Sulfhydryl Oxidation Overrides Mg^{2+} Inhibition of Calcium-Induced Calcium Release in Skeletal Muscle Triads

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ABSTRACT We studied the effect of oxidation of sulfhydryl (SH) residues on the inhibition by Mg^{2+} of calcium-induced calcium release (CICR) in triad-enriched sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle. Vesicles were either passively or actively loaded with calcium before eliciting CICR by dilution at pCa 4.6–4.4 in the presence of 1.2 mM free [ATP] and variable free [Mg^{2+}]. Native triads exhibited a significant inhibition of CICR by Mg^{2+}, with a $K_{0.5} \approx 50$ μM. Partial oxidation of vesicles with thimerosal produced a significant increase of release rate constants and initial release rates at all [Mg^{2+}] tested (up to 1 mM), and shifted the $K_{0.5}$ value for Mg^{2+} inhibition to 101 or 137 μM in triads actively or passively loaded with calcium, respectively. Further oxidation of vesicles with thimerosal completely suppressed the inhibitory effect of [Mg^{2+}] on CICR, yielding initial rates of CICR of 2 μmol/(mg $\times$ s) in the presence of 1 mM free [Mg^{2+}]. These effects of oxidation on CICR were fully reversed by SH reducing agents. We propose that oxidation of calcium release channels, by decreasing markedly the affinity of the channel inhibitory site for Mg^{2+}, makes CICR possible in skeletal muscle.

INTRODUCTION

The ryanodine receptor/calcium release channels (RyR channels) from skeletal muscle sarcoplasmic reticulum (SR) are regulated by multiple cellular components. These comprise several ions and molecules, such as Ca^{2+}, Mg^{2+}, H^+, ATP, and cyclic ADP-ribose, metabolic reactions including phosphorylation and oxidation, and interaction with other proteins, among them the dihydropyridine receptors (DHPR), calmodulin, FKBP12, triadin, and calsequestrin (Coronado et al., 1994; Meissner, 1994; Franzini-Armstrong and Protasi, 1997; Zucchi and Ronca-Testoni, 1997).

The redox state of the channel protein has a marked effect on the activity of RyR channels from skeletal and cardiac muscle. Oxidation of sulfhydryl (SH) groups induces the release of calcium from SR vesicles (Trimm et al., 1986; Zaidi et al., 1989; Prabhu and Salama, 1990; Salama et al., 1992; Abramson et al., 1995), activates RyR channels incorporated in planar lipid bilayers (Abramson et al., 1995; Favero et al., 1995; Eager et al., 1997; Marengo et al., 1998), and modifies ryanodine binding to SR membranes (Abramson et al., 1995; Favero et al., 1995; Aghdasi et al., 1997; Suko and Hellman, 1998). Highly reactive SH groups of the channel protein participate in interactions between homotetrameric channel subunits (Wu et al., 1997) in the formation of high-molecular-weight complexes with triadin (Liu et al., 1994; Liu and Pessah, 1994) and in calmodulin binding (Zhang et al., 1999; Moore et al., 1999). All protein-protein interactions that comprise or are mediated by SH groups could be part of the normal RyR channel gating mechanism.

The calcium release channels are localized in an ordered array in the SR terminal cisternae, in which every other channel molecule is associated with a DHPR of the transverse tubule membrane (Franzini-Armstrong and Kish, 1995). The transient depolarization of the transverse tubules during muscle stimulation induces a DHPR conformational change that is in turn sensed by the associated RyR channels, which open and allow calcium release from the SR (Ríos and Pizarro, 1991). It has been proposed that the ensuing increase in local [Ca^{2+}] at the triad opens the nonassociated RyR channels through calcium-induced calcium release (CICR), amplifying the overall release process (Ríos and Stern, 1997). However, the existence of CICR in mammalian skeletal muscle has been questioned (Shirokova et al., 1998). In addition, in vitro studies indicate that Mg^{2+} is a potent inhibitor of CICR at the concentrations found in skeletal muscle (Meissner et al., 1986; Moutin and Dupont, 1988; Donoso and Hidalgo, 1993). Accordingly, for CICR to be physiologically relevant in vivo, some mechanism(s) should exist to overcome the powerful inhibitory effect that Mg^{2+} exerts on this process.

We have recently shown that oxidation of SH groups modifies the calcium-dependence of RyR channels incorporated in planar bilayers, particularly decreasing the inhibition of skeletal muscle channels by 0.5 mM [Ca^{2+}] (Marengo et al., 1998). Because the inhibitory sites for Ca^{2+} seem to be the same as those for Mg^{2+} (Meissner et al., 1986, 1997; Laver et al., 1997a, b), we investigated in this work whether SH oxidation decreased the Mg^{2+} inhibition of skeletal RyR channels. For this purpose, the time courses of calcium release from native and oxidized skeletal SR vesicles were determined as a function of free Mg^{2+} concentration. The results obtained indicate that oxidation suppressed the inhibitory effect of Mg^{2+} on CICR, thus pro-

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viding a potential physiological mechanism of RyR regulation.

MATERIALS AND METHODS

Isolation and oxidation of triads

Triads were isolated from rabbit fast skeletal muscle in the presence of a combination of protease inhibitors, as described previously (Donoso and Hidalgo, 1993). Protein was determined according to Hartree (1972) using bovine serum albumin as standard. Triads were stored at −80°C for up to one month.

SH groups were oxidized at 25°C by incubating triads (passively or actively loaded with calcium) with 0.25–0.5 mM thimerosal for variable lengths of time. For passive calcium loading, triads (1 mg/ml) were equilibrated at 25°C for 3 h with calcium in a solution containing (in mM) 2 $CaCl_2$, 150 KCl, and 20 imidazole-MOPS, pH 7.2. Thimerosal was added after this time and incubation was continued at 25°C for up to 15 min. Triads were actively loaded with calcium at 25°C by incubating vesicles (1 mg/ml) in a solution containing (in mM) 0.05 $CaCl_2$, 150 KCl, 5 $MgCl_2$, 20 imidazole-MOPS, pH 7.2, 5 ATP, and 10 phosphocreatine plus 15 U/ml creatine kinase. Thimerosal was added 5 min after initiating active loading, and oxidation was continued during active loading for up to 15 min at 25°C.

Calcium release kinetics

We measured calcium release kinetics in a SX.18MV fluorescence stopped-flow spectrometer from Applied Photophysics Ltd. (Leatherhead, UK). The increase in extravesicular [Ca^{2+}] was determined by measuring the fluorescence of different calcium indicators, Fluo-3, Calcium Green-2, Calcium Green-5N, or Fluo-5N (Molecular Probes, Eugene, OR) selected according to the pCa range required (see figure legends). The fluorescent emission of these dyes was measured through a 515 nm cutoff long-pass filter, using an excitation wavelength of 488 nm. Calcium release was initiated by mixing 1 volume of the solution containing calcium loaded triads, native or oxidized, with 10 volumes of releasing solution containing (mM): 150 KCl, 20 imidazole-MOPS, pH 7.2, plus 1 μM of the respective fluorescent calcium indicator. To obtain after mixing 1.2–1.4 mM free [ATP] and variable free [Ca^{2+}] and free [Mg^{2+}], varying concentrations of ATP and $MgCl_2$, calculated as detailed below, were added to the releasing solution. Varying [ATP] and Mg^{2+} did not cause differences in ionic strength beyond 25%; these differences were taken into account when calculating the free Mg^{2+}, Ca^{2+}, and ATP concentrations of the releasing solutions.

Total calcium released

To determine the effects of increasing [Mg^{2+}] on the initial rates of calcium release $v_i = kN_{max}$, where k is the rate constant and N_{max} the total amount of calcium released, the values of k and N_{max} at different [Mg^{2+}] should be known. The values of k were obtained from the kinetic experiments described above. The values of N_{max} were measured directly by filtration of triads, native or oxidized with 500 μM thimerosal for 10 min, previously loaded with $^{45}CaCl_2$ and diluted as in the kinetic experiments. For this purpose, vesicles (4 mg/ml) were passively equilibrated in 2 mM [Ca^{2+}] as above, adding $^{45}CaCl_2$ to the incubation solution at a specific activity of 3–6 mCi/mmol. Release was induced by 1:10 dilution of calcium-loaded vesicles with releasing solution containing (mM): 150 KCl, 20 imidazole-MOPS, pH 7.2, plus ATP and $MgCl_2$ to yield after mixing pCa 4.6–4.4 (measured with Calcium Green-5N), 1.2 mM free [ATP], and varying free [Mg^{2+}] (calculated as below). Vesicles were filtered immediately under vacuum through Millipore filters (AA, 0.8 μm). Dilution plus filtration lasted 3 s, long enough to complete release even for the slowest rates. The filters were dried without further washing and the amount of calcium remaining in the vesicles was determined in a liquid scintillation counter. The radioactivity nonspecifically associated to the filters was <4% of the total radioactivity.

Free [Ca^{2+}] measurements

All calcium buffers were calculated with the WinMaxC program (www.stanford.edu/~cpatton/winmaxc2.html) using the constants provided in the file bers.ccm. The free [Ca^{2+}] of releasing solutions containing calcium but not Mg^{2+} was checked with a calcium electrode (Orion, Beverly, MA) using a standard commercial kit to calibrate the electrode (WPI, Sarasota, FL). The pCa of releasing solutions containing both calcium and Mg^{2+}, which ranged from 4.6 to 4.4, was determined with Calcium Green-5N. The K_D for Ca^{2+} of Calcium Green-5N was determined in calcium buffered solutions containing (mM): 150 KCl, 20 imidazole-MOPS, pH 7.2.

Materials

All reagents used were of analytical grade. Thimerosal, dithiothreitol, and protease inhibitors (Leupeptin, Pepstatin A, benzamidine, and phenylmethylsulfonyl fluoride) were obtained from Sigma Chemical Co. (St. Louis, MO). All fluorescent dyes were obtained from Molecular Probes, Inc. (Eugene, OR).

RESULTS

Calcium-induced calcium release in the presence of ATP

Triads were passively equilibrated with 2 mM $CaCl_2$ and were diluted 1:10 with solutions that, after mixing, had variable free [Ca^{2+}] and 1.20 mM free [ATP]. The effects of increasing free [Ca^{2+}] from 0.1 μM to 290 μM (pCa 7.0–3.5) on the rate constants k of calcium release are shown in Fig. 1. The bell-shaped calcium dependence found presented maximal values of k, ranging from 45 to 50 s^{-1}, in the pCa range 5–4.4; decreasing or increasing free [Ca^{2+}] beyond this range produced a significant decrease in k, with values <3 s^{-1} at pCa 7 or 3.5. These results indicate that μM [Ca^{2+}] markedly enhanced calcium release even in the constant presence of 1.2 mM free [ATP], demonstrating that in our experimental conditions CICR was maximally stimulated when working in the pCa range 5–4.4. Accordingly, free [Ca^{2+}] within this pCa range was used to measure the effects of increasing [Mg^{2+}] on CICR, as detailed below.

Effects of increasing free [Mg^{2+}] on calcium-induced calcium release

We selected Calcium Green-5N to measure calcium release in the pCa range 5–4.5 because, in our assay conditions, its fluorescence increased swiftly upon mixing the dye with calcium-containing solutions with a K_D of 27.5 μM. In addition, the fluorescence response of this dye toward cal-

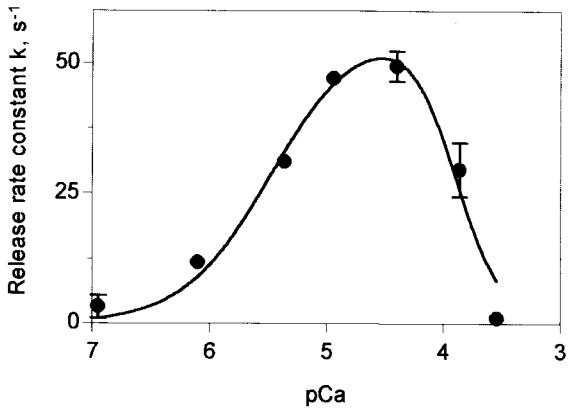

FIGURE 1 Effect of increasing extravesicular [Ca^{2+}] on the rate constants of calcium release. Triads were passively equilibrated with 2 mM $CaCl_2$ and were diluted 1:10 with solutions that, after mixing, had the variable free [Ca^{2+}] indicated in the figure plus 1.20 ± 0.02 mM free [ATP] (mean ± SD). The rate constants of calcium release were obtained from single exponential fitting of experimental records such as those shown in Fig. 2. To cover the pCa range indicated in the figure, different calcium indicators were used. Fluo-3 (K_D = 0.39 μM) was used in the pCa range 7.0–6.0; Calcium Green-2 (K_D = 0.59 μM) for the pCa range 6.0–4.9; Calcium Green-5N (K_D = 27.5 μM) for the pCa range 4.9–3.9, and Fluo-5N (K_D = 90 μM) for pCa values ranging from 3.9 to 3.5. For further details, see text.

cium was not affected by addition of up to 2 mM free [Mg^{2+}] (data not shown).

The time courses of CICR from native and oxidized triads, passively loaded with calcium and measured at free [Mg^{2+}] ranging from 21 to 352 μM, are illustrated in Fig. 2. In all cases fluorescent signals increased with time following a single exponential function with a rate constant *k*, producing a small increment in free [Ca^{2+}] < 2 μM. Increasing free [Mg^{2+}] had a strong inhibitory effect on native triads but less markedly inhibited CICR from triads oxidized with 500 μM thimerosal for 5 min. In native triads increasing free [Mg^{2+}] from 21 to 352 μM produced a substantial decrease in *k*, from 42 s^{-1} to 3 s^{-1} (Fig. 2 *A*). Triads incubated with thimerosal showed higher release rate constants at every [Mg^{2+}] tested (Fig. 2 *B*), yielding a *k* value as high as 88 s^{-1} in 21 μM [Mg^{2+}]. The inhibition by Mg^{2+} was less pronounced in oxidized triads than in native triads. Thus, in the experiment depicted in Fig. 2, increasing free [Mg^{2+}] to 352 μM decreased *k* to 32 s^{-1} in oxidized triads and to 3 s^{-1} in native triads.

A detailed comparison of the effects of increasing free [Mg^{2+}] on release rate constants is illustrated in Fig. 3. In native triads passively loaded with calcium (Fig. 3, *top panel*) the release rate constants *k* were strongly dependent on [Mg^{2+}] with a $K_{0.5}$ of 47.3 ± 2.5 μM (n = 4). Oxidation with 500 μM thimerosal for 5 min produced a marked increase in *k* at all [Mg^{2+}] tested (Fig. 3, *middle panel*), and shifted the $K_{0.5}$ for Mg^{2+} inhibition to 137.2 ± 21.1 μM (n = 4). Whereas in the absence of Mg^{2+} native triads had *k* values of 37 s^{-1}, values of *k* as high as 90 s^{-1} were obtained in triads oxidized with 500 μM thimerosal for 5 min. Further oxidation for 10 min with 500 μM thimerosal annulled both the above stimulation of *k* values and the inhibitory effect of Mg^{2+} on CICR, yielding in the free [Mg^{2+}] range from 0 to 440 μM a mean *k* value of 41.2 ± 5.5 (n = 11) (Fig. 3, *bottom panel*).

To test whether endogenous phosphorylation modified the inhibitory effects of Mg^{2+} on channel activity, we measured CICR on triads actively loaded with calcium, exploiting the fact that extensive phosphorylation of triad proteins by endogenous kinases occurred during active calcium loading (G. Barrientos and C. Hidalgo, unpublished observations). Fig. 4 (*top panel*) illustrates the effects of increasing free [Mg^{2+}] on release rate constants in triads endogenously phosphorylated during active calcium loading. These triads had a $K_{0.5}$ value for Mg^{2+} inhibition of 45.7 ± 9.2 μM (n = 3). This value is comparable to the $K_{0.5}$ value of 47.3 ± 2.5 μM obtained for Mg^{2+} inhibition in triads passively loaded with calcium, that presumably were minimally phosphorylated. Oxidation of actively loaded triads with 250 μM thimerosal for 1 min (Fig. 4, *middle panel*) shifted the $K_{0.5}$ value for Mg^{2+} inhibition to 101.2 ± 22.6 μM (n = 3) and increased *k* values at all free [Mg^{2+}] tested. Extensive oxidation with 500 μM thimerosal for 10 min (*bottom panel*) annulled both the stimulatory effects of partial oxidation and the inhibitory effect of Mg^{2+} on CICR. A mean *k* value of 24.5 ± 4.0 s^{-1} (n = 21) was obtained in triads more extensively oxidized with thimerosal in the free [Mg^{2+}] range from 24.8 μM to 1 mM (Fig. 4, *bottom panel*).

Different vesicular preparations presented somewhat different kinetics of oxidation with thimerosal. Thus, partial or complete suppression of Mg^{2+} inhibition of CICR were not always obtained in the same conditions of thimerosal concentration or time of incubation, albeit moderate variations among preparation were found. In the triad preparation actively loaded with calcium illustrated in Fig. 5, incubation with 250 μM thimerosal for 1 min increased $K_{0.5}$ from 64.1 to 135.8 μM; additional incubation with 250 μM thimerosal for 5 or 10 min increased $K_{0.5}$ to 394.3 or 433.2 μM, respectively. In the same preparation, incubation with 500 μM thimerosal for only 1 min increased the $K_{0.5}$ value to 432.6 μM, and incubation for 5 min, to 931.3 μM. Further incubation with 500 μM thimerosal for 10 min completely suppressed the inhibitory effect of Mg^{2+} on CICR, so that a $K_{0.5}$ value could not be calculated. These results indicate that oxidation of SH residues with thimerosal produced a progressive reduction in the affinity of the Mg^{2+} inhibitory site.

The effects of thimerosal on calcium release were fully reversed by dithiothreitol (DTT). Fig. 6 shows the time course of calcium release from oxidized triads passively loaded with calcium in the presence of 129 μM free [Mg^{2+}], which had a rate constant of 66 s^{-1}. If, after

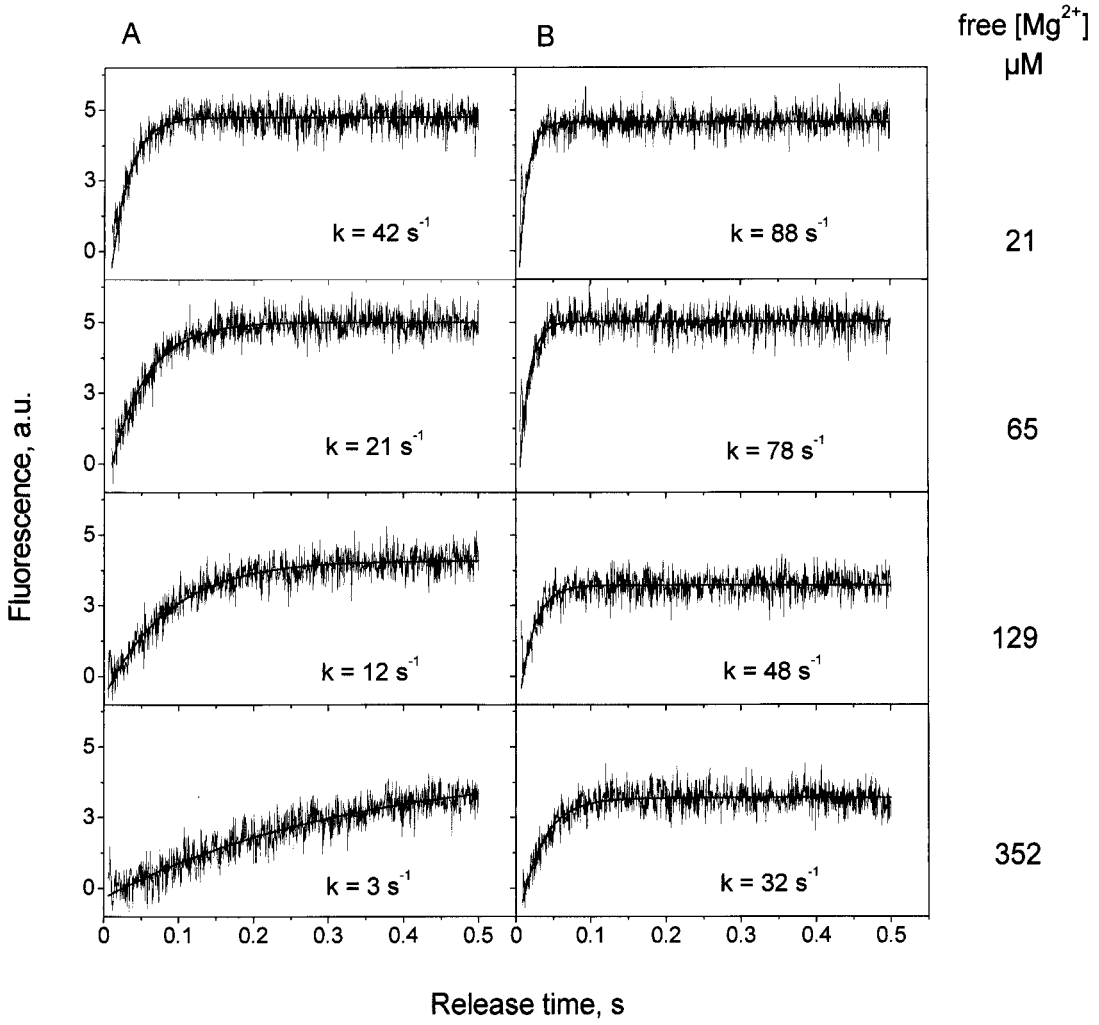

FIGURE 2 Effect of $[Mg^{2+}]$ on calcium release kinetics in native and oxidized triads. Triads (1 mg/ml) equilibrated in 2 mM $CaCl_2$, 150 mM KCl, 20 mM imidazole-MOPS, pH 7.2 were mixed (1:10) in a stopped-flow fluorescence spectrometer with a solution containing 150 mM KCl, 20 mM imidazole-MOPS, pH 7.2, 1 μM Calcium Green-5N, and the required [ATP] and $[MgCl_2]$ to obtain (after mixing) pCa 4.6–4.4, 1.2 mM free ATP, and the indicated free $[Mg^{2+}]$. (*A*) Native triads; (*B*) triads oxidized for 5 min with 500 μM thimerosal. Each trace was obtained by averaging seven different traces. The solid line represents the fitting to a single exponential function.

oxidation of triads with thimerosal, 5 mM DTT was added for 5 min before initiating CICR, the time course of release became slower, with $k = 13\ s^{-1}$. This latter value is practically the same as the rate constant of $12\ s^{-1}$ displayed by native vesicles at the same free $[Mg^{2+}]$ (Fig. 2 *A*). Incubation of native triads with 5 mM DTT alone did not modify the rate constant at all $[Mg^{2+}]$ tested (data not shown).

The data shown in Table 1 indicate that the total amount of calcium released by native or oxidized triads passively loaded with calcium was fairly constant, irrespective of the free $[Mg^{2+}]$ present during release. On average, 47.6 ± 0.7 nmol of calcium/mg protein were released in native triads and 49.5 ± 2 nmol/mg protein in oxidized triads. As a consequence, higher initial rates of calcium release were calculated in oxidized than in native triads at all free $[Mg^{2+}]$ tested. The initial release rates were 1.7 μmol/(mg × s) in native triads in the absence of Mg^{2+} and decreased to 0.2 μmol/(mg × s) at the highest free $[Mg^{2+}]$ tested, 440 μM. The calculated initial rates in triads oxidized with 500 μM thimerosal for 5 min increased to 4.3 μmol/(mg × s) in the absence of Mg^{2+} and decreased to 1 μmol/(mg × s) in 440 μM $[Mg^{2+}]$. Triads oxidized with 500 μM thimerosal for 10 min had constant initial rates of CICR of 2 μmol/(mg × s) in the 0 to 440 μM $[Mg^{2+}]$ range.

DISCUSSION

To characterize the effects of Mg^{2+} on CICR, we established first that calcium stimulation of calcium release in

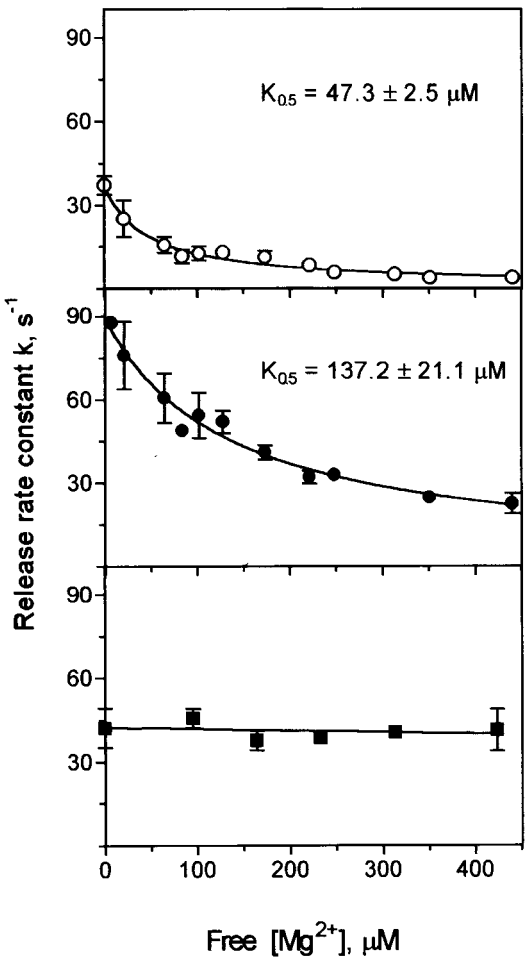

FIGURE 3 Effect of [Mg^{2+}] on calcium release rate constants in native and oxidized triads passively loaded with calcium. The rate constants k of calcium release were calculated from single exponential fitting of experiments such as those shown in Fig. 2. The solid line represents the best fitting to the equation: $k = k_{max}\ K_{0.5}/([Mg^{2+}] + K_{0.5})$, where k_{max} is the value of k in the absence of Mg^{2+} and $K_{0.5}$ represents the concentration of Mg^{2+} that produced 0.5 k_{max}. *Open symbols:* native triads; *filled circles:* triads oxidized with 500 μM thimerosal for 5 min; *filled squares:* triads oxidized with 500 μM thimerosal for 10 min. Data are given as mean ± SEM.

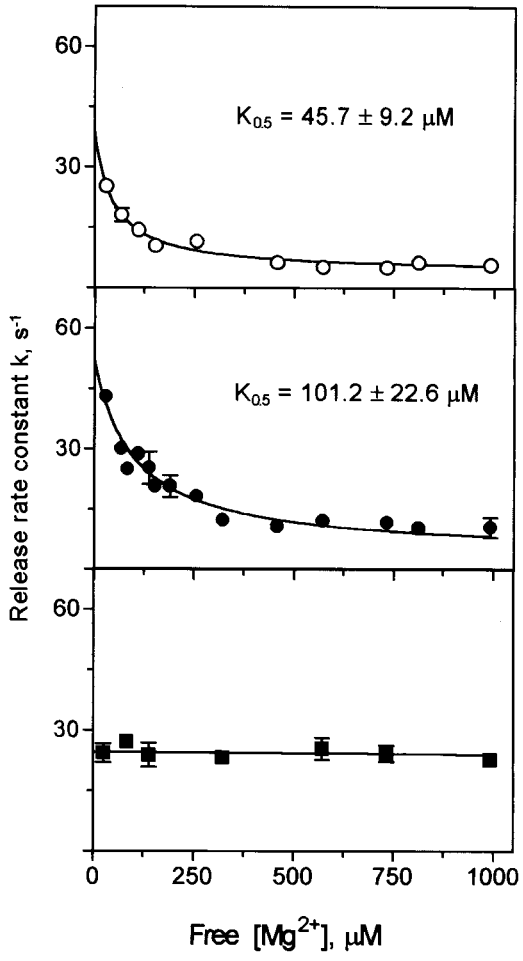

FIGURE 4 Effect of [Mg^{2+}] on calcium release rate constants in native and oxidized triads that were endogenously phosphorylated during active calcium loading. The rate constants k of calcium release were calculated from single exponential fitting of experiments such as those shown in Fig. 2. The solid line represents the best fitting to the equation: $k = k_{max}\ K_{0.5}/([Mg^{2+}] + K_{0.5})$, where k_{max} is the value of k in the absence of Mg^{2+} and $K_{0.5}$ represents the concentration of Mg^{2+} that produced 0.5 k_{max}. *Open symbols:* native triads; *filled circles:* triads oxidized with 250 μM thimerosal for 1 min; *filled squares:* triads oxidized with 500 μM thimerosal for 10 min. Data are given as mean ± SEM.

native triads took place in the presence of 1.2 mM free [ATP]. We found that CICR was maximal in the pCa range 5.0–4.4, 1.2 mM free [ATP], pH 7.2. It has been previously reported that these concentrations of Ca^{2+} and ATP induce full activation of calcium release channels, producing release of 70–80% of the passively equilibrated calcium in the millisecond time range (Moutin and Dupont, 1988; Donoso and Hidalgo, 1993; Donoso et al., 1995).

Effects of [Mg^{2+}] on CICR in native triads

The results presented in this work show that Mg^{2+}, in the presence of 1.2–1.4 mM free ATP and in the pCa range optimal for CICR, 4.6–4.4, was a potent inhibitor of calcium release from native triads. Thus, native triads passively loaded with calcium displayed a $K_{0.5}$ for Mg^{2+} inhibition of 47.3 ± 2.5 μM, whereas native triads endogenously phosphorylated during active calcium loading had a $K_{0.5}$ of 45.7 ± 9.2 μM. These values are not statistically different and indicate that, at the concentrations of free ATP and ≈1 mM [Mg^{2+}] present in skeletal muscle cells (Konishi, 1998), CICR would be completely inhibited in vivo after activation of voltage-induced calcium release.

Although there are many reports describing the inhibitory effect of Mg^{2+} on CICR, only few reports have described inhibition constants for Mg^{2+}, giving values ranging from

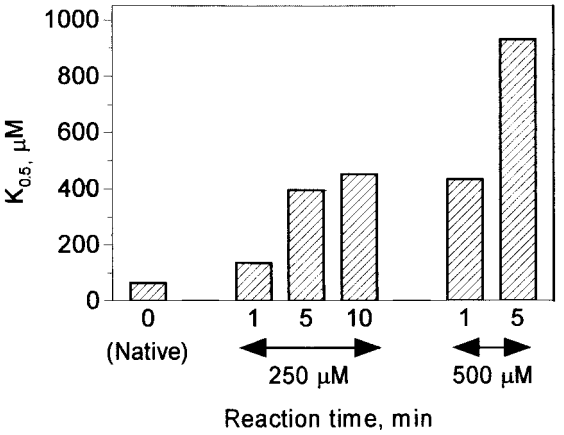

FIGURE 5 Effects of progressive SH oxidation on the rate constants of CICR. Triads were actively loaded with calcium as detailed in the text before oxidation with 250 or 500 μM thimerosal for the times indicated in the figure. Calcium release rate constants were calculated from single exponential fitting of experiments such as those shown in Fig. 2, and $K_{0.5}$ values for Mg^{2+} inhibition were calculated as described in the legend for Fig. 3.

15 to 230 μM (Meissner et al., 1986; O'Brien, 1986; Moutin and Dupont, 1988; Carrier et al., 1991). However, a comparison among these values is encumbered by the fact that a variety of calcium and nucleotide concentrations that affect channel activity by themselves were used in these determinations. Furthermore, according to current models (Laver et al., 1997a; Lamb and Laver, 1998) binding of Mg^{2+} to two independent sites causes inhibition of calcium release. At low cytoplasmic calcium concentration ($<$0.1 μM for skeletal muscle) Mg^{2+} competes with calcium for the high-affinity activator site. At higher calcium concentrations ($>$10 μM) Mg^{2+} binds to the inhibitory site that

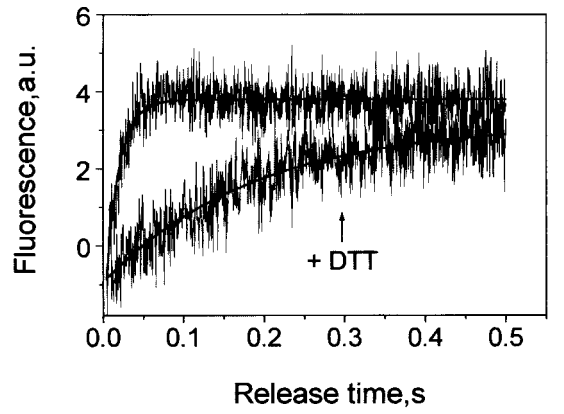

FIGURE 6 Reversibility of the oxidation of triads with thimerosal. Triads passively loaded with calcium were oxidized with 500 μM thimerosal for 5 min and were further incubated with 5 mM DTT for 5 min before inducing CICR as in Fig. 2. The free [Mg^{2+}] of the releasing solution was 129 μM. Each trace was obtained by averaging three different traces. The solid line represents the fitting to a single exponential function.

TABLE 1 Effects of SH oxidation on the total amount of calcium released by triads passively loaded with calcium

Free [Mg^{2+}] (μM)	Calcium released (N_{max}) (nmol/mg protein)	
	Native	Oxidized
7.7	46.9 ± 2.4	47.6 ± 0.4
99	48.9 ± 2.8	48.8 ± 4.0
240	48.2 ± 1.4	53.1 ± 1.7
440	47.6 ± 2.7	48.8 ± 2.0
Average	47.9 ± 0.7	49.6 ± 2.1

The amount of calcium released was measured by filtration of native or oxidized triads, passively loaded with 2 mM $^{45}CaCl_2$, 3 s after dilution in solutions with a final pCa of 4.6–4.4, 1.2 mM free ATP, and the indicated free [Mg^{2+}]. Values represent the average ± SEM of two determinations performed in triplicate on different preparations. The total amount of calcium loaded by the vesicles was 67.5 ± 0.6 nmol/mg protein. For further details, see the Materials and Methods section.

also binds calcium and is responsible for the inhibition observed at mM concentrations of Ca^{2+} or Mg^{2+} (Meissner et al., 1986; Laver et al., 1997a).

It has been reported that endogenous phosphorylation decreases the activity of single RyR channels from skeletal muscle incorporated in lipid bilayers (Hain et al., 1994). However, in our conditions native triads that were extensively phosphorylated during active calcium loading displayed the same release kinetics and high-affinity Mg^{2+} inhibitory sites as native triads passively loaded with calcium that were presumably not phosphorylated. Accordingly, RyR channels in vesicles behave differently in this regard from RyR channels incorporated in planar lipid bilayers.

SH oxidation modified the inhibitory effect of Mg^{2+} on CICR

Partial oxidation of SH residues with thimerosal produced a marked increase of the rate constants of CICR at all free [Mg^{2+}] tested, and shifted the $K_{0.5}$ for Mg^{2+} inhibition from 46–47 μM to 101–137 μM. Thus, k values as high as 90 s^{-1} were obtained in the absence of Mg^{2+} in triads passively loaded with calcium and partially oxidized with thimerosal. More extensive oxidation of SH groups with thimerosal reversed the stimulation of release rate constants produced by partial oxidation and completely suppressed the Mg^{2+} inhibitory effect on CICR. Thus, triads actively loaded with calcium and extensively oxidized with thimerosal displayed even in 1 mM [Mg^{2+}] the same k values that were obtained in the absence of Mg^{2+}. These findings agree with our previous report (Donoso et al., 1997) showing that oxidation of SR vesicles with 500 μM 2,2′-dithiodipyridine does not increase the rate constants of ATP-induced calcium release measured without Mg^{2+} at pCa 5.

Oxidation of SH groups with thimerosal or 2,2′-dithiodipyridine modifies the calcium dependence of single RyR

channels incorporated in planar lipid bilayers, and removes the inhibition of skeletal muscle channel activity exerted by $\geq$0.1 mM [Ca^{2+}] (Marengo et al., 1998). Our data showing that SH oxidation reduced at pCa 5 the inhibition of calcium release caused by Mg^{2+} give further support to the idea that Ca^{2+} and Mg^{2+} bind to the same inhibitory site on the calcium release channels.

Inasmuch as the effects of oxidation were fully reversed by subsequent reduction of the oxidized triads with DTT, we can discard nonspecific damage to the vesicles due to thimerosal. It is known that multiple classes of SH residues regulate RyR channel activity (Aghdasi et al., 1997). Accordingly, we interpret the different results obtained with partial and more extensive oxidation with thimerosal as due to sequential reaction of different SH residues of the RyR channel protein, or of accessory proteins (Liu et al., 1994). Oxidation of highly reactive SH residues with thimerosal would enhance channel activity presumably by modifying the channel sites involved in activation by Ca^{2+} (Abramson et al., 1995; Marengo et al., 1998), and at the same time would decrease the affinity of the Mg^{2+} inhibitory site. More extensive oxidation would target less reactive SH residues in progressive fashion, leading eventually to complete suppression of Mg^{2+} inhibition. Additionally, more extensive oxidation would produce a structural rearrangement of the RyR protein, reversing the stimulatory effect of CICR caused by the initial oxidation of the highly reactive SH residues.

Physiological implications

A decrease of Mg^{2+} inhibition by oxidation has been reported in single RyR channels from cardiac muscle incorporated in lipid bilayers (Eager and Dulhunty, 1998). However, to our knowledge, this is the first report that oxidation decreased the inhibitory effect of Mg^{2+} on skeletal RyR channels. As a consequence of the increase in rate constants, the initial rates of calcium release also increased markedly after oxidation. Initial release rate values as high as 4 μmol/(mg $\times$ s) were obtained in partially oxidized triads in the absence of Mg^{2+}. Furthermore, from the $K_{0.5}$ values obtained for Mg^{2+} inhibition, it can be calculated that at the physiological [Mg^{2+}] of 1 mM native or endogenously phosphorylated triads would have release rates $<$0.1 μmol/(mg $\times$ s). These rates are too slow to make a significant contribution to the cytoplasmic [Ca^{2+}] increase required for a single muscle contraction, and may explain why calcium sparks have not been detected in mammalian muscle (Shirokova et al., 1998). Oxidized triads, however, would have release rates of 2 μmol/(mg $\times$ s) at physiological [Mg^{2+}]. These rates are within the range of calcium release fluxes that precede muscle contraction in amphibian skeletal muscle (see Donoso and Hidalgo, 1993, for a discussion of this point).

It has been proposed that the physiological activation of RyR channels by the transverse tubule voltage sensors involves a decrease in the affinity of the Mg^{2+} inhibitory site (Lamb and Stephenson, 1991, 1992, 1994; Lamb and Laver, 1998). Our results indicate that partial oxidation of SH groups decreased the affinity of the skeletal RyR channels for Mg^{2+}, whereas more extensive oxidation completely suppressed this inhibition. Whether channel oxidation takes place during the physiological activation of RyR channels by the voltage sensors, allowing therefore the amplification of the release process by CICR, remains to be tested in vivo.

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