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# Redox-sensitive stimulation of type-1 ryanodine receptors by the scorpion toxin maurocalcine

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ABSTRACT

The scorpion toxin maurocalcine acts as a high affinity agonist of the type-1 ryanodine receptor expressed in skeletal muscle. Here, we investigated the effects of the reducing agent dithiothreitol or the oxidizing reagent thimerosal on type-1 ryanodine receptor stimulation by maurocalcine. Maurocalcine addition to sarcoplasmic reticulum vesicles actively loaded with calcium elicited $Ca^{2+}$ release from native vesicles and from vesicles pre-incubated with dithiothreitol; thimerosal addition to native vesicles after $Ca^{2+}$ uptake completion prevented this response. Maurocalcine enhanced equilibrium [$^3$H]-ryanodine binding to native and to dithiothreitol-treated reticulum vesicles, and increased 5-fold the apparent $K_i$ for $Mg^{2+}$ inhibition of [$^3$H]-ryanodine binding to native vesicles. Single calcium release channels incorporated in planar lipid bilayers displayed a long-lived open sub-conductance state after maurocalcine addition. The fractional time spent in this sub-conductance state decreased when lowering cytoplasmic [$Ca^{2+}$] from 10 μM to 0.1 μM or at cytoplasmic [$Mg^{2+}$] ≥ 30 μM. At 0.1 μM [$Ca^{2+}$], only channels that displayed poor activation by $Ca^{2+}$ were readily activated by 5 nM maurocalcine; subsequent incubation with thimerosal abolished the sub-conductance state induced by maurocalcine. We interpret these results as an indication that maurocalcine acts as a more effective type-1 ryanodine receptor channel agonist under reducing conditions.



## 1. Introduction

Ryanodine receptors (RyR) are intracellular $Ca^{2+}$ release channels with a key role in skeletal muscle excitation–contraction coupling [1]. Physiological activation of type-1 RyR (RyR1) channels, the prevalent isoform expressed in adult mammalian skeletal muscle, occurs as a consequence of conformational changes of the interacting dihydropyridine receptors (DHPR) induced by membrane depolarization [2]. Several cellular components and covalent modifications of the protein modify RyR1 activity, including ATP, cyclic ADP ribose, $H^+$ and $Mg^{2+}$, phosphorylation or changes in RyR1 redox state [1]. In particular, RyR redox state markedly influences the effects of physiological RyR agonists or inhibitors, such as $Ca^{2+}$, $Mg^{2+}$ and ATP, on RyR activity [3,4]. Skeletal RyR1 channels possess a few cysteine residues with high reactivity to redox agents at physiological pH [5,6]. Modification of essential sulfhydryl residues involved in the gating of RyR1 channels modulates the apparent $Ca^{2+}$ affinity of both, the activating high affinity and the inhibitory low affinity $Ca^{2+}/Mg^{2+}$ binding sites [7–9]. Oxidizing

*Abbreviations:* [$Ca^{2+}$], free calcium concentration; [$Mg^{2+}$], free magnesium concentration; DHPR, dihydropyridine receptor; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HEDTA, N-(2-hydroxyethyl)-ethylenediamine-triacetic acid; MCa, maurocalcine; $P_{MCa}$, fractional time spent by the channel in the sub-conductance state induced by maurocalcine; $P_o$, fractional time spent by the channel in the open state; $P_{oB}$, fractional time spent by the channel in the open state, excluding the time spent in the sub-conductance state induced by maurocalcine; RyR, ryanodine receptor; RyR1, type-1 ryanodine receptor; SE, standard error; SERCA, sarcoplasmic/endoplasmic $Ca^{2+}$-ATPase; SR, sarcoplasmic reticulum.

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agents decrease significantly the inhibitory effects of $Mg^{2+}$ on RyR1 activity [10,11].

Under conditions of sustained or strenuous exercise, skeletal muscle cells increase the production of reactive oxygen species that may affect RyR1 activity *in vivo* [12]. Consequently, to explore if the redox changes that occur in skeletal muscle during exercise-associated contractile activity modify RyR1-mediated $Ca^{2+}$ release, it becomes important to study RyR1 channels under different redox conditions, which affect in particular their $Ca^{2+}$ activation/$Mg^{2+}$ inhibition profiles and the effects of selective agonists. Cell permeable compounds that bind with high-affinity to RyR channels and modify their function are useful tools to study RyR1 function in a cellular context. Yet, to use these agents in muscle cells under varying redox conditions it is essential to investigate if their effects on RyR1 channel function are redox-independent. The scorpion toxin maurocalcine (MCa), a 33-mer basic peptide cross-linked by three disulfide bridges that crosses cell membranes within 1–2 min [13,14] is a high-affinity and reversible RyR1 agonist. Addition of MCa at nanomolar concentrations to RyR1 channels incorporated in planar lipid bilayers induces a well-defined channel sub-conductance state, with long-lasting openings to a level equivalent to 50–60% of maximal current [15]. Maurocalcine also activates channels modified with ryanodine or lacking the FK506-binding protein [16,17]. At 10 μM free $Ca^{2+}$ concentration ($[Ca^{2+}]$), MCa concentrations in the nM range ($EC_{50}$ = 12 nM) stimulate [$^3$H]-ryanodine binding to RyR1 [16]. Maurocalcine shifts the stimulation of [$^3$H]-ryanodine binding by $Ca^{2+}$ to lower concentrations and decreases the inhibitory effects of high $[Ca^{2+}]$ [17]. These findings correlate well with the marked stimulation of $Ca^{2+}$ release induced by MCa addition to skeletal SR vesicles ($EC_{50}$ = 17.5 nM) or cultured myotubes [17].

In this work, we used isolated skeletal SR vesicles enriched in triads to test the effects of MCa on RyR1-mediated $Ca^{2+}$ release and [$^3$H]-ryanodine binding, under the different redox conditions produced by the reducing agent dithiothreitol (DTT) or the oxidizing agent thimerosal. We also tested the effects of DTT, thimerosal and of varying free $Mg^{2+}$ concentrations ($[Mg^{2+}]$) on the sub-conductance state induced by MCa in single RyR1 channels incorporated in planar lipid bilayers. Our results strongly suggest that, under reducing conditions, MCa is an effective high-affinity RyR1 channel agonist. In addition, we show that incubation with thimerosal hinders RyR1 channel activation by MCa at resting $Ca^{2+}$ concentration.

## 2. Materials and methods

### 2.1. Materials

All reagents used were of analytical grade. Lipids were from Avanti Polar Lipids, Inc. (Birmingham, AL). Bovine serum albumin, DTT, ryanodine, thimerosal, and protease inhibitors (leupeptin, pepstatin A, benzamidine, trypsin inhibitor, phenylmethylsulfonyl fluoride) were from Sigma Chemical Co. (St. Louis, MO). Calcium Green-2 was from Molecular Probes, Inc. (Eugene, OR) and [$^3$H]-ryanodine from NEN Life Sciences (Boston, MA). Maurocalcine was synthesized as described [15].

### 2.2. Membrane preparations

SR vesicles were isolated from fast skeletal muscle of New Zealand rabbits as described [18]. Aliquots of vesicle-containing suspensions frozen in liquid nitrogen were stored at −80 °C for up to 30 days. The Bioethics Committee for Investigation in Animals of the Facultad de Medicina, Universidad de Chile approved all experimental protocols used in this work.

### 2.3. Calcium release experiments

$Ca^{2+}$ release was determined in a fluorescence spectrometer, using the fluorescent probe Calcium Green-2 to detect extravesicular $Ca^{2+}$ concentration changes. To this end, triad-enriched SR vesicles were diluted to 0.2 mg per ml in a solution containing (in mM): 100 KCl, 10 phosphocreatine plus 15 IU/ml creatine phosphokinase, 20 MOPS/Tris pH 7.2 and 0.09 μM Calcium Green-2. After addition of SR vesicles, the $[Ca^{2+}]$ of the above medium ($Mg^{2+}$-free) was determined with a $Ca^{2+}$ electrode (Orion, Beverly, MA) calibrated with a commercial kit (WPI, Sarasota, FL). $Ca^{2+}$ uptake was initiated at 25 °C by simultaneous addition of 2.0 mM $MgCl_2$ plus 2.0 mM ATP ($[Mg^{2+}]$ = 0.35 mM). $Ca^{2+}$ release was induced by addition of MCa, or caffeine. Addition of MCa to test its effects as $Ca^{2+}$ releasing agent was done 250–500 s after completion of $Ca^{2+}$ uptake. Assuming a $K_d$ of 0.4 μM, the $[Ca^{2+}]$ was calculated from Calcium Green-2 fluorescence ($F$) according to the equation:

$$[Ca^{2+}] = \frac{K_d(F_{max} - F)}{F - F_{min}}$$

The maximal ($F_{max}$) and the minimal ($F_{min}$) values of fluorescence were determined after addition of 1.0 mM $CaCl_2$ or 10 mM ethyleneglycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), respectively. In $Mg^{2+}$-free solutions the values of $[Ca^{2+}]$ calculated according to the above equation from determinations of Calcium Green-2 fluorescence, coincided with the $[Ca^{2+}]$ values determined with the $Ca^{2+}$ electrode. Caffeine addition produced some quenching of Calcium Green-2 fluorescence (10% at 5 mM caffeine), which was corrected to calculate extravesicular $[Ca^{2+}]$ levels.

### 2.4. Circular dichroism spectra

Circular dichroism (CD) spectra from 190 to 260 nm were recorded at 20 °C on a Jasco Corp J-810 dichrograph using 0.2 nm steps. MCa was dissolved in water (50 μM), water plus 50 μM thimerosal or water plus 1 mM DTT. Before recording CD spectra, MCa was preincubated at 37 °C for 15 min or 150 min, in the case of addition of thimerosal or DTT, respectively. The spectra were corrected for water, water plus thimerosal or water plus DTT signal and smoothed using a third order least squares polynomial fit.

### 2.5. Equilibrium [$^3$H]-ryanodine binding

SR vesicles (1 mg/ml) were incubated at 37 °C for 2 h in a solution containing (in mM): 150 KCl, 20 MOPS-Tris, pH 7.2, 1 EGTA, 1 $CaCl_2$, 10 nM [$^3$H]-ryanodine, plus/minus MCa at the concentrations indicated in the text. In some experiments, vesicles were pre-incubated for 15–30 min with DTT (0.5 or 1 mM). Non-specific binding was determined by addition of 10 μM ryanodine to the above incubation solution. After incubation, [$^3$H]-ryanodine binding was determined by filtration as described [19].

### 2.6. Single channel experiments

Channel recordings were obtained as reported [20–22]. The *cis* (cytoplasmic) solution contained 0.5 mM $Ca^{2+}$-HEPES and 225 mM HEPES–Tris, pH 7.4. To set the desired $[Ca^{2+}]$ and/or $[Mg^{2+}]$, N-(2-hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA) and/or EGTA were added to the cis compartment. Total concentrations of HEDTA, EGTA, $Ca^{2+}$ and $Mg^{2+}$ required for each $[Ca^{2+}]$ and/or $[Mg^{2+}]$ were calculated with the WinMAXC program (www.stanford.edu/~cpatton). The *trans* (luminal) solution contained 40 mM $Ca^{2+}$-HEPES, 15 mM Tris–HEPES, pH 7.4; therefore, in all experiments the charge carrier was $Ca^{2+}$. The lipid bilayer was held at 0 mV; channel recordings were obtained at 22 ± 2 °C.

Current data were filtered at 400 Hz (−3 dB) using an eight-pole low-pass Bessel type filter (902 LPF, Frequency Devices Inc., Haverhill, MA). Data were digitized at 2 kHz with a 12-bit A/D converter (Labmaster DMA interface, Scientific Solutions, Inc., Solon, OH) using the commercial software Axotape (Molecular Devices Corporation, Sunnyvale, CA).We defined $P_o$ as the fraction of time a single RyR1 channel spent in the open state in the absence of MCa. Addition of MCa induced a well-defined sub-conductance state; we defined $P_{MCa}$ as the fractional time the channel spent in this sub-conductance state, and $P_{oB}$ as the fractional time a channel not dwelling in the MCa-induced sub-conductance state spent in the open state (see Fig. S1). Fractional times were computed with the pClamp commercial software (Molecular Devices Corporation, Sunnyvale, CA). Nonlinear fitting of data was performed using the SigmaPlot software (Systat Software Inc., Richmond, CA).

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### 2.7. Data expression and statistical analysis

Data are expressed as mean ± standard error (SE). Unpaired Student's *t*-test, one-tailed, was used for statistical analysis, unless specified otherwise.

## 3. Results

### 3.1. Effects of maurocalcine and caffeine on vesicular $Ca^{2+}$ release

Active $Ca^{2+}$ uptake by the sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) was started by adding Mg-ATP to triad-enriched SR vesicles isolated from rabbit fast skeletal muscle. Addition of Mg-ATP to native SR vesicles produced a fast decrease in extravesicular [$Ca^{2+}$] (Fig. 1), which decreased on average from 23.8 ± 5.9 μM ($N$ = 7, measured with a calcium electrode) to <0.15 μM, calculated from the decrease in probe fluorescence. Addition of MCa to native SR vesicles actively loaded with calcium induced fast $Ca^{2+}$ release, as illustrated in the fluorescence records corresponding to representative experiments from two different SR preparations (Fig. 1, top panel). Four out of five different SR preparations displayed this same behavior. To favor the reduced state of RyR1, SR vesicles were pre-incubated with the reducing agent DTT before initiating $Ca^{2+}$ uptake. Addition of MCa effectively promoted $Ca^{2+}$ release from calcium-loaded SR vesicles pre-incubated with DTT, as evidenced by the rapid increase in probe fluorescence observed in representative records from two different SR preparations (Fig. 1, center panel). Addition of MCa elicited $Ca^{2+}$ release in six different vesicular preparations pre-incubated with DTT. Of note, the native SR preparation that did not release $Ca^{2+}$ after addition of 200 nM MCa displayed effective $Ca^{2+}$ release after pre-incubation with DTT (Fig. S2), indicating that DTT restored MCa-induced $Ca^{2+}$ release in this SR preparation. The release of $Ca^{2+}$ from native or DTT-treated vesicles was transient and lasted on average <100 s, indicating that RyR1 channels closed/inactivated rapidly after activation with MCa. The transient nature of the $Ca^{2+}$ release induced by MCa also indicates that pre-incubation with DTT did not modify SERCA activity, since SR vesicles readily took up all the $Ca^{2+}$ released by MCa decreasing [$Ca^{2+}$] to levels <0.15 μM. Addition of caffeine induced $Ca^{2+}$ release in all native and DTT-treated SR preparations tested ($N$ = 3; see Fig. S3).

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To test the effects of RyR1 oxidation, we added thimerosal (10 μM) after $Ca^{2+}$ uptake completion, to prevent possible effects of thimerosal on SERCA pump function and ensure efficient $Ca^{2+}$ uptake. In contrast to the behavior displayed by native or DTT-treated vesicles, subsequent addition of MCa (200 nM) did not stimulate release, whereas further addition of caffeine effectively induced transient $Ca^{2+}$ release, as evidenced by the representative records obtained from two different SR preparations (Fig. 1, lower panel). The fact that caffeine elicited transient $Ca^{2+}$ release after thimerosal addition indicates that thimerosal did not inhibit RyR1 or SERCA function, since caffeine released $Ca^{2+}$ *via* RyR1 and then the SR vesicles re-accumulated *via* SERCA all the $Ca^{2+}$ released by caffeine. Six different SR preparations displayed the same lack of $Ca^{2+}$ release following MCa addition after thimerosal. Addition of a higher concentration of MCa (400 nM) did not induce $Ca^{2+}$ release from thimerosal-treated vesicles (not shown), suggesting that – at the low [$Ca^{2+}$] present in the extravesicular solution after $Ca^{2+}$ uptake – oxidized RyR1 channels have significantly decreased MCa affinity compared to native or reduced channels. An alternative explanation of these results would be that an oxidizing agent such as thimerosal interacted with MCa rendering it inactive. Yet, this possibility is unlikely because pre-incubation with 50 μM thimerosal or 1 mM DTT did not modify the CD spectrum of MCa (Fig. S4). Moreover, the CD spectrum of MCa-Abu, a disulfide-less analog of MCa that does not induce $Ca^{2+}$ release in skeletal fibers, differs greatly from MCa [23]. The CD spectrum of MCa obtained after incubation with either DTT or thimerosal did not show the changes exhibited by the MCa analog without disulfide bridges (Fig. S4, inset).

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The results illustrated in Fig. 1 show that the reducing agent DTT did not hinder MCa-induced $Ca^{2+}$ release through RyR1 channels while incubation with the SH-oxidizing agent thimerosal prevented MCa-induced but not caffeine-induced $Ca^{2+}$ release. To test the hypothesis that redox agents modify the effectiveness of MCa as RyR1 agonist, we measured under different redox conditions the effects of MCa on vesicular [$^3$H]-ryanodine binding and on the activity of single RyR1 channels incorporated into planar lipid bilayers.

### 3.2. Maurocalcine increases equilibrium [$^3$H]-ryanodine binding: effects of DTT and $Mg^{2+}$

The plant alkaloid ryanodine is a highly selective agent, considered the gold standard to monitor RyR channel function. A good correlation between single RyR1 channel activity and [$^3$H]-ryanodine binding density to skeletal SR vesicles has been reported [24,25]. Maurocalcine stimulated [$^3$H]-ryanodine binding density when added to native and DTT-treated SR vesicles (Fig. 2A), which correlates with the stimulation of $Ca^{2+}$ release described above. Maurocalcine increased equilibrium [$^3$H]-ryanodine binding in both native (6.3 ± 0.5 fold) and DTT-treated vesicles (11.1 ± 1.4 fold) relative to the values determined in the absence of MCa. Irreversible RyR1 inhibition induced by long exposure to thimerosal, such as that required to measure equilibrium [$^3$H]-ryanodine binding density, precluded a detailed study of the effects of MCa on thimerosal treated vesicles.

Several reports indicate that $Mg^{2+}$ is a potent inhibitor of RyR1 activity [1]. Interestingly, oxidizing reagents decrease the inhibitory effects of $Mg^{2+}$ on RyR1 activity [11] while reducing agents increase the $Mg^{2+}$ inhibition of [$^3$H]-ryanodine binding to endoplasmic reticulum vesicles isolated from rat brain cortex [3]. Here, we investigated if MCa modified the inhibition of [$^3$H]-ryanodine binding to RyR1 produced by $Mg^{2+}$. As illustrated in Fig. 2B, incubation of native skeletal SR vesicles with 200 nM MCa significantly decreased the inhibitory effects of $Mg^{2+}$ on equilibrium [$^3$H]-ryanodine binding. Native SR vesicles incubated with MCa displayed a 5-fold higher $K_i$ value for $Mg^{2+}$-mediated

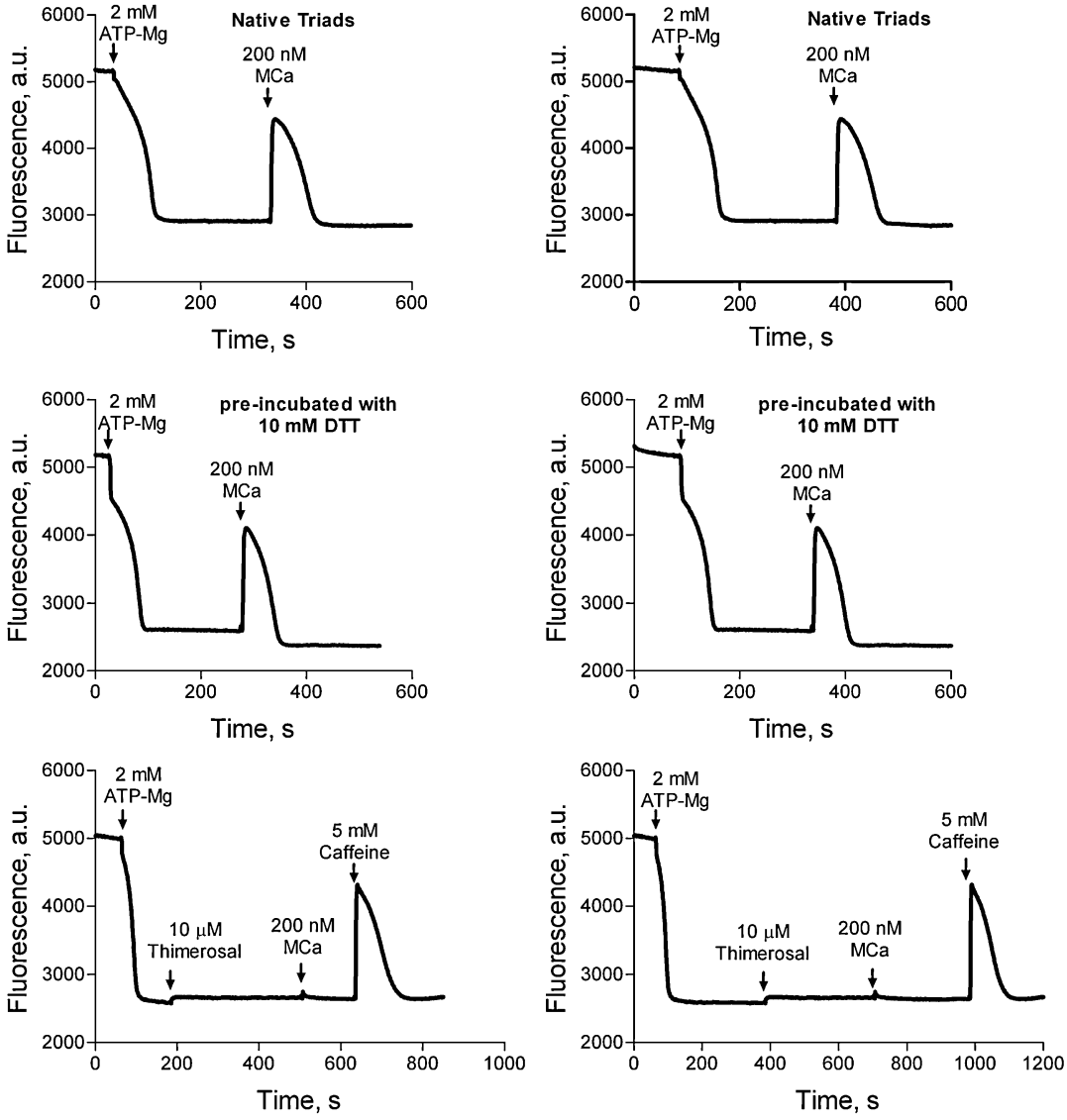


**Fig. 1.** Effects of MCa on $Ca^{2+}$ release from triad-enriched SR vesicles actively loaded with calcium. Active loading with $Ca^{2+}$ was initiated by addition of ATP-Mg, as indicated in the figure. Changes in extravesicular [$Ca^{2+}$] were determined with Calcium Green-2, as detailed in Section 2. In the six representative experiments illustrated in the figure, addition of ATP-Mg promoted fast $Ca^{2+}$ uptake, and decreased probe fluorescence to a steady value corresponding to an extravesicular [$Ca^{2+}$] < 150 nM (see Section 3). Subsequent addition of MCa caused effective and transient $Ca^{2+}$ release from native vesicles (top panel) corresponding to two different SR preparations, and from vesicles from two SR preparations pre-incubated for 30 min with 10 mM DTT (center panel). Addition of the sulfhydryl oxidizing agent thimerosal to two SR preparations after completion of $Ca^{2+}$ uptake prevented $Ca^{2+}$ release induced by MCa but not by caffeine (lower panel).

inhibition of [$^3$H] ryanodine binding relative to the $K_i$ value for $Mg^{2+}$ exhibited by control vesicles ($p = 0.016$; see Fig. 2B).

### 3.3. *Effects of maurocalcine on RyR1 single channel activity*

An earlier study showed that MCa induces a distinct long-lasting sub-conductance state in native RyR1 channels incorporated in planar lipid bilayers [15]. We have previously reported that single RyR1 channels display three different responses to cytoplasmic [$Ca^{2+}$] changes (Fig. S5), which depend on RyR channel redox state [3,8,22]. Incubation with reducing agents favors the appearance of channels with "low activity", characterized by $P_o$ values <0.1 in the [$Ca^{2+}$] range 0.1–500 μM. Partial RyR oxidation of "low activity" channels usually generates the "moderate activity" response, the typical bell-shaped response to cytoplasmic calcium previously reported for native skeletal RyR1 channels [26], with maximal activity in the 10–30 μM [$Ca^{2+}$] range and $P_o$ values <0.1 at 0.1 or 500 μM [$Ca^{2+}$]. Further oxidation increases channel activity at low *cis* [$Ca^{2+}$], increases markedly $P_o$ to values as high as 0.99 at [$Ca^{2+}$] > 100 μM and suppresses the inhibitory effect of 500 μM cytoplasmic [$Ca^{2+}$]; we call this channel behavior the "high activity" response. Here, we tested the effects of MCa on RyR1 single channels incorporated into lipid bilayers that displayed either the low or the moderate activity behavior (Fig. S5). The effects of MCa were evaluated using two parameters, (i) $P_{MCa}$ that represents the fractional time the channel spends in the MCa induced sub-conductance state and (ii) $P_{oB}$ that represents the fractional time spent in the open state in the presence of MCa, during the intervals bridging two openings at the sub-conductance level (see Fig. S1).

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The low activity single RyR1 channel depicted in Fig. 3A displayed at 10 μM [$Ca^{2+}$] a $P_o < 0.01$. Addition of 5 nM MCa induced channel openings to a well-defined sub-conductance state, with $P_{MCa} = 0.50$ and $P_{oB} = 0.01$ (Fig. 3B, top trace). In the continuous presence of MCa, lowering cytoplasmic [$Ca^{2+}$] to 1 μM by addition of

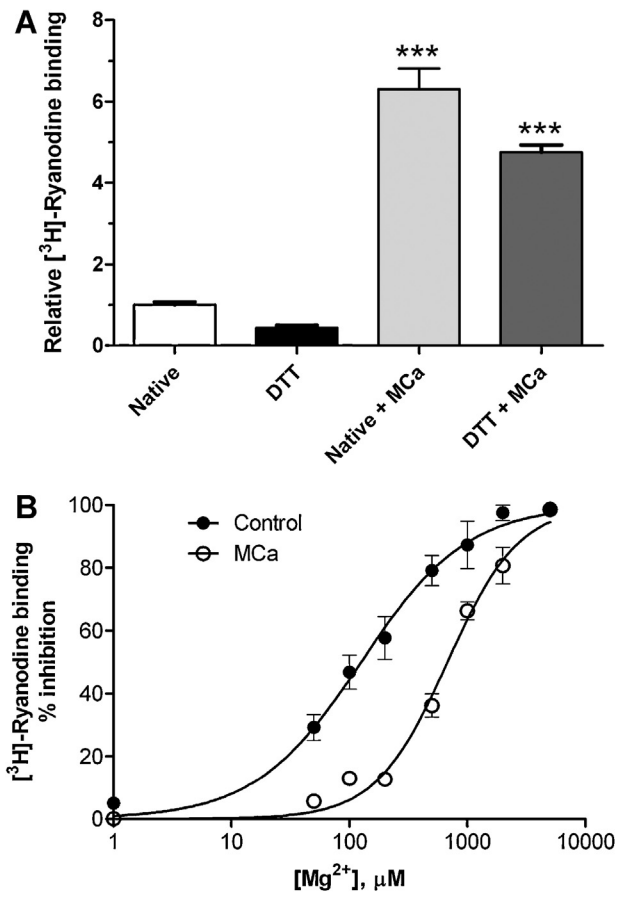


**Fig. 2.** Maurocalcine enhances [$^3$H]-ryanodine binding to triad-enriched SR vesicles and decreases the inhibitory effects of $Mg^{2+}$. As illustrated in Panel A, incubation of native vesicles with 50 nM MCa stimulated equilibrium [$^3$H]-ryanodine binding density (light gray bar) relative to the values determined in the absence of MCa (empty bar). Vesicles incubated with DTT displayed lower [$^3$H]-ryanodine binding relative to native vesicles (Panel A, black bar). Addition of 50 nM MCa to DTT-treated SR vesicles increased [$^3$H]-ryanodine binding relative to vesicles incubated only with DTT (Panel A, dark gray bar). $^{***}p < 0.0001$ calculated by One-way ANOVA followed by Dunnett's multiple comparison test. As illustrated in Panel B, incubation of native skeletal SR vesicles with 200 nM MCa significantly decreased the inhibitory effects of $Mg^{2+}$ on equilibrium [$^3$H]-ryanodine binding. Fitting the experimental points to a non-linear Hill function yielded $n_{Hill} = 1.0 \pm 0.1$ and $K_i = 125.3 \pm 8.8$ μM for native vesicles, and $n_{Hill} = 1.4 \pm 0.1$ and $K_i = 676.4 \pm 49.0$ μM for native SR vesicles incubated with 200 nM MCa.Values represent Mean ± SE ($N = 3$).

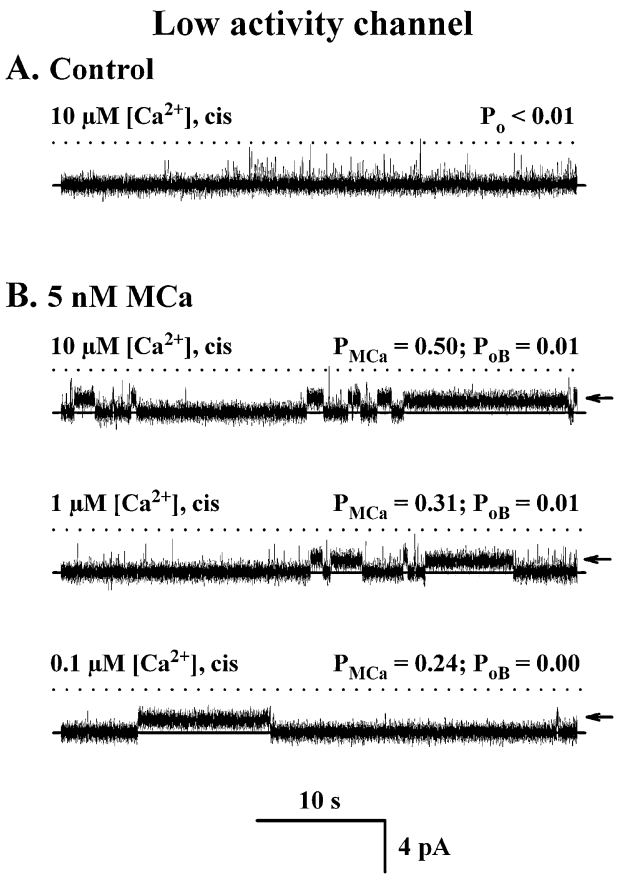


**Fig. 3.** Effects of MCa on a low activity RyR1 channel. Representative current recordings obtained with the same low activity single RyR1 channel in the absence (Panel A) or at 5 nM MCa (Panel B). $P_{MCa}$ and $P_{oB}$ values, calculated for the whole recorded periods (at least 180 s) and the free cytoplasmic [$Ca^{2+}$] are depicted above each current trace. Addition of MCa induced channel openings to a well-defined sub-conductance current level, indicated by an arrow at the right of the current traces. Sequential reduction of cytoplasmic [$Ca^{2+}$] using HEDTA and/or EGTA (see Section 2), progressively reduced both $P_{MCa}$ and $P_{oB}$ (second and third trace).

HEDTA as chelating agent decreased $P_{MCa}$ to a value of 0.31 while $P_{oB}$ stayed at 0.01 (Fig. 3B, center record). Further lowering cytoplasmic [$Ca^{2+}$] to 0.1 μM decreased $P_{MCa}$ even more, to a value of 0.24, while $P_{oB}$ decreased to 0.00 (Fig. 3B, lower record).

The single moderate activity RyR1 channel, illustrated in Fig. 4A, displayed $P_o$ values of 0.01 at 500 μM [$Ca^{2+}$] (top trace) and of 0.65 at 10 μM [$Ca^{2+}$] (lower trace), respectively. Addition of 5 nM MCa at 10 μM cytoplasmic [$Ca^{2+}$] prompted the emergence of the sub-conductance state, with $P_{MCa} = 0.84$ and $P_{oB} = 0.71$ (Fig. 4B, top trace). In the continuous presence of MCa, lowering cytoplasmic [$Ca^{2+}$] to 1 μM decreased $P_{MCa}$ to a value of 0.60 and $P_{oB}$ to 0.32 (Fig. 4B, center record). Further lowering cytoplasmic [$Ca^{2+}$] to 0.1 μM decreased both $P_{MCa}$ and $P_{oB}$ to 0.00 (Fig. 4B, lower record). Therefore, the reduction in cytoplasmic [$Ca^{2+}$] in the range of 10–0.1 μM decreased both $P_{MCa}$ and $P_{oB}$.

Average $P_{MCa}$ and $P_{oB}$ values obtained at different cytoplasmic [$Ca^{2+}$] from several single channels that spontaneously displayed either low or moderate activity are illustrated in Fig. 5. At 10 μM [$Ca^{2+}$], addition of 5 nM MCa induced the sub-conductance state in both low and moderate activity channels, which displayed $P_{MCa}$ values of $0.51 \pm 0.10$ ($N = 6$) and $0.78 \pm 0.05$ ($N = 5$), respectively. In contrast, addition of 5 nM MCa at near resting [$Ca^{2+}$] (0.1 μM) modified only low activity channels, which reached $P_{MCa}$ values of $0.24 \pm 0.04$ ($N = 11$) that differed significantly from the $P_{MCa}$ values of $0.03 \pm 0.03$ ($N = 4$) exhibited by moderate activity channels ($p = 0.002$). The $P_o$ values were similar to the $P_{oB}$ values obtained in low or moderate activity channels, respectively, suggesting that 5 nM MCa had no effect on channel activity between the sub-conductance events (Fig. 5). As expected, however, low activity channels displayed much lower $P_o$ and $P_{oB}$ values than moderate activity channels at 10 μM [$Ca^{2+}$], but not at 0.1 μM [$Ca^{2+}$] (see Fig. 5).

In previous work, we showed that thimerosal increases markedly the response of low activity RyR1 channels to cytoplasmic [$Ca^{2+}$] and sequentially promotes the moderate and the high activity responses, increasing the apparent affinity for RyR activation by [$Ca^{2+}$] and reducing the apparent affinity for RyR inhibition by [$Ca^{2+}$] [8]. To test if thimerosal affects the single channel response to MCa, we added thimerosal to low activity channels treated with MCa. The single low activity channel illustrated in Fig. 6 displayed at 10 μM cytoplasmic [$Ca^{2+}$] a $P_o < 0.01$ (Fig. 6A, top trace), a value typical of low activity channels. After lowering cytoplasmic [$Ca^{2+}$] to 0.1 μM and adding 5 nM MCa, the channel exhibited the MCa-induced sub-conductance state, with $P_{MCa} = 0.08$ and $P_{oB} = 0.01$ (Fig. 6A, lower trace). Thimerosal was added to the cytoplasmic side to a final concentration of 200 μM;

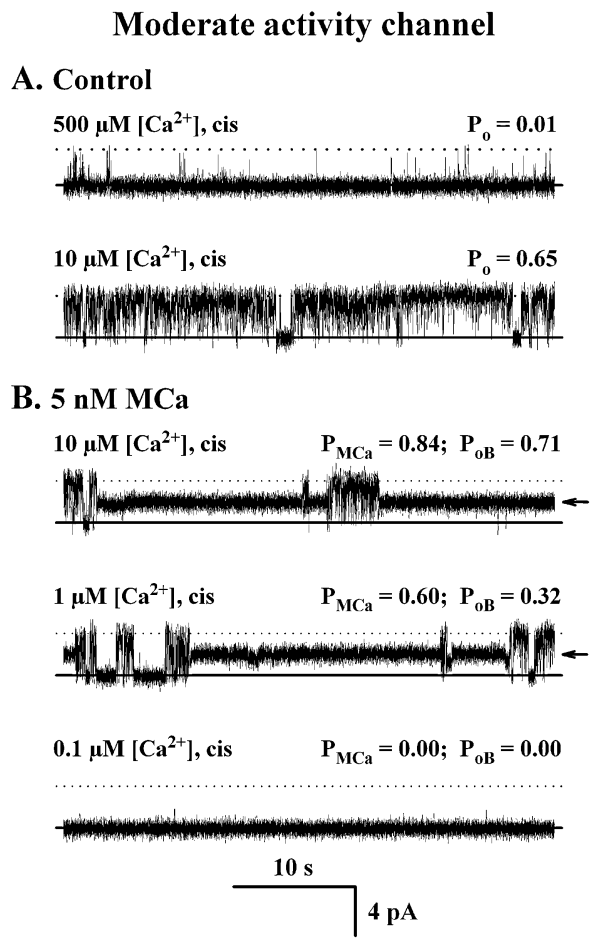


**Fig. 4.** Effects of MCa on a moderate activity RyR1 channel. Representative current recordings of the same moderate activity single RyR1 channel obtained in the absence (Panel A) or at 5 nM MCa (Panel B). $P_{MCa}$ and $P_{oB}$ values, calculated for the whole recorded periods (at least 150 s) and the free cytoplasmic [$Ca^{2+}$] are depicted above each current trace. Addition of MCa prompted the emergence of the sub-conductance current level, as indicated by an arrow at the right of the top and middle traces of Panel B. Sequential reduction of cytoplasmic [$Ca^{2+}$] using HEDTA and/or EGTA (see Section 2), progressively reduced both $P_{MCa}$ and $P_{oB}$ (second and third trace). Notice that $P_{MCa}$ and $P_{oB}$ values were equal to 0.00 at 0.1 μM [$Ca^{2+}$].

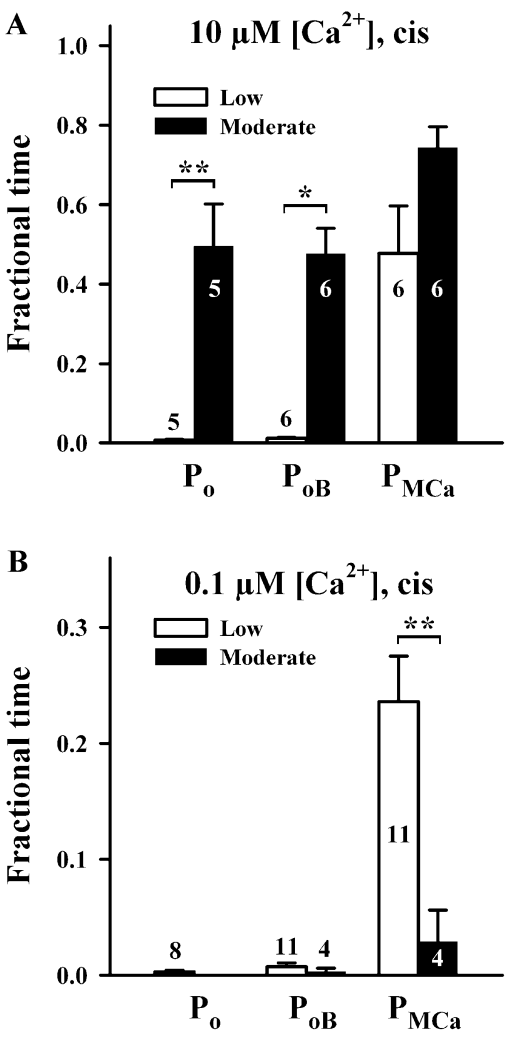


**Fig. 5.** Comparative effects of MCa on low and moderate activity RyR1 channels. Bars represent mean $P_o$, $P_{oB}$ and $P_{MCa}$ values (±SE) obtained from several single channels that spontaneously displayed either low (empty bars) or moderate activity (black bars). As illustrated in Panel A, addition of 5 nM MCa at 10 μM [$Ca^{2+}$] induced the sub-conductance state in both low and moderate activity channels. In contrast, as illustrated in Panel B, addition of 5 nM MCa at 0.1 μM [$Ca^{2+}$] modified only low activity channels. Numbers above or on each bar represent the number of single channel data included in each condition. $^{*}p < 0.05$; $^{**}p < 0.005$.

after 80 s, the cytoplasmic chamber was perfused with 20 ml of 225 mM HEPES–Tris, pH 7.4. Subsequent addition of 5 nM MCa at 0.1 μM cytoplasmic [$Ca^{2+}$] failed to elicit the sub-conductance state observed before incubation with thimerosal, although $P_{oB}$ reached 0.02, a value never observed in low activity channels at this [$Ca^{2+}$] (Fig. 6B, lower trace, see Fig. S5). In three independent experiments carried out at cytoplasmic [$Ca^{2+}$] = 0.1 μM, single low activity RyR1 channels displayed average values of $P_{MCa} = 0.07 \pm 0.01$ and $P_{oB} = 0.00 \pm 0.00$ after addition of 5 nM MCa. Subsequent addition of thimerosal as above decreased $P_{MCa}$ to $0.01 \pm 0.01$ ($p = 0.02$), whereas $P_{oB}$ value reached $0.02 \pm 0.01$. Therefore, incubation of low activity channels with thimerosal, which favors RyR activation by [$Ca^{2+}$], precludes RyR1 activation by 5 nM MCa at 0.1 μM cytoplasmic [$Ca^{2+}$], a response presumably due to modification of RyR1 cysteine residues by thimerosal.

### 3.4. $Mg^{2+}$ inhibits the effects of maurocalcine on RyR1 single channel activity

In view of the fact that SR vesicles incubated with MCa displayed 5-fold higher $K_i$ for $Mg^{2+}$ inhibition of [$^3$H]-ryanodine binding when compared to control vesicles (Fig. 2B), we investigated next the joint effects of $Mg^{2+}$ and MCa on low activity single RyR1 channels. As illustrated in Fig. 7A, after addition of 5 nM MCa at 10 μM cytoplasmic [$Ca^{2+}$] a low activity channel displayed $P_{MCa} = 0.68$ and $P_{oB} = 0.06$ (top record). Subsequent increase of cytoplasmic [$Mg^{2+}$] to 30 μM, decreased $P_{MCa}$ to 0.35 and $P_{oB}$ to 0.02 (center record). Increasing cytoplasmic [$Mg^{2+}$] to 300 μM further decreased $P_{MCa}$ to 0.09 and $P_{oB}$ to 0.00 (lower record). In this experiment, increasing cytoplasmic [$Mg^{2+}$] reduced $P_{MCa}$ with $K_{0.5} = 30 \pm 8$ μM. Similar results were obtained with another low activity channel.

The moderate activity channel illustrated in Fig. 7B displayed $P_{MCa} = 0.95$ and $P_{oB} = 0.28$ in 10 μM cytoplasmic [$Ca^{2+}$] plus 10 μM cytoplasmic [$Mg^{2+}$] and 5 nM MCa (top record). Subsequent increase of cytoplasmic [$Mg^{2+}$] to 30 μM had negligible effect on both $P_{MCa}$ and $P_{oB}$ (center record), whereas further increase to 100 μM free [$Mg^{2+}$] decreased $P_{MCa}$ to 0.24 and $P_{oB}$ to 0.09 (lower record). In three independent experiments with moderate activity channels, increasing cytoplasmic [$Mg^{2+}$] reduced $P_{MCa}$ and $P_{oB}$ with $K_{0.5}$ of $72 \pm 19$ μM and $61 \pm 13$ μM, respectively. Therefore, the apparent affinity values of cytoplasmic $Mg^{2+}$ for inhibition of $P_{MCa}$ and $P_{oB}$ were comparable ($p = 0.662$), favoring the

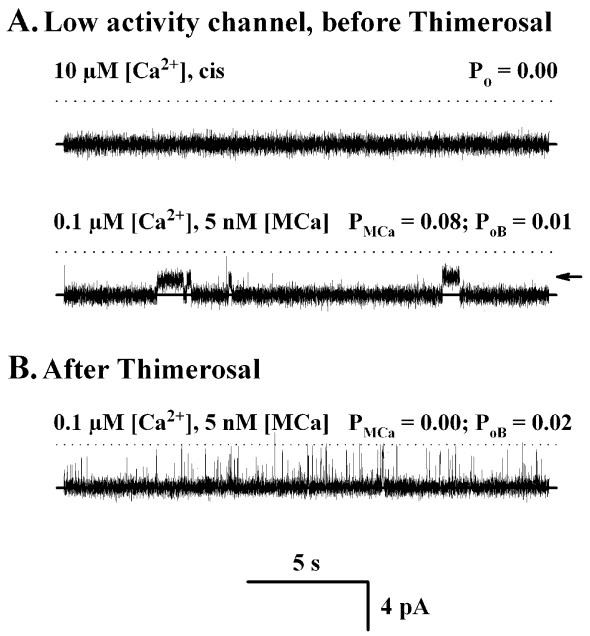


**Fig. 6.** Thimerosal treatment inhibited MCa activation of a single RyR1 channel at 0.1 μM [$Ca^{2+}$]. Panel A shows representative current recordings obtained from a single low activity channel at the indicated cytoplasmic [$Ca^{2+}$] before (upper trace) and after addition of MCa (lower trace). Sub-conductance current level is indicated, as usual, with an arrow at the right of lower trace. $P_{MCa}$ and $P_{oB}$ values, calculated for the entire recorded periods (at least 70 s) and cytoplasmic [$Ca^{2+}$] are displayed above each trace. Panel B was obtained with the same channel recorded in Panel A, after incubation with 200 μM thimerosal for 80 s followed by extensive perfusion of the cis chamber to eliminate non reacted thimerosal (for further details, see Section 3). At 0.1 μM [$Ca^{2+}$], addition of 5 nM MCa did not elicit the sub-conductance state displayed by the channel before incubation with thimerosal (compare trace in Panel B with lower trace in Panel A).

possibility that both effects are related. Moreover, the apparent affinity of $Mg^{2+}$ inhibitory effects is seemingly higher in low than in moderate activity RyR1 channels.

## 4. Discussion

As pointed out earlier [27], $Ca^{2+}$ release from the SR is significantly more sensitive to redox agents than $Ca^{2+}$ uptake. The remarkable effects of redox agents on SR $Ca^{2+}$ release reside in the great sensitivity of RyR1 channels to oxidizing/reducing agents, which by changing RyR1 redox state define the response of the channel to agonists and inhibitors. Several authors have proposed that RyR channels act as cellular redox sensors [27–30]. The redox state of a few highly reactive cysteine residues, defined as such because they react with redox agents at physiological pH, determines RyR channel activation/inhibition by physiological agonists/inhibitors, and in particular, it conditions how RyR channels respond to changes in cytoplasmic [$Ca^{2+}$] or [$Mg^{2+}$] [4].

The results presented in this work show that redox reagents modify the agonist effects of MCa on skeletal RyR1 in a complex manner. The sulfhydryl-reducing reagent DTT, which strongly inhibits RyR1 channel activation by $Ca^{2+}$ [8], did not prevent the stimulation of $Ca^{2+}$ release, or the opening of RyR1 single channels to a sub-conductance state, produced by MCa addition at near resting [$Ca^{2+}$] levels (0.1 μM [$Ca^{2+}$]), or the increase in [$^3$H]-ryanodine binding density to skeletal SR vesicles. In contrast, at 0.1 μM [$Ca^{2+}$] the oxidizing reagent thimerosal prevented both MCa-induced $Ca^{2+}$ release from SR vesicles and the emergence of the typical sub-conductance state induced by MCa in single RyR1 channels. Thus, the present results concerning the effects of redox agents on the agonist action of MCa on $Ca^{2+}$ efflux, ryanodine binding and single

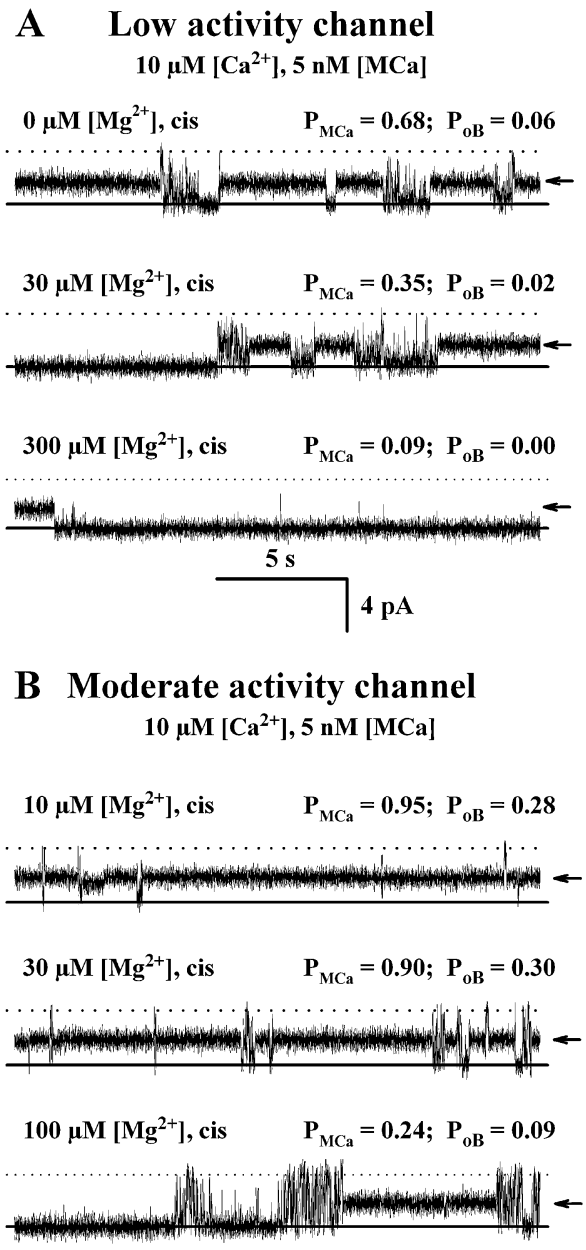


**Fig. 7.** $Mg^{2+}$ inhibits the effects of Maurocalcine on low and moderate activity RyR1 channels. Representative current recordings were obtained, at 10 μM cytoplasmic [$Ca^{2+}$] and in the presence of 5 nM MCa, from single channels that spontaneously displayed either low (Panel A) or moderate activity (Panel B). In each case, the same channel was sequentially exposed to increasing cytoplasmic [$Mg^{2+}$], as indicated near each current trace. $P_{MCa}$ and $P_{oB}$ values, calculated from the entire recorded periods (at least 180 s), are depicted above the recordings. The arrows indicate the sub-conductance current level induced by MCa.

channel sub-conductance opening, provide a consistent framework.

The fact, however, that $P_{MCa}$ decreased as single channel activity diminished when lowering cytoplasmic [$Ca^{2+}$] (Figs. 3 and 4), discards the possibility that the redox-sensitivity of the agonist effects of MCa is simply due to an increase in the apparent affinity of $Ca^{2+}$ binding site(s) involved in RyR1 activation. If this were the case, an oxidizing agent such as thimerosal that favors channel activation by low [$Ca^{2+}$], should have increased $P_{MCa}$; likewise, moderate activity channels should have displayed more activation by MCa than low activity channels, exactly the opposite effects as those observed. Therefore, we propose that in spite of the

inhibition of RyR1 channel activation by $Ca^{2+}$ produced by reducing agents, the reduction of RyR1 cysteine residues enhances the binding affinity of MCa to RyR1. Accordingly, the stimulation by MCa of RyR1-mediated $Ca^{2+}$ release at 0.1 μM [$Ca^{2+}$] under reducing conditions would arise from increased MCa binding to reduced RyR1 (or low activity) channels, which are poorly activated by low [$Ca^{2+}$] but would have high affinity for MCa binding. On the contrary, at resting [$Ca^{2+}$] oxidized RyR1 (or moderate activity) channels would exhibit scant MCa binding in spite of their higher activity, due to their lower affinity for MCa.

The simplest way to explain how both low and moderate activity channels decreased $P_{MCa}$ when channel activity diminished on lowering [$Ca^{2+}$] or increasing [$Mg^{2+}$] (see below) is to propose that the binding site(s) for MCa in RyR1 are freely accessible in the open channel state. This feature may explain why MCa does not elicit $Ca^{2+}$ release when injected into resting skeletal muscle fibers [31], in which RyR1 channels are closed due to their mechanical interaction with the II-III loop of the neighboring DHPR [32–34]. A previous study suggested that MCa binds to RyR1 channels that open on membrane depolarization and that this interaction specifically alters the process of repolarization-induced closure of the channels [31]. Furthermore, voltage-activated low-amplitude local $Ca^{2+}$ signals persist after fiber repolarization in the presence of MCa [35]. Based on these combined results, we propose that MCa binding to open RyR1 channels gives rise to the MCa-induced subconductance state that transiently keeps the channels refractory to DHPR-induced closing after repolarization. A note of caution emerges from the present results regarding the use of MCa to test RyR1 channel function in muscle cells under physiological conditions that promote RyR1 oxidation, since we have shown here that in these conditions MCa is not an efficient RyR1 agonist.

Single channel experiments show that cytoplasmic [$Mg^{2+}$] reduced the agonist effect of MCa on RyR1. Since the apparent affinity of $Mg^{2+}$ for the inhibitory effects on $P_{MCa}$ and on $P_{oB}$ were comparable, the most straightforward mechanism of inhibition of the agonist effect of MCa on RyR1 channel is the inhibition of $P_o$ by $Mg^{2+}$, with no modification in RyR1 affinity for MCa binding. We report here that MCa induces a significant decrease in $Mg^{2+}$ inhibition of [$^3$H]-ryanodine binding to native RyR1 channels, suggesting that MCa binding to the RyR1 protein produces a decrease in the $Mg^{2+}$ affinity of its $Ca^{2+}$/$Mg^{2+}$ inhibitory sites. Previous studies indicate that RyR1 oxidation also decreases RyR1 sensitivity to $Mg^{2+}$ inhibition [9,11]. Moreover, reducing agents seem to increase markedly the affinity of RyR1 channels for MCa, suggesting the presence of cysteine residues in the MCa binding site(s). Maurocalcine binds to two discrete RyR1 regions, fragment 3 (residues 1021–1631) and fragment 7 (residues 3201–3661) [36]. Noteworthy, one cysteine residue susceptible to *S*-glutathionylation (Cys1591) is present in fragment 3 whereas fragment 7 contains a cysteine residue (Cys3635) that is susceptible to *S*-glutathionylation, *S*-nitrosylation and disulfide oxidation [6]. In addition, another cysteine residue (Cys3193), situated only eight amino acids away from the beginning of fragment 7, is also susceptible to *S*-glutathionylation. It would be of interest to test in future studies if MCa binding to RyR1 modifies the environment of one or more of these cysteine residues, resulting in a decreased affinity for $Mg^{2+}$. Conversely, modification of these residues by oxidation with thimerosal might decrease the affinity of MCa binding to RyR1. Accordingly, it would worthwhile to test in future studies if mutations of these three particular cysteine residues affect the stimulation of RyR1 activity by MCa. Nevertheless, with the present information we cannot rule out other possibilities, such as redox modifications of accessory proteins present in the RyR1 complex that might accompany RyR1 after incorporation in the bilayer.

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