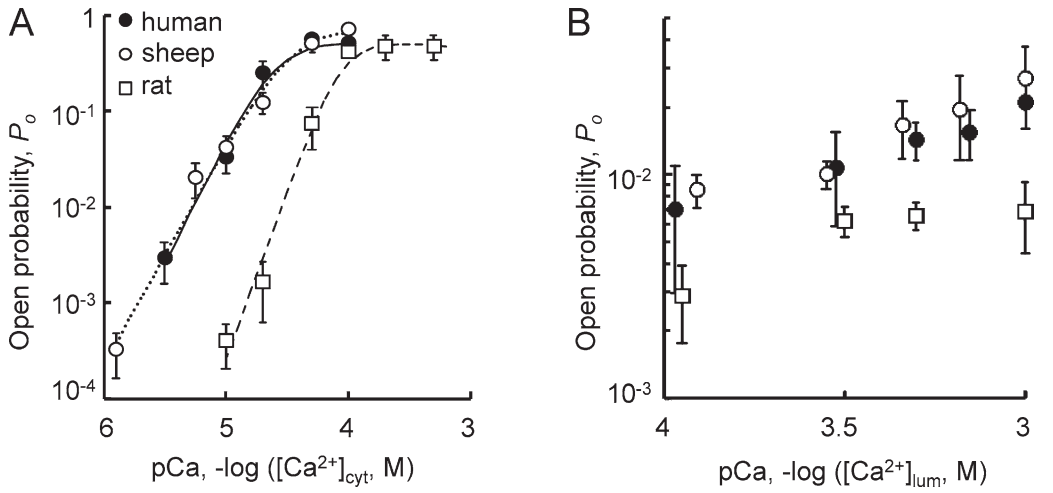

Figure 3. Ca^{2+} regulation of RyR2 in the presence of 1 mM Mg^{2+}. (A) Cytoplasmic [Ca^{2+}] activation response of RyR2 in the presence of 1 mM free Mg^{2+} and 2 mM ATP in the cytoplasm (luminal pCa 4). RyR2 from human, sheep, and rat are indicated in the legend (legend applies to A and B). (B) Luminal [Ca^{2+}] activation response of RyR2 in the presence of 1 mM luminal Mg^{2+} (cytoplasmic pCa 7 and 2 mM ATP). Data points show the mean ± SEM. The solid and dashed curves show Hill fits to the data. The numbers of experiments and the Hill parameters are listed in Table 4. Data for rat RyR2 in A were obtained from Cannell et al. (2013). Numbers of experiments in B were three to six for human, two to four for rat, and four for sheep.

with RyR2 from rat and sheep, two commonly used animal models for RyR2 function. The rationale for the experimental conditions in the three figures in this study is as follows: The cytoplasmic and luminal regulatory sites on the RyR2 (Figs. 1 and 2, respectively) involve regulation by both Mg^{2+} and Ca^{2+}. Hence, we initially show regulation by Ca^{2+} in the absence of Mg^{2+} (Fig. 1, A and B; and Fig. 2, A–C) and then regulation by Mg^{2+} with certain fixed levels of [Ca^{2+}] (Fig. 1, C and D; and Fig. 2 D). Fig. 3 then shows cytoplasmic and luminal regulation by Ca^{2+} at a physiological level of Mg^{2+}.

RyR2 from human, sheep, and rat were strongly activated by cytoplasmic Ca^{2+} alone (i.e., in the virtual absence of luminal Ca^{2+}, ∼1 nM; Fig. 1 A). The P_o of RyR2 from the three species was $\sim 10^{-4}$ at submicromolar Ca^{2+} (i.e., pCa 9 to 7) and increased to near unity at

TABLE 2

The Hill fit parameters for cytoplasmic regulation data shown in Fig. 1

Species	$[Ca^{2+}]_L$ pCa	$[Ca^{2+}]_C$ pCa	P_{max}	P_{min} (×10^3)	K_a/K_i	H_a/H_i	n
					μM		
Activation by cytoplasmic Ca^{2+}							
Human	9	-	0.9 ± 0.3	0.14 ± 0.02	4.0 ± 1.0	2.5 ± 0.4	4–6
Sheep	9	-	0.90 ± 0.03	0.15 ± 0.08	2.0 ± 0.3	2.6 ± 1.3	4–5
Rat	9	-	0.60 ± 0.10	0.20 ± 0.05	3.2 ± 0.5	2.8 ± 0.5	4
Inhibition by cytoplasmic Mg^{2+}							
Human	4	7	0.03 ± 0.02	0[a]	15 ± 4	1.2 ± 0.5	4
Sheep	4	7	0.19 ± 0.06	0[a]	18 ± 3	1.4 ± 0.3	3–26
Rat	4	7	0.019 ± 0.015	0[a]	13 ± 6	1.3 ± 0.7	10
Human	4	4	0.90 ± 0.06	0[a]	5,400 ± 800	0.8 ± 0.2	4–10
Sheep	4	4	0.98 ± 0.10	0[a]	1,600 ± 300[b]	0.7 ± 0.3	4–7
Rat	4	4	0.95 ± 0.03	0[a]	5,500 ± 600	1.6 ± 0.7	5

n indicates the number of experiments. The Hill equation for Ca^{2+} activation is

$$P_o = \frac{P_{max}\left(\left[Ca^{2+}\right]/K_a\right)^{H_a}}{\left(1+\left(\left[Ca^{2+}\right]/K_a\right)^{H_a}\right)} + P_{min}.$$

The Hill equation for Mg^{2+} inhibition is

$$P_o = \frac{P_{max}}{\left(1+\left(\left[Mg^{2+}\right]/K_i\right)^{H_i}\right)}.$$

[a]Parameters that were not adjusted during fitting.
[b]Significantly different to human ($P < 0.01$).

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micromolar concentrations (pCa 5 and 4). Activation of RyR2 showed no significant differences between species in their sensitivity to cytoplasmic Ca^{2+} with K_a values of 4 µM, 3.2 µM, and 2.0 µM for human, rat, and sheep, respectively (Fig. 1 B and Table 2).

The inhibitory effects of cytoplasmic Mg^{2+} at cytoplasmic [Ca^{2+}] of 100 nM (pCa 7) and 0.1 mM (pCa 4) were measured because previous studies using sheep RyR2s (Laver et al., 1997b) show that Mg^{2+} inhibition under these conditions is determined by different and independent mechanisms. At cytoplasmic pCa 7, Mg^{2+} is a competitive antagonist at the cytoplasmic Ca^{2+} activation site (A site), whereas at pCa 4, Mg^{2+} inhibits by binding to the low-affinity, cytoplasmic Mg^{2+}/Ca^{2+} inhibition site (I_1 site; Laver et al., 1997a). At cytoplasmic pCa 7, human RyR2s were strongly inhibited by micromolar cytoplasmic Mg^{2+} (Fig. 1 C). RyR2 from human, sheep, and rat were all similarly inhibited by cytoplasmic Mg^{2+} at pCa 7 (Fig. 1 D). However, at pCa 4, RyR2s from sheep were threefold more sensitive to cytoplasmic Mg^{2+} inhibition than those from rat and human.

Human RyR2 could be activated by luminal Ca^{2+} alone (i.e., at subactivating cytoplasmic Ca^{2+}, pCa 7; Fig. 2 A). RyR2s from all three species could be activated by luminal Ca^{2+} (Fig. 2 B) with a bell-shaped Ca^{2+} response. However, there were differences in the luminal Ca^{2+} responses between species. Thus, although RyR2 from human and sheep exhibited similar peak activation, rat RyR2 had a peak activation that was 10-fold less ($P < 0.001$). Hill fits to the data (Fig. 2 B, solid and dashed curves) give K_a values of 35 µM, 12 µM, and 10 µM for human, sheep, and rat RyR2s, respectively. The declining phase of the luminal Ca^{2+} response was less pronounced in human and could only be detected at luminal pCa 2. The magnitude of the luminal Ca^{2+} response depended on cytoplasmic Ca^{2+} as shown in Fig. 2 C. At cytoplasmic pCa 7, increasing luminal [Ca^{2+}] (from pCa 9 to pCa 4) caused increases in open probability in RyR2 from human, sheep, and rat, but the effect of luminal Ca^{2+} on RyR2 activity was negligible once RyRs were appreciably activated by cytoplasmic Ca^{2+} at pCa 5.5 (3 µM).

Millimolar concentrations of Mg^{2+} added to the luminal bath inhibited RyRs from human, sheep, and rat at cytoplasmic pCa 7, and the luminal [Mg^{2+}] dependencies of P_o are shown in Fig. 2 D and Table 3. RyR2s from human and sheep heart showed a similar dependence on luminal Mg^{2+}, whereas rat RyR2 showed a markedly different dependence. Hill fits to the data show K_i values for luminal Mg^{2+} inhibition of 550 µM, 650 µM, and 78 µM for human, sheep, and rat RyR2s, respectively. Interestingly, the higher Hill coefficient for the luminal [Mg^{2+}] response of human and sheep RyRs compensated for the differences in K_i, leaving no significant difference in the levels of inhibition at physiological (1 mM) luminal Mg^{2+} for all species.

Ca^{2+} activation in the presence of [Mg^{2+}]

To assess the gating properties of RyR2 at physiological (systolic) concentrations of Ca^{2+} and Mg^{2+}, we measured the activation of RyR2 by luminal and cytoplasmic Ca^{2+} in the presence of 1 mM Mg^{2+} (Fig. 3). Fig. 3 A and Table 4 show the cytoplasmic [Ca^{2+}] dependence of P_o in the presence of cytoplasmic 1 mM free Mg^{2+} and 2 mM ATP. The lower limit to the experimental cytoplasmic [Ca^{2+}] range was set by the minimum P_o that we could reliably measure ($P_o \sim 10^{-4}$). Under these conditions, human RyR2 had a K_a for Ca^{2+} activation of 25 ± 8 µM and a maximum P_o of 0.55 ± 0.1. In the presence of 1 mM cytoplasmic Mg^{2+}, RyR2 from human and sheep have similar Ca^{2+} activation properties, whereas RyR2 from rat is threefold less sensitive to cytoplasmic Ca^{2+} ($P < 0.01$).

The luminal Ca^{2+} activation properties of RyR2 channels from human, sheep, and rat in the presence of 1 mM luminal Mg^{2+} (without cytoplasmic Mg^{2+}) are shown in Fig. 3 B. The near linear luminal Ca^{2+} dependencies of RyR P_o seen here are substantially different to the strongly

TABLE 3

The Hill fit parameters for luminal regulation data shown in Fig. 2

Species	$[Ca^{2+}]_L$ pCa	$[Ca^{2+}]_C$ pCa	P_{max}	P_{min} (×10^3)	K_a/K_i	H_a/H_i	n
					µM		
Activation by luminal Ca^{2+}							
Human	-	7	0.14 ± 0.03	0.8 ± 0.3	35 ± 12	1.3 ± 0.2	3–17
Sheep	-	7	0.19 ± 0.05	0.9 ± 0.4	12 ± 3	2[a]	3–11
Rat	-	7	0.013 ± 0.003[b]	0.3 ± 0.1	10 ± 7	2[a]	3–16
Inhibition by luminal Mg^{2+}							
Human	4	7	0.09 ± 0.02	0[a]	550 ± 30	3.8 ± 1.8	5–6
Sheep	4	7	0.10 ± 0.04	0[a]	650 ± 80	2.0 ± 1.0	4–11
Rat	4	7	0.023 ± 0.011[c]	0[a]	78 ± 14[b]	1.3 ± 0.5	7

n indicates the number of experiments. Hill parameters for luminal Ca^{2+} inhibition at luminal approximately pCa 3 were poorly determined and not shown.

[a]Parameters that were not adjusted during fitting.

[b]Significantly different to human ($P < 0.01$).

[c]Significantly different to human ($P < 0.05$).

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saturating dependencies measured in the absence of luminal Mg^{2+} (Fig. 2). In the presence of luminal Mg^{2+}, the RyR2 channels from human and sheep exhibit identical responses, which were not significantly ($P > 0.1$) different than that shown for RyR2 from rat.

DISCUSSION

Mechanisms for Ca^{2+} and Mg^{2+} regulation of RyR2

This study presents a detailed analysis of the regulation of human RyR2 by intracellular Ca^{2+} and Mg^{2+}. We report the first measurements of the concentration dependencies of native human RyR2 regulation by luminal and cytoplasmic Ca^{2+} and Mg^{2+}, and we compare these properties with those of RyR2s from sheep and rat, which have been isolated and measured under identical conditions. We find that human RyR2 displayed the same Ca^{2+}/Mg^{2+} regulation phenomena as seen in rat and sheep. RyR2 was activated by cytoplasmic and luminal Ca^{2+} and inhibited by Mg^{2+} in the lumen and cytoplasm. However, although the sensitivity of human and sheep RyR2 to luminal Ca^{2+} and Mg^{2+} were similar, the rat RyR2 was less sensitive to activation by luminal Ca^{2+} and was more sensitive (i.e., a lower K_i) to inhibition by luminal Mg^{2+}.

The molecular mechanism for these differences is not yet clear. They could arise from differences in the amino acid sequences of the RyR2s, which share 96–98% similarity(ExPASy: human Q92736; rat B0LPN4; and sheep Q9MZD9). However, the sensitivity to cytoplasmic Ca^{2+} of isolated human native RyR2 reported here ($K_a = 4$ µM; Table 2) is nearly 10-fold lower than that seen in recombinant RyR2 in the presence of 50 mM luminal Ca^{2+} (K_a between 0.4 and 0.7 µM [Wehrens et al., 2004]). The difference could be caused by either (a) factors other than amino acid sequence being important in determining RyR2 activity or (b) the different concentrations of luminal Ca^{2+} used. Species-specific posttranslational modifications or differing degrees of association with co-proteins such as FKBP12/12.6 (Galfré et al., 2012) could be responsible. For example, the adrenergic tone of rat hearts is known to affect the activity of RyR2 isolated from these hearts and incorporated into artificial lipid bilayers (Li et al., 2013). It is also possible that variations in adrenergic tone (and thus protein phosphorylation) between species could underlie the different RyR2 properties from human, sheep, and rat.

The cytoplasmic Ca^{2+} activation and Mg^{2+} inhibition of RyR2 observed in several species has been attributed to the combined action of a high-affinity Ca^{2+} activation site (Smith et al., 1986; Hymel et al., 1988) and a low-affinity divalent cation inhibition site (Meissner, 1986; Laver et al., 1995, 1997a) located in the cytoplasm-facing domains of the RyR2. These sites are referred to here as the A site and I_1 site after the nomenclature of Balog et al. (2001). Mg^{2+} inhibition that is apparent at submicromolar (diastolic) cytoplasmic Ca^{2+} occurs because Mg^{2+} binding occludes the A site and prevents Ca^{2+} from binding and activating the RyR2, and unlike Ca^{2+}, Mg^{2+} does not cause channel opening. Competition between Ca^{2+} and Mg^{2+} for the A site reduces the potency of this form of Mg^{2+} inhibition at high (systolic) Ca^{2+}, revealing another Mg^{2+} inhibition mechanism caused by Mg^{2+} binding to the I_1 site. We found that RyR2s from human, rat, and sheep are similarly activated by cytoplasmic Ca^{2+} and inhibited by Mg^{2+} at cytoplasmic pCa 7 (Fig. 1), suggesting that the A sites could be strongly conserved between species. However, the different RyR2 sensitivities to cytoplasmic Mg^{2+} seen at cytoplasmic pCa 4 between human and sheep indicated interspecies differences in the I_1 sites.

Interestingly, there are substantial differences in the luminal regulation of RyR2 from rat compared with that seen in human and sheep (Fig. 2), where it can be seen that rat RyR2 is less activated by luminal Ca^{2+} and more inhibited by luminal Mg^{2+}. Interpreting the precise mechanism for this phenomenon is complicated by the fact that luminal Ca^{2+} can pass through the channel and act via the cytoplasmic facing A site (Tripathy and Meissner, 1996; Laver, 2007). However, because the cytoplasmic sites produce similar regulation of RyR2 across species (Fig. 1), the observed differences in RyR2 inhibition by luminal Mg^{2+} and activation by luminal Ca^{2+} indicate a divergence in the properties of the L site of rat RyR2s from human and sheep.

The importance of luminal Mg^{2+}

The excitability of the SR was shown in early studies to be substantially increased by increasing luminal [Ca^{2+}]

TABLE 4

The Hill fit parameters for cytoplasmic Ca^{2+} activation of RyR2 in the presence of 1 mM Mg^{2+} shown in Fig. 3 A

Species	Luminal pCa	P_{max}	P_{min}	K_a	H_a	n
				µM		
Activation by cytoplasmic Ca^{2+}						
Human	4	0.55 ± 0.10	0[a]	25 ± 8	2.6 ± 0.6	4–8
Sheep	4	0.70 ± 0.10	0[a]	34 ± 5	2.2 ± 0.2	5–10
Rat	4	0.54 ± 0.06	0[a]	88 ± 9[b]	3.5 ± 0.4	4–7

n indicates the number of experiments.
[a]Parameters that were not adjusted during fitting.
[b]Significantly different to human ($P < 0.01$).

(Fabiato and Fabiato, 1977). It is now understood that luminal Ca^{2+} has a direct activating effect on the RyR2 Ca^{2+} release channel (Sitsapesan and Williams, 1997; Györke et al., 2002) and that in addition to Ca^{2+} influx through the plasma membrane, Ca^{2+} in the SR can trigger Ca^{2+} release and so contribute to pacemaking and rhythmicity in cardiac muscle (Vinogradova et al., 2005; Imtiaz et al., 2010). The importance of luminal Ca^{2+} as a determinant of cardiac rhythm is highlighted by arrhythmias associated with excessive luminal [Ca^{2+}] (Schlotthauer and Bers, 2000). Fig. 3 B shows that RyR2 P_o increases from 0.007 to almost 0.02, (approximately threefold increase) when luminal [Ca^{2+}] increases from 0.1 to 1 mM. This is in agreement with the luminal Ca^{2+} dependence of Ca^{2+} spark frequency in cardiomyocytes (Zima et al., 2010). We also show that this dependence on luminal Ca^{2+} over the physiological [Ca^{2+}] range requires the presence of physiological [Mg^{2+}]. The removal of luminal Mg^{2+} in our bilayer experiments markedly decreased the K_a for luminal Ca^{2+} activation from ~1 mM to <0.1 mM, causing RyR2 P_o to become nearly constant over the physiological range of luminal Ca^{2+} (Fig. 2 B). These results suggest that luminal Mg^{2+} may play an important role in the cell in shaping the dose–response of RyR2s to store Ca^{2+} load during diastole.

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