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Differential Activation by Ca^{2+}, ATP and Caffeine of Cardiac and Skeletal Muscle Ryanodine Receptors after Block by Mg^{2+}

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Abstract. The block of rabbit skeletal ryanodine receptors (RyR1) and dog heart RyR2 by cytosolic [Mg^{2+}], and its reversal by agonists Ca^{2+}, ATP and caffeine was studied in planar bilayers. Mg^{2+} effects were tested at submaximal activating [Ca^{2+}] (5 μM). Approximately one third of the RyR1s had low open probability (''LA channels'') in the absence of Mg^{2+}. All other RyR1s displayed higher activity (''HA channels''). Cytosolic Mg^{2+} (1 mM) blocked individual RyR1 channels to varying degrees (32 to 100%). LA channels had residual $P_o < 0.005$ in 1 mM Mg^{2+} and reactivated poorly with [Ca^{2+}] (100 μM), caffeine (5 mM), or ATP (4 mM; all at constant 1 mM Mg^{2+}). HA channels had variable activity in Mg^{2+} and variable degree of recovery from Mg^{2+} block with Ca^{2+}, caffeine or ATP application. Nearly all cardiac RyR2s displayed high activity in 5 μM [Ca^{2+}]. They also had variable sensitivity to Mg^{2+}. However, the RyR2s consistently recovered from Mg^{2+} block with 100 μM [Ca^{2+}] or caffeine application, but not when ATP was added. Thus, at physiological [Mg^{2+}], RyR2s behaved as relatively homogeneous Ca^{2+}/caffeine-gated HA channels. In contrast, RyR1s displayed functional heterogeneity that arises from differential modulatory actions of Ca^{2+} and ATP. These differences between RyR1 and RyR2 function may reflect their respective roles in muscle physiology and excitation-contraction coupling.

Key words: Calcium release channel — Ryanodine receptors — Sarcoplasmic reticulum — Excitation-contraction coupling — Intracellular calcium

Introduction

The ryanodine receptor (RyR)/calcium release channels are the main pathway for releasing calcium from the sarcoplasmic reticulum (SR) into the cytosol, as required for activation of the contractile machinery of striated muscles [8, 13, 30, 37, 41]. In heart, calcium entry through cardiac dihydropyridine receptors (DHPR) activates the RyRs (calcium-induced calcium release, CICR), whereas in skeletal muscle fibers, the macroscopic phenomenology of EC-coupling is referred as depolarization-induced calcium release (DICR) and the DHPR in the T-tubule acts as a voltage sensor [10, 11, 15, 20, 32, 42]. The scheme in skeletal muscle is that depolarization of the T-tubule induces interaction between ''voltage-sensitive domains'' of the DHPR and cytosolic segments in ''coupled'' RyRs, which release Ca (DICR), and in turn activate ''uncoupled'' RyRs by CICR [4, 10, 14, 17, 20, 32, 41–43]. Coexistence of RyR1 channels ''coupled'' or ''uncoupled'' to DHPRs in the same skeletal fiber implies that individual RyR1 channel function is heterogeneous. Thus, there are noticeable differences in how cardiac and skeletal RyR channels interact ''in vivo'' with the DHPR channels during their respective modes of excitation-contraction (EC) coupling.

There is also evidence, from ''in vitro'' channel measurements, for functional differences between the RyR1 (skeletal form) and RyR2 (heart form) isoforms [reviewed in 6, 8, 37, and 41]. For example, we have shown that the RyR1 channel, but not the RyR2 channel, is modulated by FK-506 binding protein 12 (FKBP12) and FKBP 12.6 [1, 44–46]. The FKBP proteins are tightly associated to the RyR channels and may be involved in the excitation-contraction

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coupling process [42, 44–46]. RyR1 channels, when reconstituted into planar lipid bilayers, behave more heterogeneously than RyR2 channels in their response to Ca^{2+} [6]. We have also found heterogeneity in the response of RyR1 channels to ATP [39, 40]. Strikingly, almost half of the RyR1 channels were activated with 0.5 mM ATP at nanomolar [Ca^{2+}] (i.e., a virtually Ca^{2+}-free solution). This was not the case for RyR2 channels [1, 39, 40].

In this report, we compare individual skeletal and cardiac muscle RyR channel function with regard to their sensitivity to inhibition by Mg^{2+}. Cytosolic free [Mg^{2+}] affects "in vivo" RyR activity both at rest and in response to excitatory events [22, 23]. In vitro studies show that free [Mg^{2+}], at physiological levels [33], inhibit RyR channels [8, 30, 37, 38]. There appear to be differences between RyR1 and RyR2 channels sensitivity to Mg^{2+} [8, 30], as well as their interaction with Ca^{2+} when Mg^{2+} is present [6, 25].

In light of RyR1 channel functional heterogeneity [24–27, 31, 39, 40], the focus here is on individual channels rather than averages of data from several channels. Since cardiac and skeletal RyR channels differ in their sensitivity to ATP, caffeine and Ca^{2+} [1, 6, 8, 30, 39, 40], we also studied the recovery from block by Mg^{2+} with these agonists. We find that RyR2 channels, despite their variable sensitivity to Mg^{2+}, behaved similarly to Ca^{2+} and caffeine-gated channels. By contrast, individual RyR1 channels displayed functional heterogeneity in their response to ATP, Ca^{2+} and caffeine.

Materials and Methods

Drugs and Chemicals

DibromoBAPTA(1,2-Bis(2-amino-5-bromophenoxy)ethane-N,N, N′,N′]-tetraacetic acid), obtained from Fluka (Buchs, Switzerland) was dissolved at 10 mM in aqueous buffer containing 125 mM Tris, and pH was titrated to 7.4 with HEPES. Calmodulin was a gift from Dr. Roger Colbran (Department of Molecular Physiology & Biophysics, Vanderbilt University Medical School, Nashville, TN) or obtained from Alexis (San Diego, CA). Recombinant FKBP12 and FKBP12.6 proteins were prepared in our laboratory. The FK506 analogue L-683, 590, which we referred to as FK590, was obtained from Dr. Greg Wiederrecht of Merck Research Laboratories (Rahway, NJ). Phospholipids were obtained from Avanti (Alabaster, Alabama), and decane from Aldrich (Milwaukee, WI). All other drugs and chemicals were from Sigma (St. Louis, MO) or were reagent grade.

Isolation of Membrane Fractions

Six preparations of rabbit skeletal muscle terminal cisternae (TC) vesicles were used. Tissue for these preparations was obtained predominantly from white muscle of the leg and back of the rabbit as described previously [35]. Data on cardiac ryanodine receptors were also obtained by using six different preparations of sarcoplasmic reticulum microsomal fractions from canine heart, which were isolated from fresh dog heart ventricular muscle as described by Chamberlain et al [5].

Bilayer Technique

Planar lipid bilayers were formed on 100- or 180-μM diameter circular holes in Teflon septa, separating two 1.3-ml compartments, as previously described [6, 36]. Briefly, the trans compartment was filled with HEPES-Ca [containing HEPES 250 mM, and $Ca(OH)_2$ 53 mM (pH 7.4)] and was clamped at 0 mV by the EPC-7 (List electronics, Darmstadt, Germany) or Axopatch 200B (Axon Instruments, Foster City, CA) patch-clamp amplifier.The cis compartment (ground) was filled with HEPES-Tris solution [HEPES 250 mM and Tris 140 mM (pH 7.4)]. Bilayers of a 5:3:2 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (50 mg/ml in decane) were painted onto the holes of the bilayer septum from the cis side, and subsequently 500–1000 mM CsCl and 1 mM $CaCl_2$ were added to the cis solution to promote vesicle fusion. TC vesicles from rabbit skeletal muscle (1–5 μg) or dog cardiac SR (5–15μg) were added to the cis solution. After Cl^-currents were observed, the cis chamber was perfused for 5 min at 4 ml/min with HEPES-TRIS solution. Channel currents are depicted as negative (downward deflections) in figures, and reflect cation flux from trans (luminal) to cis (cytosolic) compartments.

Data were filtered though a low-pass Bessel filter at 0.8–1 kHz, digitized at 2–5 kHz a 12-bit analog-to-digital converter, and stored on an optical disk for computer analysis, using the pClamp6.03 software (Axon Instruments). In most cases, open probabilities (P_o) were calculated by 50%-threshold analysis. In experiments where the channels have very short open times, P_o had to be estimated as proportion of areas in amplitude histograms. In multichannel experiments, the global open probability (nP_o) was estimated with the program "NPO" (by Jinliang Sui, M.D., Ph.D., Dept. of Physiology & Biophysics, Mount Sinai School of Medicine, New York, NY). In the figures, the P_o (for single channels) or nP_o/x (for multiple channels) are shown, with x representing the maximal number of current levels observed. RyR channels were identified by their current amplitudes at 0 mV (from 3.5 to 4.4 pA), slope conductance (from 80 to 110 pS), and reversal potential (+30 to 40 mV; trans–cis) [6]. When tested, ryanodine (2 μM) always induced long openings of about 1/3 maximal current amplitude. RyR channels were biphasically gated by Ca^{2+}, activated by ATP, and blocked by Ruthenium Red. Since single RyR1 channels reconstituted in planar lipid bilayers represent a functionally heterogeneous population [1, 6, 7, 24, 27, 31], comparison of RyR channel activity of under different conditions was based on paired observations.

As before [1, 6, 7], dibromoBAPTA was utilized to buffer free Ca^{2+} because of its very good buffering capacity in the range where RyR channels are activated (0.5–10 μM). DibromoBATPA also has a very low affinity for Mg^{2+}. Free Ca^{2+} levels were determined by measuring fluorescence of Fura2 or with a calcium-sensitive electrode. Free [Ca^{2+}] and [Mg^{2+}] were calculated with the program MaxChelator 1.70 [3].

Results

The block by Mg^{2+} of single RyR channels of rabbit skeletal muscle and dog heart was studied after reconstitution of SR membranes into planar lipid

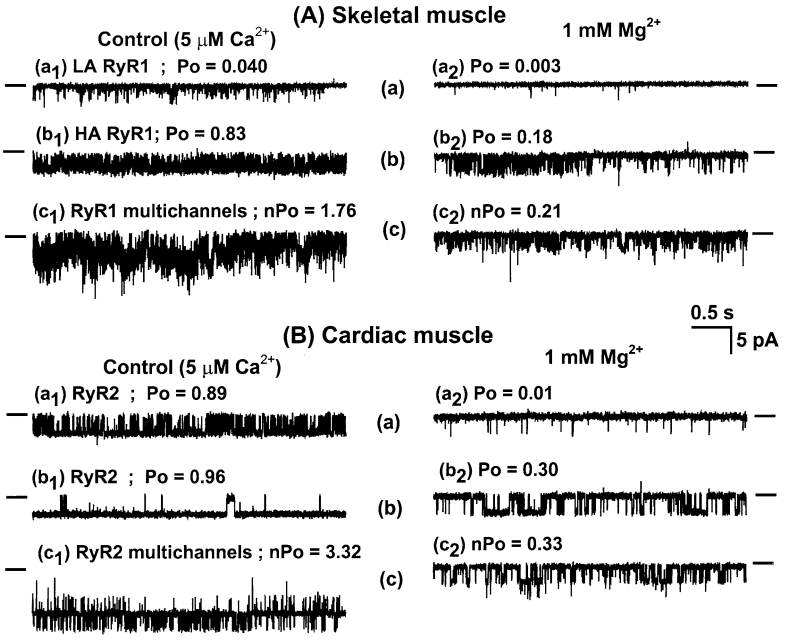

Fig. 1. Effect of Mg^{2+} on single-channel activity of ryanodine receptors. (*A*) Examples of skeletal RyRs: (*a*) "low activity" (LA) RyR; (*b*) "high activity" (HA) RyR; (*c*) multichannels ($n = 3$ channel levels). (*B*) Examples of cardiac RyRs: (*a*) and (*b*) single cardiac RyR channels; (*c*) multichannels ($n = 4$ current levels observed). All channel recordings were carried out at 0 mV (holding potential) after reconstituting rabbit skeletal muscle terminal cisternae or dog cardiac sarcoplasmic reticulum into planar lipid bilayers. In all the studies except Fig. 6, the cytosolic $[Ca^{2+}]$ (*cis chamber*) was maintained constant at 5 μM with dibromoBAPTA. Representative traces and open probability values (index of channel activity) for all experiments are shown in the absence (control) and in the presence of 1 mM Mg^{2+}. Channel openings are shown as downward deflections. The closed state is indicated to the left or right of the channel recordings.

bilayers. In all experiments the solutions were at pH 7.4, the bilayer membrane was clamped at 0 mV, and Ca^{2+}(50 mM in trans) was used as current carrier.

Individual RyR Channels from Cardiac and Skeletal Muscle are Blocked by Magnesium to Varying Degrees

It was previously described that single RyR1 channels, but not RyR2 channels, are functionally heterogeneous. The RyR1 population consists of at least two channel types, low activity (LA) and high activity (HA) [6, 27, 31]. The low activity (LA) RyR1 channels (~30%) had $P_o \leq 0.1$ and activated with an EC_{50} for $[Ca^{2+}]_{free}$ of 60–200 μM. The high activity (HA) RyR1 channels, although more heterogeneous, reached much higher maximal P_o values ranging from 0.2 to 0.9 with an EC_{50} for $[Ca^{2+}]_{free}$ of 0.6–10 μM [6]. Consequently, in order to better understand RyR1 channel response to agonists and antagonists, the behavior of individual channels, rather than just average channel behavior, was examined.

Individual channel recordings at submaximally activating free cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{free}$) of 5 μM before (control) and after addition of 1 mM $[Mg^{2+}]$ to the cytosolic face (cis) are shown in Fig. 1*A* and 1*B*, respectively. Individual RyR1 and RyR2 channels displayed variable activity in control condition (no Mg^{2+} added). The heterogeneity is much more apparent in the RyR1 case, as previously reported [6]. In all cases the activity of both RyR1 and RyR2 channels was reduced by 1 mM $[Mg^{2+}]$ (1 mM approximates cytosolic $[Mg^{2+}]$) [33] (Fig. 1*A* and 1*B*).

The results of several experiments are illustrated in Fig. 2. The P_o values of individual channels at 5 μM Ca^{2+} (no added Mg^{2+}) are plotted against the % degree of inhibition by 1 mM Mg^{2+}. The % inhibition is defined as

$$100(1 - P_o^{(1\text{ mM } Mg^{2+})}/P_o^{(\text{no } Mg^{2+} \text{ added})})$$

Note that the control P_o values in the absence of Mg^{2+} varied from 0.01 to 0.95 for RyR1 channels (Fig. 2*A*), which is a consequence of the existence of at least two functionally distinct channel types in the population, low activity (LA) and high activity (HA), as previously described [6, 27, 31]. The LA RyR1 channels (Fig. 2*A*, ○) had a mean P_o of 0.065± 0.013 ($n = 12$). The inhibition by 1 mM Mg^{2+} was 94 ± 2% (ranging from 84 to 100%) corresponding to a mean P_o of 0.005 ± 0.003. For the data in Fig. 2*A*, the mean P_o of the HA RyR1 was 0.498 ± 0.065 (●; $n = 34$). On average, the inhibition by 1 mM Mg^{2+} was 87 ± 3% (ranging from 32 to 100%, $n = 34$ paired observations), corresponding to a mean P_o of 0.062 ± 0.014.

RyR2 channel behavior was more homogeneous with most channels being highly active (Fig. 2*B*). For RyR2 channels, only HA-like behavior was observed [6]. The P_o values in absence of added Mg^{2+} (control condition) ranged from 0.37 to 0.99 (Fig. 2*B*). Mean P_o values for RyR2 channels were on average higher than those for HA RyR1 channels ($p < 0.01$), both in control (0.747 ± 0.030, $n = 37$) and 1 mM Mg^{2+} (0.108 ± 0.018). The inhibition by 1 mM $[Mg^{2+}]$ (86 ± 2%, range of 45 to 100%, $n = 37$, paired observations) was similar to that found in RyR1 channels.

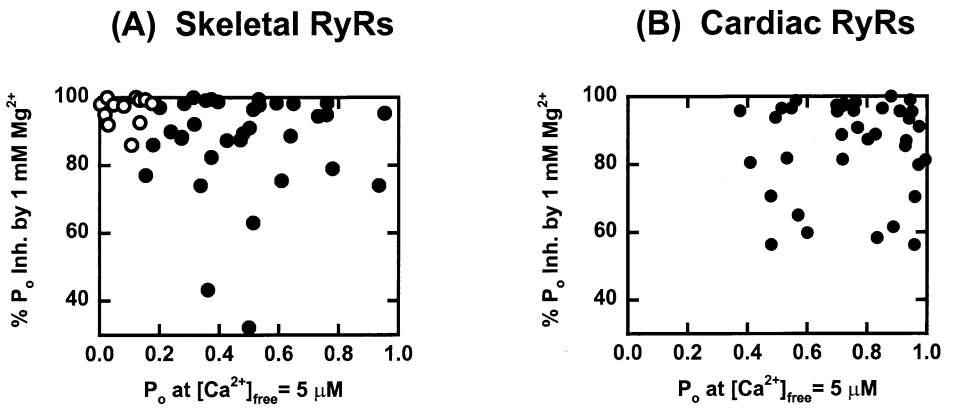

Fig. 2. Effect of addition of 1 mM Mg^{2+} on open probability of RyRs. (*A*) Open probability (P_o) data from $n=46$ RyRs from skeletal muscle (○ and ● for LA and HA skeletal RyRs, respectively), and (*B*) from $n = 37$ RyRs (○) from heart. Cytosolic [Ca^{2+}] was buffered at 5 μM using dibromoBAPTA. The *X* axis shows the open probability (P_o) of the channels before the addition of 1 mM Mg^{2+} (control values). The *Y* axis shows the % of inhibition on P_o induced by 1 mM Mg^{2+}, defined as $\{1-[P_o^{(1mM\ Mg^{2+})}])/[P_o^{(control)}]\}$. 100.

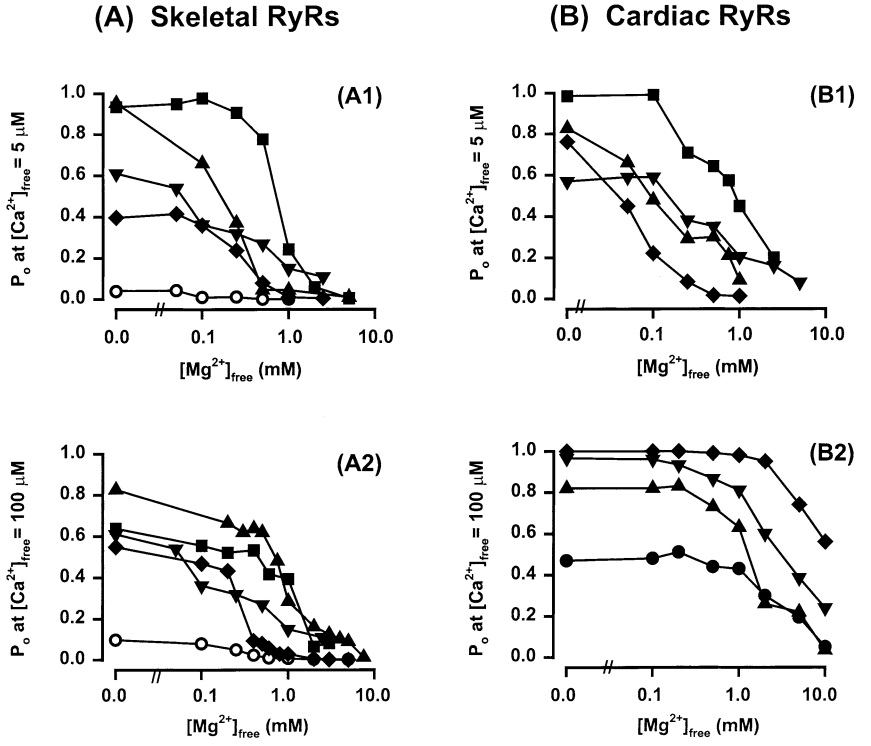

Fig. 3. Effect of cumulative addition of Mg^{2+} on the open probability of RyRs. (*A*) Summary of experiments with measurement of open probability *vs.* [Mg^{2+}], carried out with skeletal RyRs bathed with 5 μM [Ca^{2+}] (*A1*) or with 100 μM [Ca^{2+}] (*A2*); in both cases examples of HA RyRs (filled symbols) and LA RyRs (○) are shown. (*B*) Summary of experiments with cardiac RyRs in 5 μM [Ca^{2+}] (*B1*) or with 100 μM [Ca^{2+}] (*B2*). Each curve represents a different channel with data collection of 4 minutes or longer for each point.

For a number of RyR1 and RyR2 channels exposed to 5 μM Ca^{2+} (submaximally activating levels), we determined the changes in open probability elicited by addition of Mg^{2+} between 0.05 and 5 mM (Fig. 3*A1* and 3*B1*). For LA RyR1 channels (with $P_o < 0.1$ in control conditions), the sensitivity to Mg^{2+} was always high with average inhibition >90%, ($n=10$); mean value half maximal inhibitory [Mg^{2+}] (IC_{50}) for Mg^{2+} was 140 ± 25 μM (range from 95 to 200 μM, $n=4$). For HA RyR1 channels, Mg^{2+} sensitivity was more variable, ranging from 130 to 750 μM but average IC_{50} was 280 ± 50 μM ($n=9$). For RyR2 channels, mean value for IC_{50} was 340 ± 120 μM (range 70 – 990 μM, $n=8$). Statistically, in 5 μM Ca^{2+} the

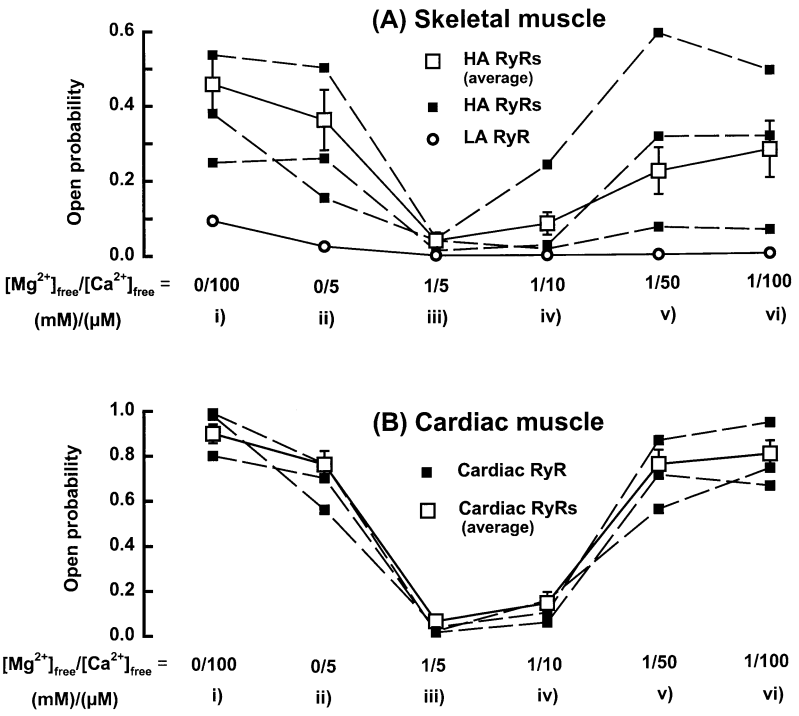

Fig. 4. Effect of cytosolic Ca^{2+} on the block of RyRs by Mg^{2+}. P_o of the channels was determined in the absence of Mg^{2+} at $[Ca^{2+}]$ of 100 and 5 μM (by adding dibromoBAPTA), indicated in the figure as 0/100 (*i*) and 0/5 (*ii*) for $[Mg^{2+}]$ (millimolar)/$[Ca^{2+}]_i$; (micromolar). Then 1 mM Mg^{2+} was added, followed by additions of Ca^{2+} to final concentrations of 5, 10, 50 and 100 μM Ca^{2+} (indicated 1/5 (*iii*); 1/10 (*iv*); 1/50 (*v*) and 1/100 (*vi*)). (*A*) Experiments with skeletal muscle; three individual HA RyR1, (■), one LA RyR (○) and average of eleven HA RyR experiments (□). (*B*) Experiments with cardiac RyR; (■) three different channels, and (□) average of six experiments.

average IC_{50} for LA RyR1 ≤ IC_{50} for HA RyR1 ≤ IC_{50} RyR2. Even though LA RyR1 channels apparently displayed high sensitivity to Mg^{2+}, there were no significant differences in the means when the three populations of channels were compared (because of the wide variation of sensitivities within the HA and RyR2 populations).

Ca^{2+} Reactivates RyR2 and HARyR1 Channels after Mg^{2+} Block, but not LARyR1 Channels

Figure 4*A* shows the action of increasing $[Ca^{2+}]$ on Mg^{2+} block in RyR1 channels (no ATP added). The LA RyR1 channels were previously defined by its overall low activity and its sensitivity to cytosolic $[Ca^{2+}]$ [6] (*see* above). For three LA RyR1 channels, P_o was low in all conditions (e.g., Figure 4*A*, ○). With $[Ca^{2+}]$ of 100 μM, P_o was 0.061 ± 0.022 ($n=3$). Decreasing $[Ca^{2+}]$ to 5 μM decreased P_o to 0.014 ± 0.005. Subsequent addition of 1 mM Mg^{2+} resulted in $P_o \sim 0$ in all channels tested. The Mg^{2+} inhibited the channel even when $[Ca^{2+}]$ was elevated again to 100 μM (0.001 ± 0.001, $n=3$) or 1.4 ± 1.4% of control P_o values) (control = 100 μM Ca^{2+}, no Mg^{2+}). Other RyR1 channels ($n=11$) were classified as HA channels and data from three of those are shown in Fig. 4*A* (■). Changing $[Ca^{2+}]$ from 100 to 5 μM, decreased P_o from 0.459 ± 0.077 to 0.362 ± 0.081 ($n=11$). Addition of Mg^{2+} block further decreased P_o to 0.040 ± 0.021. However, in contrast to LA RyR1 channels, HA RyR1 channels recovered to different extents with increasing $[Ca^{2+}]$ to 100 μM (0.251 ± 0.075) or 54 ± 12% (range 20 to 130%) of control P_o values (100 μM Ca^{2+}, no Mg^{2+}). The pooled values of HA RyR channels ($n=11$;□) are also shown (Fig. 4*A*).

Similar experiments were performed with RyR2 channels. Three single-channel examples (■) and the pooled values ($n=6$; □) are given in Fig. 4*B*. The P_o averaged 0.899 ± 0.042 in control (100 μM Ca^{2+}) but decreased to 0.760 ± 0.060 ($n = 6$) with addition of 5 μM Ca^{2+}. The P_o decreased further to 0.0625 ± 0.027 when Mg^{2+} was added. After subsequent increase in $[Ca^{2+}]$ to 100 μM, these channels reactivated (6/6 experiments) from the Mg^{2+} block, reaching a P_o value of 0.800 ± 0.059, or 89 ± 6% (range: 66 – 106%) of the control. This % of P_o recovery for cardiac RyR2 was significantly higher than the 54 ± 12% observed for skeletal HA RyR1 ($p < 0.05$).

We also measured the cumulative effects of Mg^{2+} on channels incubated with 100 μM Ca^{2+}. Figure 3 *A2* and *B2* show changes in P_o of RyR1 and RyR2 channels after addition of Mg^{2+} (0.1 to 10 mM). For LA RyR1 channels (Fig. 3*A2*, ○), the half maximal inhibitory $[Mg^{2+}]$ (IC_{50}) ranged from 170 to 300 μM with a mean value of 230 ± 30 μM, ($n=4$). HA RyR1 channels (Fig. 3*A2*, filled symbols) in 100 μM Ca^{2+} were less sensitive ($p < 0.02$) to $[Mg^{2+}]$ and their IC_{50}'s ranged from 300 μM to 1.5 mM with a mean value of 860 ± 140 μM ($n=7$). For RyR2 channels (Fig. 3*B2*), sensitivity to Mg^{2+} was even lower. Mean value for IC_{50} was 4.55 ± 1.4 mM (range from 1.8 to 11 mM, $n=9$), substantially higher than that of the RyR1 channels ($p < 0.05$). Thus, for RyRs studied with maximal activating Ca^{2+} (100 μM Ca^{2+}), the

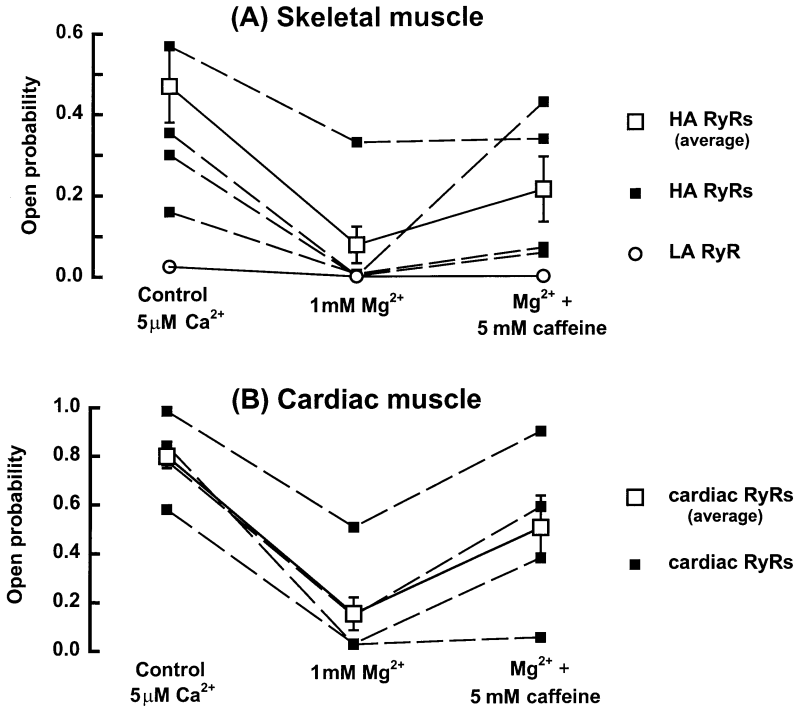

Fig 5. Effect of caffeine (5 mM) on Mg^{2+} block of RyRs. Cytosolic [Ca^{2+}] was buffered using dibromoBAPTA at 5 μM throughout the experiment. P_o of RyR was measured first in the absence of Mg^{2+}, ATP and caffeine (*control*). Then, 1 mM Mg^{2+} was added (Mg^{2+}), which partially or fully blocked the channel. After 5–10min, 5 μM caffeine was added to the cytosolic (cis) chamber (Mg^{2+} + caffeine). (*A*) Experiments with skeletal muscle RyR1; HA RyRs (■), LA RyR (○), and average of $n = 7$ experiments with HA channels (□). (*B*) Experiments with cardiac RyR2s, (■) individual data, and (□) average of eight experiments.

IC_{50}'s for Mg^{2+} inhibition were in the following order: LA RyR1 < HA RyR1 < RyR2. This correlates closely with the IC_{50}'s for Ca^{2+} inhibition reported previously in these three channel types [6].

In Presence of Mg^{2+}; Caffeine Activates RyR2 and HA RyR1 Channels, but not LA, RyRl Channels

Caffeine appears to activate RyR channels by increasing their sensitivity to Ca^{2+} [8, 30, 34, 37]. We tested the action of 5 mM caffeine on Mg^{2+} block (5 μM [Ca^{2+}], no ATP) and these data are shown in Fig. 5. Not surprisingly, RyR1 channels responded quite heterogeneously to the caffeine challenge. LA RyR1 channels (Fig. 5*B*, ○, $n = 3$) closed with Mg^{2+} added and displayed only occasional short openings with high levels of Ca^{2+}. The same behavior was observed after the caffeine challenge (i.e., LA RyR1 channels did not recover after caffeine application). By contrast, caffeine with variable efficacy reversed Mg^{2+} block of HA RyR1 channels in 5/7 experiments (Fig. 5*A*, ■; 4 individual HA RyR1 channels are shown). On average, caffeine increased P_o values from 13.8 ± 8.8% of control with 1mM Mg^{2+} (ranging from 0.5 to 65%) to 45 ± 15% (range 4 to 113%). The pooled data for HA RyR1 channels (Fig. 5*A*, □) are also shown.

With RyR2 channels, recovery from Mg^{2+} block with 5 mM caffeine was observed in 7 of 8 channels. Four examples for individual channels (■) and the pooled data (□) are shown in Fig. 5*B*. Caffeine (5 mM) increased the P_o values in 1 mM Mg^{2+} from 19 ± 7% of control (range 0 to 51%) to 66 ± 18% (range 13 to 147%). Thus, RyR2 channels as well as HA RyR1 channels (but not LA RyR1 channels) reactivated with caffeine to variable degrees. The response to caffeine of the RyR2 channel reveals some heterogeneity among the channel population.

In Presence of Mg^{2+}, ATP Activates HA RyR1 Channels but not LA RyR1 or RyR2 Channels

Figure 6 and Fig. 7 show the effect of adding ATP to skeletal RyRs incubated with 1 mM Mg^{2+}. Similarly, Fig. 8 shows ATP modulation of cardiac RyR2 channels in 1 mM Mg solutions. ATP is a known activator of RyR channels [8, 30, 37, 39, 41]. In muscle, the cytosolic [ATP] is ~4–5 mM [16]. Here, we test whether increasing total [ATP] to 4 mM reactivates channels blocked by [Mg^{2+}]. In all these experiments (Fig. 6 to Fig. 8), the "cytosolic" free [Mg^{2+}] (1 mM) and free [Ca^{2+}] (5 μM) were kept constant. Two examples of LA RyR1 channel behavior are given in Fig. 6 and Fig. 7*B* (O). As usual, these channels had low activity in control (5 μM Ca^{2+}) with P_o near 0.026 ± 0.010 (range 0.007 to 0.045, $n = 4$) and openings were even less frequent after 1 mM Mg^{2+} was added (P_o was always < 0.001; Figs. 6, 7*B*, O). The frequency of short openings increased slightly with ATP application ($P_o = 0.001 \pm 0.001$ with 1 mM ATP; range 0 to 0.003) ($P_o = 0.002 \pm 0.002$ with 4 mM ATP; range 0 to 0.004). The ATP was added up to 4 mM ATP, keeping free [Mg^{2+}] and

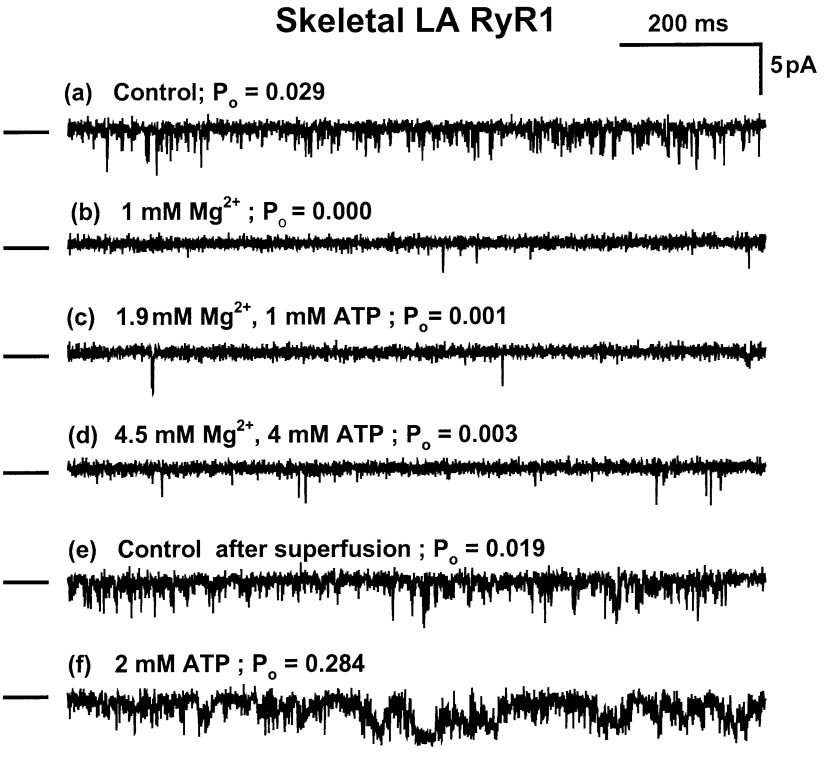

Fig 6. Response of LA SkM RyR channels to Mg^{2+} and ATP. The changes of activity in the same LA channel are shown throughout this figure, and it was studied at cytosolic [Ca^{2+}] = 5 μM (buffered with dibromoBAPTA). (*a*) The low-activity channel had low P_o at control conditions (no added Mg^{2+}), and (*b*) P_o decreased dramatically after addition of 1 mM Mg^{2+}. (*c*) and (*d*) Cumulative increase of total [Mg^{2+}]/[ATP] to 1.9/1 and 4.5/4 mM (for *b*, *c*, and *d*, the free [Mg^{2+}] is ~ 1 mM) did not reactivate the channel. (*e*) Perfusion of the cytosolic bath with HEPES-TRIS (4 min. at 5 ml/min) restored channel activity. (*f*) Addition of 2 mM ATP, in the absence of Mg^{2+}, activated the LA channel.

free [Ca^{2+}] constant (Figs. 6, 7*B*, ○). This Mg^{2+} block was reversible if Mg^{2+} and ATP were removed (P_o = 0.029 ± 0.010, n = 4; Figs. 6, 7*B*). In the absence of Mg^{2+}, 2mM ATP increased the activity of the channels to near 0.2 (Fig. 6*f*). Similar results were found with six other LA RyR1 channels and were included in the averaged values. For all LA RyR1 channels, millimolar ATP levels are required (in the absence of Mg^{2+}) to activate the channels. With 2 mM ATP, P_o increased from 0.026 ± 0.010 (range 0.007 to 0.045, n = 6) to 0.334 ± 0.127 (range 0.040 to 0.550).

The HA RyR1 channels [6] had P_o's ranging from 0.13 to 0.80 (n = 11). The addition of 1 mM Mg^{2+} inhibited these channels (Fig. 7) from a mean P_o value of 0.384 ± 0.061 (in control) to 0.037 ± 0.017 (n = 11). In contrast to LA channels (Figs. 6 and 7*B*, ○), all HA RyR1 channel (Figs. 7*A*, 7*B*, ■) recovered, at least partially, with ATP application. On average, the mean P_o value of Mg^{2+} blocked channels increased to 0.290 ± 0.056 after addition of 4 mM ATP. This corresponds to a recovery of 81 ± 15% (range 30 to 152%, n = 11) of their initial activity (in absence of Mg^{2+}). A second exposure to Mg^{2+}, after washing out ATP, again decreased P_o, indicating that the effect of ATP was reversible (*data not shown*). The pooled P_o values for all HA skeletal RyR channels are included in Fig. 7B, □).

The RyR2 channels (11/11 cases) had high P_o (range 0.42 to 0.98) in the control condition (Fig. 8*A*). These channels were inhibited to various extents by addition of 1 mM Mg^{2+}, as shown in the three examples (Fig. 8*B*, ■). However, unlike HA RyR1 channels, [ATP]'s up to 4 mM did not appreciably increase P_o of the blocked channels (Fig. 8*B*). On average (Fig. 8*B*, □), the P_o decreased from 0.788 ± 0.053 to 0.106 ± 0.034 after Mg^{2+} addition (n = 11, 13.4 ± 4% of control) and the channels recovered only to P_o of 0.177 ± 0.04 (22% of their control activity) after 1 mM ATP application (P_o = 0.166 ± 0.038, 20 ± 4% of control after 4 mM ATP, n = 11). Channel activity recovered completely after Mg^{2+} was removed (P_o = 0.801 ± 0.072, n = 8). In summary, ATP application reversed the blocking action of Mg^{2+} only for HA RyR1 channels. The same ATP application was considerably less effective in reversing the blocking action of Mg^{2+}on RyR2 or LA RyR1 channels. These differences may be related to the different sensitivities of these channels to ATP (*see* Discussion).

In Presence of Physiological Levels of Mg^{2+} and ATP, the Action of $[Ca^{2+}]_{free}$ on RyR1 and RyR2 Channels was Different

$[Ca^{2+}]_{free}$ titration curves of RyR1 and RyR2 channels in the presence of 5 mM ATP and 1 mM free [Mg^{2+}] are shown in Fig. 9. All LA RyR1 channels had very low P_o (Fig. 9*A*, ○). However, the P_o increased significantly (p < 0.01) from P_o = 0.0003 ± 0.0003 (P_o < 0.001 in all cases) at 100 nM $[Ca^{2+}]_{free}$ to

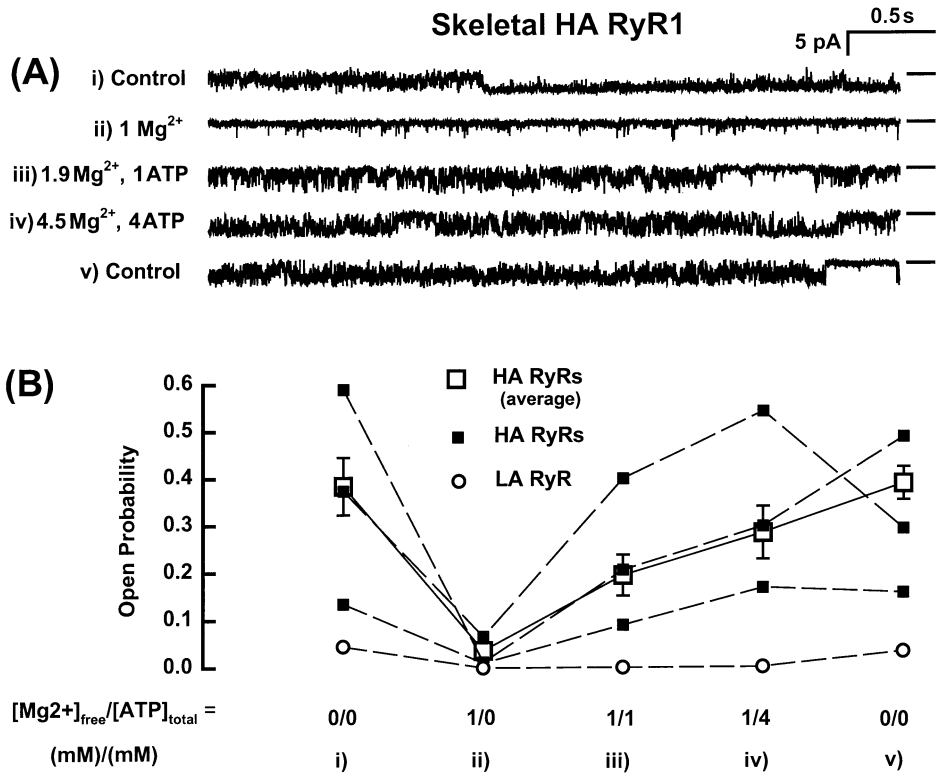

Fig. 7. Effect of addition of Mg^{2+} and ATP on P_o of skeletal muscle HA RyRS. The channels were exposed to constant cytosolic $[Ca^{2+}]$ of 5 μM throughout the experiment. (*A*) Examples of Ca^{2+} currents from a single HA RyR1. (*B*) Individual P_o values from 3 experiments with HA RyR1 (■), one LA RyR1 (○) and average values for 10 HA RyR1s (□). For both, the example in (*A*) and the data points in (*B*) conditions were:(*i*) HEPES-TRIS and $[Ca^{2+}] = 5$ μM in the absence of Mg^{2+} and ATP, which is indicated as *Control* (*A*) and with ratio (*B*) of $[Mg^{2+}]_{free}$ (estimated as indicated in Methods) to $[ATP]_{total}$ of 0/0 ; (*ii*) addition of 1 mM Mg^{2+} with no added ATP ratio = 1/0); (*iii*) presence of 1.9 total mM Mg^{2+} and 1 mM ATP (1.9 Mg^{2+}, 1 ATP; ratio = 1/1); (*iv*) presence of 4.5 mM total Mg^{2+} and 4 mM ATP ratio = 1/4; (*v*) after removal of Mg^{2+} and ATP by perfusion of the cytosolic bath with 20 ml/4 min of HEPES-TRIS (*Control* = ratio = 0/0).

0.007 ± 0.002 (range 0.004–0.015, $n = 4$) at $[Ca^{2+}]_{free}$ = 100 μM. The changes in the activity of the LA RyRs with $[Ca^{2+}]_{free}$ are better reflected in the channel recordings of Fig. 10. They illustrate that even though the increase in P_o is very modest numerically, there is a great increase in the frequency of brief LA RyR1 openings when $[Ca^{2+}]_{free}$ is raised from 100 nM to 100 μM (Fig. 10 and Fig. 10 legend).

As shown in Fig. 9*A* (●), individual HA RyR channels activated with different patterns. Some HA RyR1 channels still had substantial activity at resting $[Ca^{+2}]_{free}$ (100 nM) levels. HA RyR1 activated to different degrees with increasing $[Ca^{+2}]_{free}$ and maximal P_o observed at 100 μM $[Ca^{+2}]_{free}$ varied considerably (Fig. 9, ●). The EC_{50} for $[Ca^{+2}]_{free}$ of HA RyR1 channels was 2.5 ± 1.1 μM (range 0.3–10 μM, n = 8). As a result of averaging various patterns of channel behavior, the mean HA RyR channel (Fig. 9*A*, □) appears to activate gradually (Fig. 9*A*, □).

In contrast, RyR2 channels show a unique pattern of behavior (Fig. 9*B*). Most channels displayed infrequent openings at $[Ca^{+2}]_{free}$ < 1 μM (i.e., $P_o \leq 0.01$), with the exception of a few channels that had residual P_o's ~0.01–0.05 at resting $[Ca^{+2}]_{free}$. All the RyR2 activated sharply and to high P_o values. The EC_{50} for $[Ca^{2+}]_{free}$ RyR2 channels was 14 ± 2.0 μM (range 5–25 μM, $n = 8$), significantly higher than those of HA RyR1 channels ($p < 0.01$). Comparing the effects of Ca^{2+}on RyRs in the presence *vs* absence of ATP (Fig. *9 vs*. Fig. *4*), it is clear that physiological levels of ATP had a more profound impact on skeletal RyR1 behavior than on cardiac RyR2 channels.

Putative RyR-Channel Modulators Do not Change Gating Behavior from LA to HA Channels

We have addressed previously [6] and also consider here (*see* Discussion) the underlying basis for LA RyR channels in skeletal muscle. We also tested four potential factors that are known to modulate RyRs aiming to determine if they could induce changes in gating behavior from LA to HA types. As the results were negative, we will only list the agents utilized and we will briefly summarize some details of the studies.

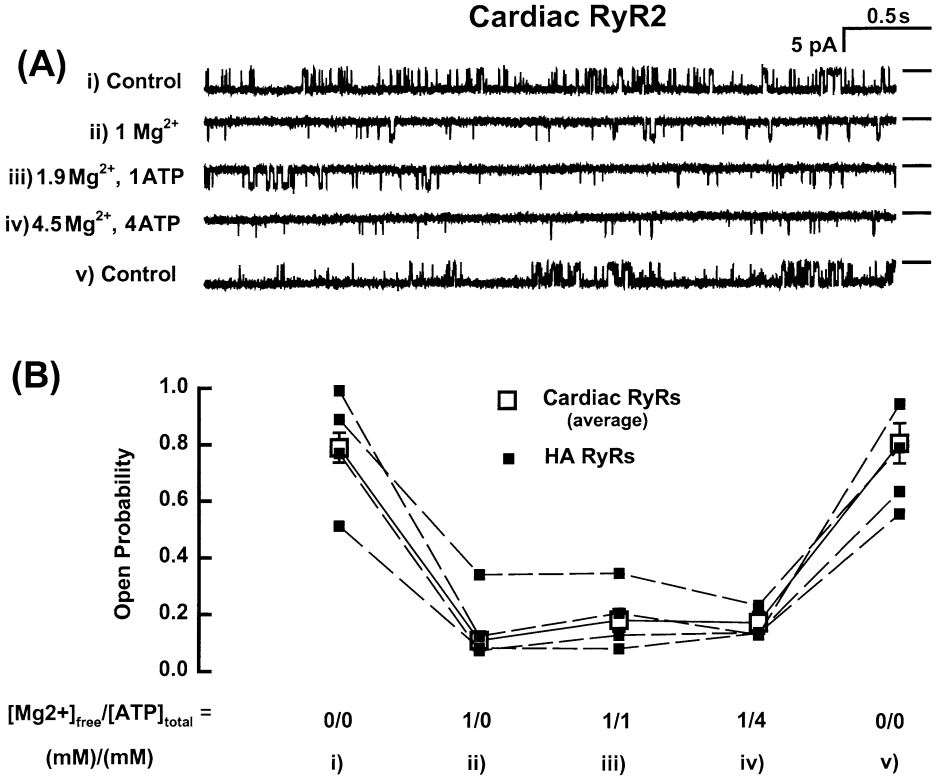

Fig 8. Effect of addition of Mg^{2+} and ATP on P_o of cardiac RyR2 exposed to constant cytosolic [Ca^{2+}] of 5 μM. (*A*) Examples of RyR2 Ca^{2+} currents and (*B*) P_o values of 4 individual RyRs and averages from 8 experiments with cardiac RyRs. For both, the example in (*A*) and the data points in (*B*) condition were: (*i*) HEPES-TRIS and [Ca^{2+}] = 5 μM in the absence of Mg^{2+} and ATP (Indicated *Control* with ratio of $[Mg^{2+}]_{free}$ to $[ATP]_{total}$ of 0/0; (*ii*) addition of 1 mM Mg^{2+} with no added ATP (ratio = 1/0); (*iii*) in the presence of 1.9 mM total Mg^{2+} and 1 mM ATP (1.9 Mg^{2+}, 1 ATP; ratio = 1/1); (*iv*) in the presence of 4.5 mM total Mg^{2+} and 4 mM ATP (ratio = 1/4);(*v*) after removal of Mg^{2+} and ATP by perfusion of the cytosolic bath with 20ml/4min of HEPES-TRIS (*Control* = ratio = 0/0).

We tested (*a*) FKBP12: The FKBPs are known be associated with and modulate RyR1 channels [1, 44–46]. In our previous work [1, 44], HA RyR channels did not become LA channels upon FKBP12 addition to the bath. Here, we find that FKBP12 addition (1 μM) does not convert LA to HA RyR1 channels ($n=6$ experiments). We also found that exposure of LA channels to 25–50 μM FK590 (or FK506) for 30 minutes reversibly increased P_o in the presence of ATP (from $P_o = 0.23 \pm 0.5$ to 0.45 ± 0.09; $p < 0.02$). In the absence of ATP, the channels displayed the LA pattern (P_o always < 0.1). (*b*) Calmodulin: This peptide is also known to associate and modulate RyR1 channels [8, 30, 46, 48]. In our hands, calmodulin addition (1 μM) slightly increased HA RyR1 channel P_o at 1 μM [Ca^{2+}] ($n=6$) but did not affect LA RyR1 channel behavior ($n=4$). (*c*) Protein Kinase A: We previously reported that PKA activated HA RyR channels in the presence of Mg^{2+} ([18, 19, 39], but *see* also 26, 29). Here, we found that exposure of LA RyR1 channels to the catalytic subunit of PKA (50 μg/ml bilayer solution) did not change LA into HA RyRs ($n=7$ experiments). (*d*) Redox status: We have tested previously various redox agents (e.g., NADP, NADH, mercaptoethanol, thimerosal, glutathion, etc.) using a calcium-loading assay, and found that only thimerosal produced substantial activation of RyR channels [7]. Exposure of single RyR channels to thimerosal (200 μM) in bilayers activated channels in 2μM [Ca^{2+}], as previously described by others [28]. However, most RyR channels, including six of nine LA RyR1 channels, inactivated after thimerosal washout. Those three LA RyR1 channels remaining active retained their usual low P_o behavior after thimerosal removal. Thus, the effect of thimerosal on LA RyR1 channels was in part reversible, similar to those of ATP effects (*see* above).

In summary, a potential physiological factor that may act to produce the observed single RyR1-channel functional heterogeneity was not found (despite testing several possibilities). Although some physiological factors tested changed P_o, all LA RyR1 channels retained their distinctive and defining func-

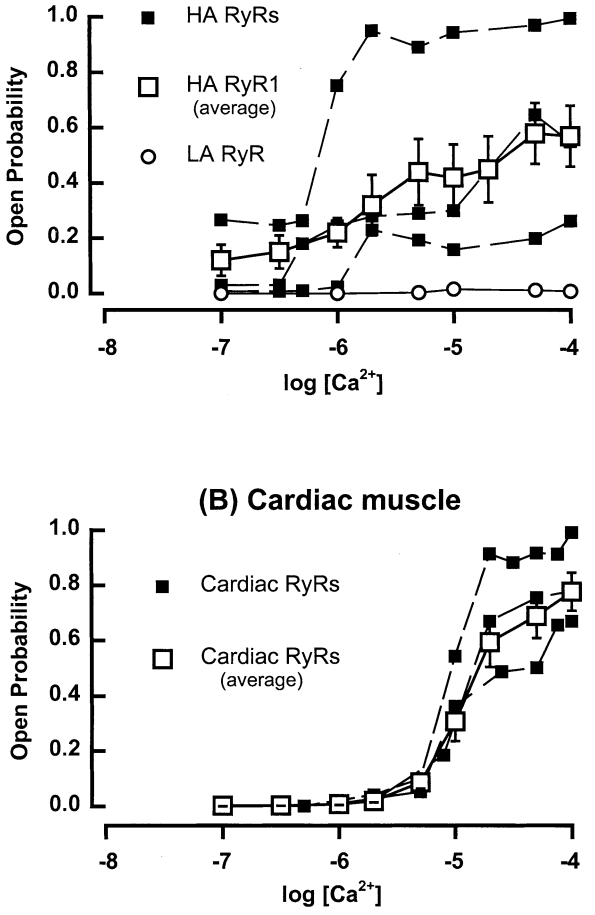

Fig. 9. Ca^{2+} activation of ryanodine receptors incubated with near physiological Mg^{2+} and ATP levels. Channels were incubated with 5.6 mM total Mg^{2+} and 5 mM ATP ($[Mg^{2+}]_{free} \sim 1$ mM) and open probabilities were determined at $[Ca^{2+}]_{free}$ ranging from 0.1 to 100 μM. (*A*) Individual P_o values from 3 experiments with HA RyR1 (■), one LA RyR (○) and average values for 8 HA RyRs (□). (*B*) Individual P_o values from 3 experiments with cardiac RyR1 (■), and average values for 7 HA RyRs (□).

tional characteristics (low P_o, low sensitivity to $[Ca^{2+}]_{free}$ and ATP).

Discussion

In this study, we compared RyR channels from skeletal muscle and from heart with regard to their inhibition by Mg^{2+} and their response to ATP, Ca^{2+}, and caffeine when Mg^{2+} is present. We detected variable sensitivity of individual RyR1 and RyR2 channels to block by near-physiological cytosolic levels of Mg^{2+}(1 mM). Such conditions display further heterogeneity in the behavior of the channels. We also found important differences between RyR1 and RyR2 channels in their response to the agonists Ca^{2+}, caffeine and ATP. Skeletal muscle displays two different types of behavior under 1 mM Mg^{2+}: (*a*) HA RyR1 channels, which could be reactivated, at least in part, by increasing cytosolic Ca, caffeine and/or ATP; (*b*) LA RyR1 channels, which recover poorly from Mg^{2+} block with the addition of those agonists (albeit these channels show some enhanced activity with ATP and high $[Ca^{2+}]$). Heart contains mainly HA-like RyR channels, which recover from Mg^{2+} inhibition after increasing $[Ca^{2+}]$ and caffeine, but not ATP. Some interesting aspects of the experimental data are discussed below.

Mg^{2+} Blocks RyR2 and RyR1 Channels. Both Channels Display Heterogeneous Behavior

Our results indicate that in the presence of 5 μM Ca^{2+}, the inhibitory action of Mg^{2+} was not significantly different in RyR2, LA RyR1, and HA RyR1 channels. Previous reports suggest that RyR2 channels are less sensitive than RyR1 channels to Mg^{2+} [8, 49]. Such differences appear to reflect the varied experimental conditions including the cytosolic $[Ca^{2+}]$ levels applied. It has been reported [25] that RyR2 channels have lower Mg^{2+}sensitivity at $[Ca^{2+}] \geq 10$ μM. This is in agreement with our observation that for channels bathed with 100 μM Ca^{2+}, sensitivities to Mg^{2+} are LA RyR1 > HA RyR1 > RyR2. Thus, at high Ca^{2+} levels, cardiac RyR2 channels are, indeed, less sensitive to block by Mg^{2+}. It may be noted, however, that in the presence of ATP some RyR1 channels are rather insensitive to Mg^{2+} or are activated at lower Ca^{2+} levels than RyR2 channels. Thus, studying RyRs with physiological levels of ATP and Mg^{2+} is important to properly correlate individual channel behavior in bilayers with local events of calcium release (Ca^{2+}-sparks) or global Ca^{2+} transients in vivo.

Ca^{2+} and Caffeine Action on Mg^{2+} Block of RyR1 and RyR2 Channels

The mechanism of Mg^{2+} block seems to be a combination of (*1*) competition with Ca^{2+} for occupation of high-affinity Ca^{2+}-activation sites and (*2*) binding to low-affinity nonspecific divalent inhibitory sites [25]. For both HA RyR1 and RyR2 channels, the half maximal activating $[Ca^{2+}]$ was variable, ranging from 1 to 10 μM [6, 8, 24], which may underlie the variation as well as the similar degree of sensitivity to Mg^{2+} observed here with 5 μM $[Ca^{2+}]$. The situation is rather different when Ca^{2+} is increased to higher levels (100 μM). For cardiac RyRs, the sensitivity to inhibition by divalent cations is much less than for skeletal RyRs [6, 8, 24, 25], which could explain why 100 μM $[Ca^{2+}]$ almost fully counteracted the inhibitory effect of Mg^{2+} in all cardiac RyRs, whereas for HA skeletal RyRs, the Mg^{2+} block is still pronounced. Such a model is also consistent with the

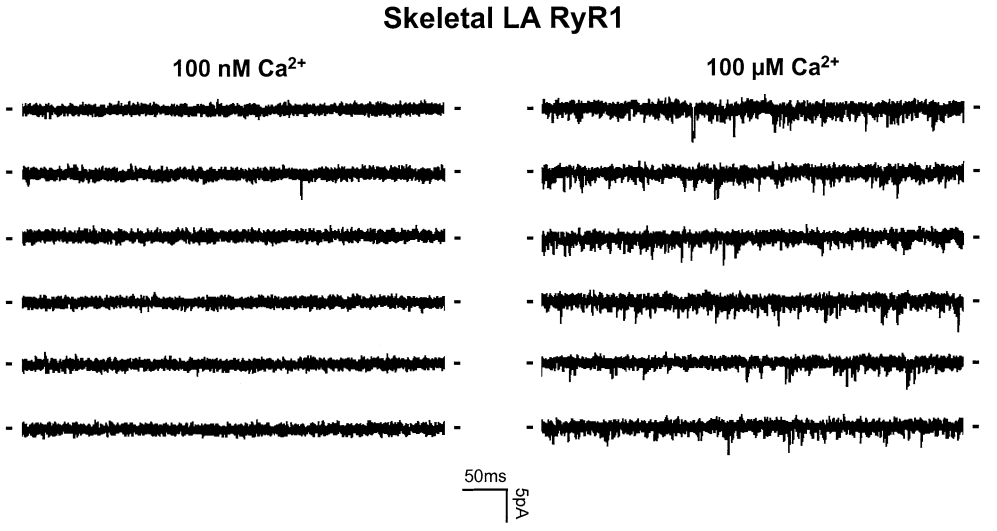

Fig. 10. Ca^{2+} greatly increased the frequency of skeletal LA RyR channel openings. The example shows single-channel recordings of a skeletal low-activity (LA) RyR channel at 100 nM and 100 μM cytosolic [Ca^{2+}]. Channel openings are shown as downward deflections. The closed state is indicated to the left and right of the channel recordings. Analysis of 5-min recordings at 0.1 μM [Ca^{2+}] of $n = 54$ detected opening events (frequency was 11 ± 4 min^{-1}), which had an average duration of 0.75 ± 0.05 msec; estimated P_o was 0.00015. At 100 μM [Ca^{2+}], the event duration was 0.83 ± 0.04 msec ($n = 2577$ events) significantly not changed. However, the frequency of openings significantly increased to 515 ± 20 min^{-1} ($p < 0.01$); the estimated P_o was 0.0071. A marked increase in the opening frequency, upon increasing [Ca^{2+}] from resting (100 nM) to maximally activating levels (100 μM), was also seen in 3 additional experiments with LA RyR1 channels.

poor recovery found in LA RyRs, where the activating [Ca^{2+}] sites are of much lower affinity (100 to 300 μM), and in a similar range as the inhibitory sites [6, 31].

The channel agonist caffeine is believed to increase calcium sensitivity of Ca^{2+}- binding sites on ryanodine receptors [8, 30, 34]. Accordingly, caffeine reactivates with variable efficacy the activity of HA RyR1 and RyR2 channels, but was ineffective in activating LA RyR1 channels.

The Basis of Single RyR1 Channel Functional Heterogeneity Remains Elusive

Even "purified" RyR1 channels, presumably devoid of any interacting molecules, exhibit functional heterogeneity, i.e., display the LA and HA profiles [6, 8, 31]. We have demonstrated in our previous work [6], that variability in channel behavior is only a minor contributor to the levels of heterogeneity observed in skeletal muscle. We also compared here (*results not shown*) duplicate determinations of P_o in the same channel (after reestablishing original conditions of the original set of measurements by superfusion). The ranges of variation between original and duplicate P_o measurements were minor compared to the levels of heterogeneity observed among individual channels. Thus, modal gating of the RyRs (spontaneous shifts in P_o during individual channel recordings) cannot explain observed differences in LA and HA behavior between channels. We also tested recently the possibility that LA RyR1 channels are gated by cyclic ADP ribose (cADPR), but we did not find that this compound modulates any type of RyR channel in vitro [7].

We tested whether the observed heterogeneous pattern of LA and HA behavior detected in skeletal muscle RyR1 could result from certain known channel modulatory factors. They included calmodulin and FKBPs, two proteins that bind and modulate RyR1 channels [1, 8, 44–46, 48]. We found, however, that FKBP and/or calmodulin do not convert LA into HA channels. We also determined that neither protein kinase A, known to modulate HA RyR channel behavior [18, 19, 29, 39, 40], nor Thimerosal, a potent redox agent that affects RyR1 channels [7, 28, 48], transform LA into HA channels. In summary, the LA RyR1 appears to be a stable "in vitro" gating status of the channels, which was not transformed to the HA status by the action of the above indicated putative modulators.

Is the Observed LA Channel Behavior a Consequence of the Degradation of "Normal" HA RyR1 Channels?

Several lines of evidence suggest that LA channels are not degraded HA channels. First, in the absence of Mg^{2+}, the LA RyR1 channels can be reversibly activated by high [ATP] (from P_o of 0.05 to $P_o > 0.2$). Furthermore, LA channels reach even higher P_o

values (~0.45) in the presence of ATP and FK590. Thus, these channels are not "dead," albeit they will display consistently low P_o values under more physiological conditions. Second, LA channel behavior is only observed in RyR1 channel populations, not in RyR2 or RyR3 channel populations [6, 21, 31]. Interestingly, LA channels appear to be more abundant in fast twitch muscle [2, 9], where Ca transients decay faster [15]. If LA channel behavior were the result of general channel degradation, then LA behavior should be observed for all channel types. Third, LA- and HA-channel behavior is stable under our experimental conditions. Transitions between HA and LA profiles are very infrequently observed in the same channel [6, 31]. Generally, degradation of RyRs is likely to be random and not a progressive change from HA $\rightarrow$ LA $\rightarrow$ inactive channel. Fourth, there is no clear correlation between "normal" HA RyR1 behavior in bilayers and physiological calcium release in cells. It is still quite uncertain what types of single RyR behavior contribute to Ca^{2+} signaling "in vivo." The "normal representative" single-channel behavior still needs to be defined.

Is Differential Activation of RyR1 and RyR2 Channels by ATP a Physiological Adaptation?

This study shows that LA RyR1 channels, in the presence of 1 mM Mg^{2+}, achieve only relatively low P_o (~0.01) levels after addition of ATP and high Ca^{2+}. The HA RyR1 channels recover from Mg^{2+} block when physiological nucleotide concentrations are applied. This difference in Mg^{2+} sensitivity correlates well with the different ATP and Ca^{2+} sensitivities of these channels [1, 6, 8, 39, 40]. The HA RyR1 channels were far more sensitive to ATP than LA channels. In our conditions, free ATP ranged from 60 to 250 μM, sufficient to activate to high P_o levels most HA RyR1 channels [39, 40]. It was not sufficient to activate LA RyR1 channels. These required free [ATP] $\geq$1 mM (no Mg^{2+} added) to reactivate. All RyR2 channels had high activity at 5 μM Ca^{2+} but, as with LA RyR1 channels, displayed lower sensitivity to ATP (at low Ca^{2+} levels) than HA RyR1 channels [1, 39]. This may explain the lack of recovery from Mg^{2+} block by increased [ATP] in both LA RyR1 and RyR2 channels.

Regulation of RyR1 channels by ATP seems advantageous for fast twitch skeletal muscle fibers, which derive their ATP largely by glycolysis. The concentration of total ATP can decrease by 30% in the fatigued muscle, concomitantly with an increase in free [Mg^{2+}] and a decrease in free [ATP] [12, 22, 23]. According to our data, single RyR channel activity would be lower and therefore SR Ca^{2+} release would be decreased. Decreased cytosolic Ca^{2+} would reduce the energy demands in the fiber. By contrast, in the healthy heart, which depends mainly on mitochondria and aerobic metabolism for ATP synthesis, [ATP] is relatively constant and such a compensatory mechanism would not be operative.

Speculations Concerning Functional Differences between RyR2 and RyR1 Channels

During EC-coupling, the RyR1 channel operates in a dynamic and complex environment where the channel is thought to open briefly for few milliseconds [15, 22, 32, 37, 41–43]. Models of alternating RyR1 channels activated either by DICR or CICR (depolarization-induced and calcium-induced calcium release) have been proposed [4, 42, 43] based on electron microscopy data [4, 14] and electrophysiological studies [10, 15, 20, 32, 42]. Thus, the notion of structural and functional RyR-channel heterogeneity in skeletal muscle is not novel. Our results support previous reports in bilayers [6, 24, 27, 31] and present further evidence that the RyR1 population in skeletal muscle is functionally heterogeneous and includes two general categories of channel behavior (LA and HA).

It is possible that some HA RyR1 channels, "open" at resting [Ca^{2+}], may be controlled "in vivo" by the DHPR voltage sensor. In this scheme, these HA RyR1 channels release Ca^{2+} in response to T-tubule depolarization (i.e., modulated by the DICR process). The other RyR1 channels (HA gated by Ca^{2+} and LA channels) would be closed at resting [Ca^{2+}]. These channels may participate in the amplification of the Ca^{2+} release process (i.e., modulated by the CICR process). Other schemes can be conceptualized as well. The point is that it is possible that the observed single RyR1 functional heterogeneity has physiological implications.

A high level of heterogeneity in RyR channel function is apparently confined to RyR1, the only RyR isoform that can activate via this complex mechanism for EC-coupling, which seems to involve DICR and CICR [32, 47]. In cardiac muscle, there is apparent lack of "coupling" between the RyR2s and the DHPR [14] and CICR is the main mechanism for EC coupling [11, 47]. Accordingly, the cardiac RyR2s display in single-channel studies a much more uniform behavior, which includes a steep [Ca^{2+}] dependency for their activation both in the presence or absence of Mg^{2+} and ATP [6, 8, 24, 39, this report]. These cardiac RyR2 channel properties may be those "adequate" for CICR. Interestingly, the RyR3 isoform, which is expressed in some muscle fibers and that we purified from bovine diaphragm, behaved homogeneously like HA Ca^{2+}-gated channels [21]. Both, RyR2 and RyR3 channels appear to be activated "in vivo" exclusively via CICR [47].

In conclusion, we find in the presence of Mg^{2+} marked differences in the response of skeletal *vs.* cardiac RyRs to changes in [ATP], and [Ca^{2+}]. Such differences in channel behavior may be the consequence of adaptation to different metabolic constraints and/or related to the different excitation contraction-coupling mechanisms in skeletal muscle *vs.* heart.

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