Involvement of Mg^{2+} in terminating Ca^{2+} release in cultured rat skeletal muscle

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Abstract Combined patch-clamp and fura-2 measurements were performed to investigate the mechanism that terminates Ca^{2+} release in rat skeletal myoballs. When cells were intracellularly perfused with solution containing 1 mM free Mg^{2+}, the caffeine (10 mM)-induced Ca^{2+} transient was abruptly terminated by membrane repolarization (−70 mV). With low intracellular Mg^{2+} (e.g. 50 μM) perfusion, however, repolarization failed to terminate the caffeine transient. The results show that intracellular Mg^{2+} is necessary for repolarization-induced closing of the Ca^{2+} release channel.

Key words: Skeletal muscle; Excitation-contraction coupling; Ryanodine receptor; Ca^{2+} release channel; Caffeine; Fura-2

1. Introduction

Contraction of skeletal muscle is triggered by a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) due to release of Ca^{2+} from the sarcoplasmic reticulum (SR) following depolarization of the transverse (T) tubular membranes [1]. It is now functionally well established that the Ca^{2+} release channel responsible for this process is the ryanodine receptor (RyR) [2,3]. Earlier studies on depolarization-induced Ca^{2+} release (or contraction) showed that Ca^{2+} release is turned off by membrane repolarization [4–8], and based on this evidence the rate of Ca^{2+} release was estimated. Since Ca^{2+} release flux started to decay within a millisecond of repolarization, there seemed to be a direct mechanical coupling between the voltage sensor and the release channel [5,9]. Recently, it was shown that caffeine-induced Ca^{2+} release could also be terminated by repolarization (repolarization-induced stop of caffeine-induced Ca^{2+} release: RISC phenomenon) [2]. RISC was observed only when the voltage sensors in T-tubular membranes were in a voltage-activated state. Since caffeine opens the RyR independent of T-tubular membrane depolarization [1,10], the RISC phenomenon suggests that repolarization somehow forces closure of the RyRs/Ca^{2+} release channels even when Ca^{2+} release not associated with the T-tubular membrane depolarization is occurring. More specifically, the deactivation of the dihydropyridine receptors (DHPR) [11] may induce structural alterations in the RyR, resulting in closure of this channel.

Intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) of skeletal muscle is estimated to be around 1 mM [12–16] and appears to be maintained rather constant [12,13,15,16]. The physiological $[Mg^{2+}]_i$ effectively inhibited Ca^{2+} release channel activity in cut muscle fibers [17], fragmented and reconstituted SR vesicles [18–20], and reconstituted RyRs in lipid bilayer membranes [20]. Furthermore, 10 mM Mg^{2+} completely inhibited depolarization-induced contraction in skinned fibers with sealed T-tubules [21,22]. Therefore, Mg^{2+} binding to the RyR induced by repolarization may be a possible step in closing the release channel, as was recently proposed by Lamb and Stephenson [21–23]. The present study was conducted to further address this idea with regard to the RISC phenomenon.

2. Materials and methods

The procedures for preparation of primary cultures of rat myotubes, for monitoring $[Ca^{2+}]_i$ and for controlling membrane potential were essentially as described previously [2]. Briefly, fore- and hind-limbs of newborn rats (1–2 days old) were used for primary cultures of myoblasts. 2 to 3 days after fusion of the myoblasts to form the myotubes, 25 nM colchicine was added for 2 days to obtain round cells. Combined patch-clamp and fura-2 $[Ca^{2+}]_i$ measurements were performed using a computer-controlled data acquisition system (EPC-9; HEKA, Lambrecht, Germany) as described by Neher [24]. The tight-seal (higher than 10–12 GΩ) whole-cell configuration was used to control membrane voltage and to measure ionic current. Liquid junction potential correction (+8 mV) was made before seal formation. For current measurements, series resistance (less than 3.5 MΩ) was partially compensated (by 50–70%). Capacitative currents were corrected before each voltage pulse using the automatic capacitance compensation feature of the EPC-9. Test current was further corrected for linear leakage and capacitative current, in which control current was elicited from −100 mV. $[Ca^{2+}]_i$ was monitored with a photomultiplier-based system where two fluorescence intensities (emission measured at 500 nm, excitation at 360 and 390 nm) were sampled at 2–5 Hz by computer-driven charting program. An in situ calibration was made to estimate the absolute value of $[Ca^{2+}]_i$ as described previously [2,24]. In the present and previous studies [2], cells were treated with colchicine to disrupt the cytoskeleton. This, together with perfusing the cells with relatively high concentrations of fura-2 (100–500 μM) through low series resistance pipettes, effectively reduced the movement artifact due to cell contraction. In fact, the change in the fluorescence intensity induced by cell contraction was less than 5% of total fluorescence, and cells were bigger than the measuring field (cell-derived autofluorescence was subtracted before obtaining the whole-cell configuration). Experiments were performed at room temperature (20–25°C). Standard saline solution contained (in mM): TEA-Cl 150, $CaCl_2$ 3, $MgCl_2$ 1, glucose 11, HEPES 10, pH 7.2. For Ca^{2+} current measurements, 3 μM TTX was added to this solution. Sylgard-coated patch pipettes had resistances between 1.4–2.2 MΩ after filling with the internal solution. The standard internal solution contained (in mM): cesium glutamate 145, fura-2 (potassium salt) 0.1–0.2, NaCl 8, Mg-ATP 8, HEPES-CsOH 10, pH 7.2. The free magnesium concentration was calculated to be around 1 mM assuming an apparent affinity of $6.9 \cdot 10^3$ M^{-1} for Mg^{2+} to ATP [21]. Low Mg^{2+} solution contained (in mM): cesium glutamate 145, fura-2 0.1–0.2, Mg-ATP 1, Na-ATP 7, NaCl 1, HEPES-CsOH 10, pH 7.2 ($[Mg^{2+}]$ = 0.05 mM). For obtaining the calcium transient versus voltage relationship (Fig. 2C,D), fura-2 concentration was 0.5 mM. For calcium current measurement,

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Abbreviations: SR, sarcoplasmic reticulum; T-tubule, transverse tubule; RyR, ryanodine receptor; RISC, repolarization-induced stop of caffeine-induced Ca^{2+} release; DHPR, dihydropyridine receptor; TTX, tetrodotoxin; EGTA, ethylene-glycol-bis-*N,N,N',N'* tetraacetic acid; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethansulphonic acid.

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the following internal solutions were used (in mM): cesium glutamate 145, fura-2 0.05–0.1, NaCl 8, EGTA 10, $MgCl_2$ 5, Mg-ATP 8, HEPES-CsOH 10, pH 7.2 (high Mg^{2+} solution); cesium glutamate 145, fura-2 0.05–0.1, NaCl 1, EGTA 10, Mg-ATP 1, Na-ATP 7, HEPES-CsOH 10, pH 7.2 (low Mg^{2+} solution). Caffeine application was made by local ejection from wide-tipped pipettes. These pipettes contained 10 mM caffeine in nominally calcium free saline solution in which Ca^{2+} was replaced with an equimolar concentration of Mg^{2+}.

3. Results

3.1. Low $[Mg^{2+}]_i$ abolishes RISC whereas RISC is present in 1 mM $[Mg^{2+}]_i$

Membrane repolarization terminates caffeine-induced SR Ca^{2+} release provided Ca^{2+} release is previously activated by depolarizing pulses of at least 0.5 to 1 s (RISC phenomenon) [2]. Fig. 1A shows a control caffeine response during a long-lasting depolarization, in which the cell was first depolarized to −30 mV to induce SR Ca^{2+} release. The transient increase in $[Ca^{2+}]_i$ was due predominantly to Ca^{2+} release from the SR, since removal of extracellular Ca^{2+} or addition of calcium channel blockers such as La^{3+} (0.5 mM) or Ni^{2+} (5 mM) did not significantly alter the shape or the amplitude of $[Ca^{2+}]_i$ change [2]. After $[Ca^{2+}]_i$ had started to decline, 10 mM caffeine was applied during the depolarization, causing a secondary increase in $[Ca^{2+}]_i$, presumably due to enhancement of SR Ca^{2+} release by a Ca^{2+}-induced Ca^{2+} release mechanism [1,2]. In Fig. 1B, the same protocol as used in Fig. 1A was applied except that the membrane was repolarized immediately after the peak of caffeine transient. Upon repolarization, $[Ca^{2+}]_i$ abruptly dropped to the pre-pulse level despite the continuous presence of caffeine (see arrow). In this case, the pipette solution contained 1 mM free magnesium. Other cells that were perfused with 1 mM $[Mg^{2+}]_i$ solution invariably showed abrupt and complete termination of caffeine-activated Ca^{2+} transient upon repolarization (even 45 min after obtaining the whole-cell configuration) for (long-lasting) depolarizing pulses between −30 and −10 mV ($n = 10$).

Fig. 1C shows that repolarization terminated the caffeine-activated Ca^{2+} transient at all stages of the caffeine response. In this case, the cell was depolarized to −20 mV and repolarized to −70 mV at different stages of the caffeine transient. The result demonstrates that repolarization could change the time derivative of caffeine transient from positive to negative values and supports the previously proposed idea that repolarization somehow forces closure of the release channel and that RISC is not due primarily to depletion of stored Ca^{2+} [2].

However, the degree and the speed of abrogation of the caffeine transient after repolarization was dependent on $[Mg^{2+}]_i$. Fig. 1D,E shows that during perfusion with 0.05 mM $[Mg^{2+}]_i$ solution, the RISC phenomenon gradually disappeared. (In this type of experiment, cells were perfused with low $[Mg^{2+}]_i$ solution from the beginning through the patch-pipettes). Fig. 1D shows that at an early stage of intracellular perfusion, repolarization was effective in partially abrogating the caffeine transient (see section 3). During the early stages of low $[Mg^{2+}]_i$ perfusion, caffeine transients after repolarization had two components, a relatively fast decay followed by a slower decay (plateau) (see Fig. 1D). Immediately after breaking in, there was only the fast component, that is, $[Ca^{2+}]_i$ completely returned to the pre-pulse level after repolarization (data not shown). Then a slower component (plateau) appeared and grew with time. This plateau of $[Ca^{2+}]_i$ remaining after repolarization was due presumably to the caffeine for the following reasons: First, $[Ca^{2+}]_i$ returned to the pre-pulse levels after removal of caffeine. Second, in the absence of caffeine, $[Ca^{2+}]_i$ invariably returned to the pre-pulse levels after repolarization with a single exponential time course. In other words, only the fast component was observed (see Fig. 1E). At a later stage, the time course of $[Ca^{2+}]_i$ decay after repolarization became significantly slow and RISC was no longer observed (Fig. 1E left). It should be noted that even at later stages, repolarization abruptly terminated depolarization-induced Ca^{2+} transient in the absence of caffeine (Fig. 1E right), indicating that Ca^{2+} uptake by the SR was still maintained [21]. Similar results were obtained from 15 other cells.

It should be stressed that during perfusion with low $[Mg^{2+}]_i$ solution (0.05 mM), all the cells tested showed a gradual increase in resting $[Ca^{2+}]_i$, which occasionally led to spontaneous Ca^{2+} release (about 30% of the cells tested) when $[Ca^{2+}]_i$ reached around 0.7 μM. Since low $[Mg^{2+}]_i$ is known to potentiate Ca^{2+}-induced Ca^{2+} release [1], this indicates that $[Mg^{2+}]_i$ was controlled at a sufficiently low level that resting $[Ca^{2+}]_i$ could trigger Ca^{2+} release from the SR by Ca^{2+}-induced Ca^{2+} release mechanism [1]. Indeed, the increase in resting $[Ca^{2+}]_i$ was not observed when cells were perfused with 2 mM fura-2 instead of 0.1–0.2 mM fura-2 in order to increase the Ca^{2+} buffering capacity. However, under these strong Ca^{2+} buffering conditions, regenerative Ca^{2+} release was not observed during perfusion with 0.05 mM $[Mg^{2+}]$ solution, contrary to what was reported by Lamb and Stephenson in rat skinned muscle fibers [22]. One possible explanation for the discrepancy is that the level of $[Mg^{2+}]_i$ attained in the present study was not as low as that in the skinned fiber experiments.

Table 1
The effect of intracellular Mg^{2+} concentration on both the half decay time of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) following repolarization in the absence (DICR) and presence (CafICR, middle) of 10 mM caffeine and the level of $[Ca^{2+}]_i$ 6 s after repolarization in the presence of caffeine (CafICR, bottom)

	1 mM $[Mg^{2+}]$	0.05 mM $[Mg^{2+}]$
DICR	0.63 ± 0.15	0.93 ± 0.44
half decay time(s)	($n = 11$)	($n = 12$)
CafICR	1.56 ± 0.31	6.75 ± 3.57
half decay time(s)	($n = 13$)	($n = 10$)
CafICR	< 10	63.19 ± 14.68
$[Ca^{2+}]_i$ level 6s after repolarization (%)	($n > 30$)	($n = 10$)

The data were taken between 8 and 20 min after obtaining the whole-cell configuration. DICR denotes depolarization-induced Ca^{2+} release (−20 mV depolarization for 5 s) and CafICR denotes caffeine-induced Ca^{2+} release (RISC was induced by +20 mV depolarization for 1 s and subsequent repolarization to −70 mV). Low $[Mg^{2+}]_i$ (0.05 mM) perfusion slowed the half decay time of $[Ca^{2+}]_i$ after repolarization, irrespective of the presence or absence of 10 mM caffeine, although the effect was much stronger in the presence of caffeine. Also, the level of $[Ca^{2+}]_i$ 6 s after repolarization remained elevated in the presence of caffeine (CafICR, bottom). It should be noted that in the present and previous studies [2], fura-2 was used as an intracellular Ca^{2+} indicator. Since the kinetics of fura-2 may be much slower in myoplasm than in the in vitro [32], the half decay time presented in Table 1 may be overestimated, although the general observation may not differ even if fast indicators are used instead of fura-2.

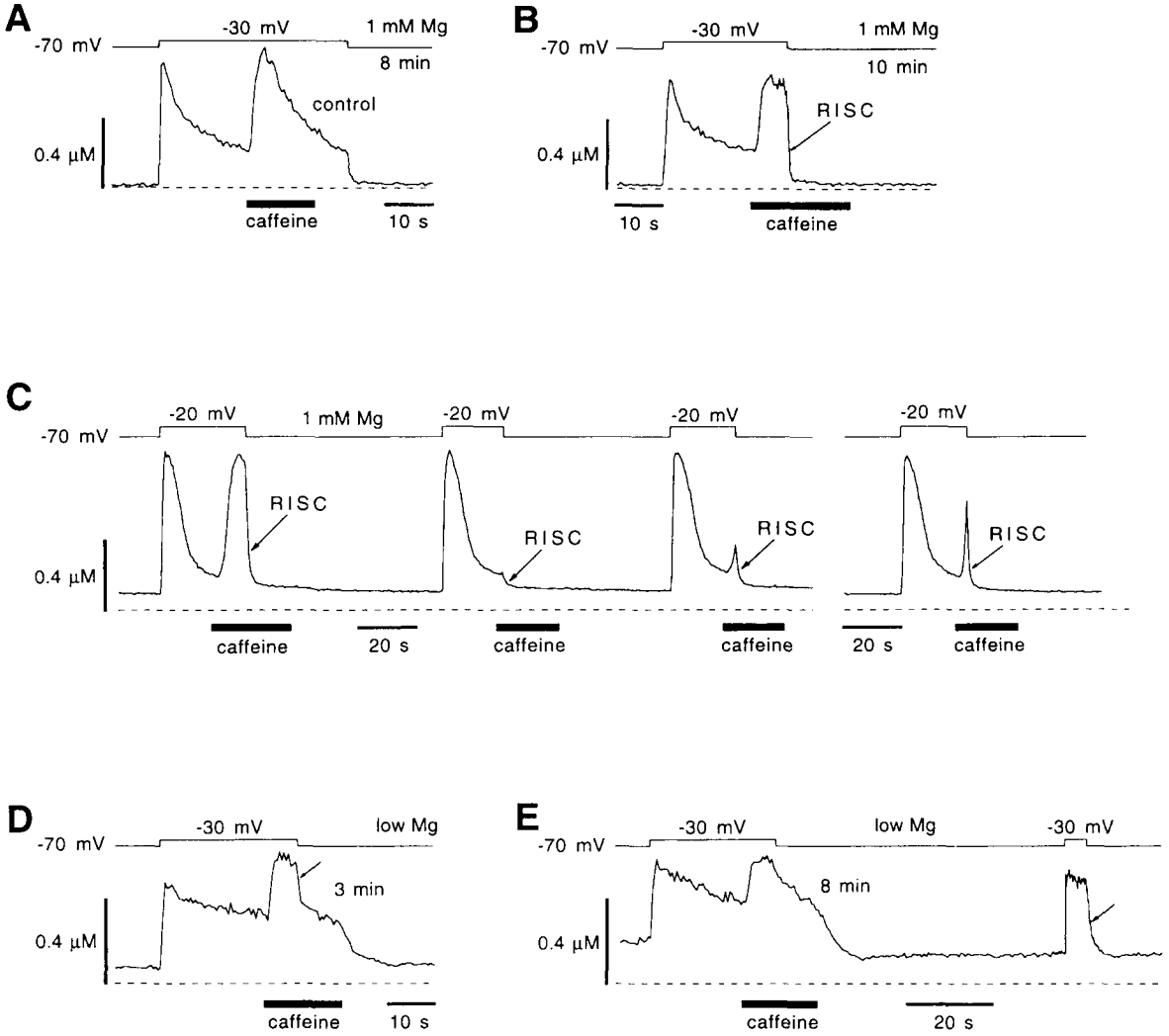

Fig. 1. The effect of $[Mg^{2+}]_i$ on repolarization-induced stop of caffeine-activated SR Ca^{2+} release. A and B were recorded from the same cell which was perfused with internal solution containing 1 mM $[Mg^{2+}]_i$. (A) Control caffeine transient during long-lasting depolarization. The cell was depolarized to −30 mV from a holding potential of −70 mV and then 10 mM caffeine was applied, while keeping the membrane depolarized. (B) Repolarization-induced stop of caffeine-activated Ca^{2+} release (RISC). The same protocol as used in A, except that repolarization was performed 30 s after depolarization. Note that $[Ca^{2+}]_i$ abruptly dropped to the pre-pulse level upon repolarization despite the continuous presence of caffeine. The linear capacitance of the cell C= 208 pF. (C) RISC was observed in all stages of the caffeine-induced Ca^{2+} release. The cell was depolarized to −20 mV and after the decay of $[Ca^{2+}]_i$ 10 mM caffeine was applied to activate SR Ca^{2+} release. Note that repolarization changed the time derivative of caffeine transient even when the rate of Ca^{2+} release was maximum. C= 100 pF. D and E were recorded from the same cell which was perfused with 0.05 mM $[Mg^{2+}]_i$ solution. (D) RISC was partially inhibited 3 min after perfusing with low $[Mg^{2+}]_i$ solution. The same protocol as used in (B). $[Ca^{2+}]_i$ did not return to prepulse level following repolarization (arrow). (E) RISC was completely abolished 8 min after perfusing with low $[Mg^{2+}]_i$ solution. The same protocol as used in B and D. 1 min after the cessation of caffeine application, the cell was further applied depolarizing pulse of −30 mV for 5 s and then repolarized to −70 mV. Note that $[Ca^{2+}]_i$ abruptly returned to the pre-pulse level after repolarization in the absence of caffeine (arrow). C= 103 pF.

3.2. Low $[Mg^{2+}]_i$ abolishes RISC over a wide range of depolarizing potentials

To investigate the effect of stronger depolarizations (>+20 mV) on caffeine-induced Ca^{2+} release, it is desirable to shorten the pulse duration (e.g. 1 s) to reduce the degree of inactivation of the voltage sensors [2]. Therefore, depolarizing pulses were applied just after the peak of caffeine transient as shown in Fig. 2A,B. Fig. 2A shows a control RISC experiment during 1 mM $[Mg^{2+}]_i$ perfusion. 10 mM caffeine was first applied to induce Ca^{2+} release and then, just after the peak of the caffeine transient, a depolarizing pulse of +20 mV was applied for 1 s, before returning the membrane potential to −70 mV (holding potential). Under these conditions, repolarization completely terminated the caffeine transient (see arrow) (n = 15). With low $[Mg^{2+}]_i$ perfusion, however, repolarization no longer curtailed the caffeine transient even for stronger depolarizations, as is shown in Fig. 2B (+20 mV, center; +60 mV, right) (n = 12). The shape of the caffeine transient with the depolarizing pulse was not noticeably different from that without pulse application (control, Fig. 2B left). The inhibitory effect of low $[Mg^{2+}]_i$ on the RISC phenomenon was assessed by monitoring the increase in half decay time, $t_{1/2}$ (the time required for reduction of $[Ca^{2+}]_i$ after repolarization to 50% of the peak value before depolarization) and also the $[Ca^{2+}]_i$ level 6 s after repolarization, expressed

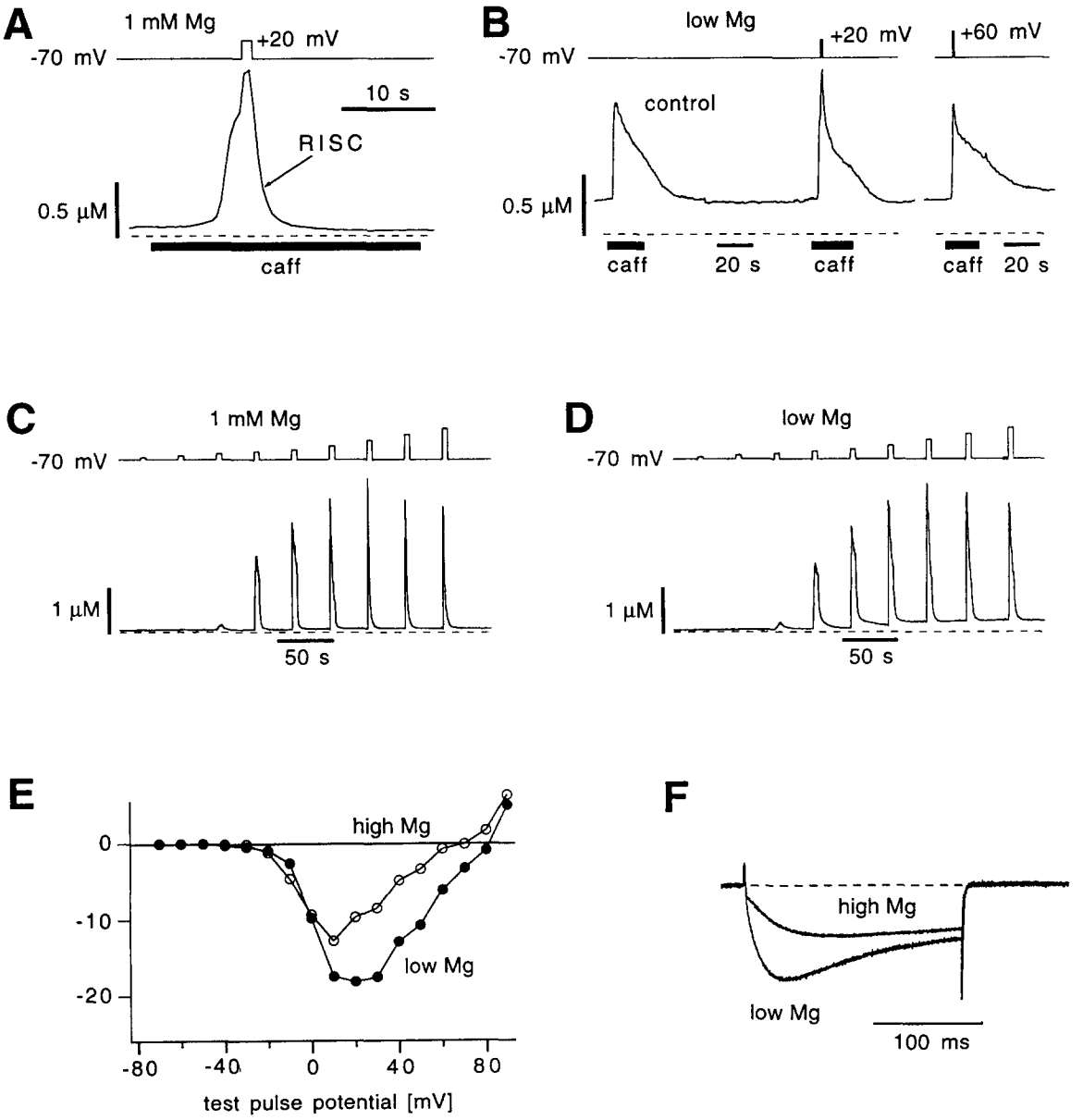

Fig. 2. Low $[Mg^{2+}]_i$ abolishes RISC even at stronger depolarizing potentials. (A) Control RISC experiment in 1 mM $[Mg^{2+}]_i$ perfusion. 10 mM caffeine was applied first, and just after the peak of caffeine transient, a depolarizing pulse of +20 mV was applied for 1 s and then repolarized to −70 mV. Note that repolarization completely terminated caffeine-induced Ca^{2+} release. C= 82 pF. It should be noted that there was a delay between the onset of caffeine application and the start of SR Ca^{2+} release. This suggests that 1 mM $[Mg^{2+}]_i$ effectively inhibited Ca^{2+}-induced Ca^{2+} release triggered by resting $[Ca^{2+}]_i$ [1]. Consistent with this, the delay was shorter in low $[Mg^{2+}]_i$ (see Fig. 2B). (B) Repolarization after strong 1 s depolarizations to +20 mV and +60 mV did not curtail the caffeine transient in low $[Mg^{2+}]_i$ (0.05 mM) perfusion. Left: control caffeine transient in low $[Mg^{2+}]_i$. The cell was applied 10 mM caffeine while keeping membrane potential at −70 mV. Center: the cell was first exposed to 10 mM caffeine to induce SR Ca^{2+} release, and just after the peak of caffeine transient, a depolarizing pulse to +20 mV was applied for 1 s and then the potential was repolarized to −70 mV. Note that repolarization did not curtail the caffeine transient. Right: the same protocol as used in B (center) except that depolarizing potential was +60 mV. C= 436 pF. (C and D) Depolarization-induced Ca^{2+} transient at different test potentials during either 1 mM (C) or 0.05 mM $[Mg^{2+}]_i$ (D) perfusion. (C) The cell was perfused with 1 mM $[Mg^{2+}]_i$ solution. After fluorescence intensity at 360 nm excitation (isosbestic point) reached steady level, depolarizing pulses (5 s) to different potentials were applied every 30 s. The sequence of test potential was (from left to right), −60, −50, −40, −30, −20, 0, +30, +60 and +90 mV. C= 326 pF. (D) The cell was perfused with 0.05 mM $[Mg^{2+}]_i$. The same protocol as used in C. There was no significant difference in the Ca^{2+} transient-voltage relationship between 1 mM $[Mg^{2+}]_i$ (C) and 0.05 mM $[Mg^{2+}]_i$ (D) perfusion, although the rate of decay in $[Ca^{2+}]_i$ during depolarization and after repolarization were slightly slowed in low $[Mg^{2+}]_i$. C= 140 pF. (E) Current–voltage relationship of DHP-sensitive calcium channels in high (≈5 mM) and low (<0.05 mM) $[Mg^{2+}]_i$ solutions. Abscissa represents membrane voltage and ordinate represents peak current amplitude normalized to the linear capacitance of the cells. Current peaked around +10 mV (12.79±3.17 pA/pF) (n = 5) in high $[Mg^{2+}]_i$ and +20 mV (18.18 ± 2.95 pA/pF) (n = 8) in low $[Mg^{2+}]_i$. (F) Low $[Mg^{2+}]_i$ increased the pulse current but did not significantly alter the tail current. Ca^{2+} currents evoked by depolarizations to +20 mV (100 ms) were obtained from different cells which were perfused with different $[Mg^{2+}]_i$ solutions. The Ca^{2+} currents were superimposed and normalized to match the peak amplitude of tail currents. C= 230 pF (high $[Mg^{2+}]_i$) and 116 pF (low $[Mg^{2+}]_i$).

as the percentage of the peak amplitude. In most cases, $[Ca^{2+}]_i$ dropped to lower than 10% of the peak caffeine transient within 5 s during 1 mM $[Mg^{2+}]_i$ perfusion. Table 1 summarizes the effect of $[Mg^{2+}]_i$ on RISC and on the decay of $[Ca^{2+}]_i$ after repolarization (−20 mV depolarization for 5 s) in the absence of caffeine.

3.3. Abolition of RISC in low $[Mg^{2+}]_i$ is not due to $[Mg^{2+}]_i$ effect on the voltage sensor

It is unlikely that the inability of repolarization to curtail caffeine-activated Ca^{2+} transient in low $[Mg^{2+}]_i$ is due to a shift in the voltage dependence of the voltage sensor for the following reasons: first, there was no significant difference in the voltage dependence of the Ca^{2+} transient evoked by 5 s depolarization to different potentials during either 1 mM $[Mg^{2+}]_i$ or 0.05 mM $[Mg^{2+}]_i$ perfusion (Fig. 2C). In this type of experiment, a higher concentration of fura-2 (0.5 mM) was used to prevent regenerative Ca^{2+} release due to Ca^{2+}-induced Ca^{2+} release. The threshold potential to induce SR Ca^{2+} release was invariably between −50 and −40 mV for both 1 mM $[Mg^{2+}]_i$ and 0.05 mM $[Mg^{2+}]_i$ perfusion ($n = 8$). Second, there was no apparent shift of the current–voltage relationship (10 mV at most) between high $[Mg^{2+}]_i$ (≈5 mM) and low $[Mg^{2+}]_i$ (<0.05 mM) perfusion (Fig. 2E). The difference would be even less between 1 mM and 0.05 mM $[Mg^{2+}]_i$ perfusion. It is also unlikely that low $[Mg^{2+}]_i$ reduced the activity of the voltage sensor (DHPR) itself since low $[Mg^{2+}]_i$ usually increased the peak current amplitude ($n = 8$) (Fig. 2E). Augmentation of Ca^{2+} current in low $[Mg^{2+}]_i$ has also been reported in cardiac muscle cells [25]. One may still argue that tail currents may contribute to terminating caffeine-induced Ca^{2+} release. However, this is unlikely for the following reasons: First, in both the present and previous studies, caffeine was applied in nominally Ca^{2+}-free solution, which reduced Ca^{2+} current (high concentrations of caffeine also block DHP-sensitive calcium current [26,27]). Second, low $[Mg^{2+}]_i$ did not significantly increase the size of the tail current relative to that of the pulse current (Fig. 2F) whereas low $[Mg^{2+}]_i$ inhibited RISC over a wide range of depolarizing potentials (−30 to +100 mV) (Fig. 2E).

4. Discussion

The present study shows that the RISC phenomenon was abolished with low $[Mg^{2+}]_i$ (0.05 mM) perfusion (Figs. 1E and 2B) whereas it was present with physiological $[Mg^{2+}]_i$ (1 mM $[Mg^{2+}]_i$) perfusion. Since low $[Mg^{2+}]_i$ is known to enhance SR Ca^{2+} release [17], the present results support the idea that RISC is not due primarily to depletion of stored Ca^{2+}, but to termination of Ca^{2+} release by repolarization as suggested previously [2]. Consistent with this, repolarization curtailed the caffeine-activated Ca^{2+} transient at all stages of the caffeine response (Fig. 1C). The inhibitory effect of low $[Mg^{2+}]_i$ on RISC was not due primarily to a reduction in SR Ca^{2+} pump activity because, under the same conditions, repolarization still abruptly terminated depolarization-induced Ca^{2+} transient in the absence of caffeine (Fig. 1E, right). One may argue that during several applications of caffeine and depolarizing pulses, the Mg^{2+} concentration inside the SR became low because of incomplete substitution of released Ca^{2+} with Mg^{2+} [13], which reduced SR Ca^{2+} pump activity [28]. However, RISC was also abolished even when caffeine and a depolarizing pulse (+20 mV, 1 s) were first applied 10 min after perfusion with low Mg^{2+} solution during which no SR Ca^{2+} release was induced. Nevertheless, I cannot completely exclude the possiblility that low $[Mg^{2+}]_i$ to some extent reduced SR Ca^{2+} pump activity as suggested by Jacquemond and Schneider [29]. It is also unlikely that low $[Mg^{2+}]_i$ directly affected the voltage sensor (DHPR) because there was no significant shift in the Ca^{2+} transient-voltage relationship or current-voltage relationship in low $[Mg^{2+}]_i$. Consistent with this was the fact that low $[Mg^{2+}]_i$ abolished RISC over a wide range of depolarizing potentials (Figs. 1E and 2B).

One possible explanation for abolition of RISC in low $[Mg^{2+}]_i$ is that repolarization may increase the affinity of the RyRs for Mg^{2+} via a mechanical interaction between the voltage sensors and the RyRs. With physiological concentrations of intracellular Mg^{2+} (1 mM), Mg^{2+} binds to the regulatory site of the RyR upon repolarization, which, in turn, induces conformational changes in the RyR, and thereby forces closure of the release channel even when caffeine is bound to the RyR. In low $[Mg^{2+}]_i$, however, repolarization may no longer close the release channel simply because $[Mg^{2+}]_i$ is not high enough to occupy the regulatory site of the RyR as proposed by Lamb and Stephenson [21]. An alternative explanation is that repolarization may induce conformational changes in the RyR via a mechanical interaction between the voltage sensor and the RyR, and thereby force closure of the release channel. In this scheme, Mg^{2+} binding to the RyR alone may not close the release channel. However, the ability of repolarization to close the release channel may be enhanced when the RyR is in Mg^{2+}-bound form.

One curious feature of the RISC time course during the early stages of low $[Mg^{2+}]_i$ perfusion was that the time course of the fast component of $[Ca^{2+}]_i$ decay (just after repolarization) was relatively constant even though the slower component of $[Ca^{2+}]_i$ decay (plateau) increased to more than half the peak amplitude of the caffeine transient (see Fig. 1D). This suggests that most of the release channels were closed just after repolarization (during the fast component) but some of the release channels were reopened again before $[Ca^{2+}]_i$ returned to the pre-pulse level, resulting in a slower component (plateau). This feature could well be explained by the former hypothesis in the last paragraph if we assume that the increased affinity of the RyR for Mg^{2+} just after repolarization decays with time before $[Ca^{2+}]_i$ returns to the pre-pulse level. (This is reasonable because, although caffeine could induce Ca^{2+} release at the resting membrane potential through the Ca^{2+}-induced Ca^{2+} release mechanism [1], it failed to do so immediately after repolarization in 1 mM $[Mg^{2+}]_i$ (RISC). This suggests that the affinity of the RyR for Mg^{2+} is higher just after repolarization than in the resting state. Actually, RISC was transient even in 0.6–1 mM $[Mg^{2+}]_i$. Notice that in the continuous presence of caffeine, some cells showed a secondary increase in $[Ca^{2+}]_i$ from the resting level after RISC (see Fig. 5 of [2]). In this scheme, most of the RyRs could still be closed just after repolarization even though $[Mg^{2+}]_i$ is low, because the affinity of the RyR for Mg^{2+} is extremely high. When the affinity of the RyR for Mg^{2+} decreases to the level where caffeine overcomes this effect, Mg^{2+} may be kicked off from the regulatory site of the RyR, resulting in rapid reopening of the release channel (notice that the RyRs/Ca^{2+} release channels that bind Mg^{2+} are in an open state whereas those that do not bind Mg^{2+} are in a closed state in this scheme). At later stages of low $[Mg^{2+}]_i$ perfusion, however,

some or most of the RyRs may, from the beginning, fail to bind Mg^{2+} upon repolarization, resulting in a slower decay in $[Ca^{2+}]_i$ after repolarization. To explain the RISC time course by the latter hypothesis, one has to know how the release channels can be rapidly reopened from a closed state.

When cells were perfused with 1 mM $[Mg^{2+}]_i$, RISC was invariably complete even 40 min after obtaining whole-cell configuration. In 1 mM $[Mg^{2+}]_i$, RyRs/Ca^{2+} release channels might be kept in a closed state until $[Ca^{2+}]_i$ completely returned to the pre-pulse level, because at this concentration, Mg^{2+}-binding effect might overcome the caffeine effect. In the previous study [2], 0.6 mM $[Mg^{2+}]_i$ solution was primarily used. Under these conditions, abrogation of caffeine-activated Ca^{2+} transient after repolarization was occasionally incomplete especially at later stages of the experiments, suggesting that $[Mg^{2+}]_i$ level is a major determinant of the effectiveness of repolarization to curtail the caffeine transient.

Simon et. al. [30] reported that, in the presence of 0.5 mM caffeine, Ca^{2+} release induced by short depolarizations (10–20 ms) continued 10–60 ms after repolarization, although in the absence of caffeine Ca^{2+} release was turned off within a few milliseconds of fiber repolarization in adult muscle. Therefore, the result obtained in adult muscle appears incompatible with RISC phenomenon observed in cultured myotubes. However, it should be noted that even in cultured myotubes, caffeine significantly slowed the decay of $[Ca^{2+}]_i$ following repolarization after simple depolarization-induced Ca^{2+} release (see Table 1). Furthermore, repolarization terminated caffeine-induced Ca^{2+} release only when cells were depolarized for longer than 0.5 to 1 s prior to repolarization [2]. Consistent with this, in the experiments of Simon et. al. [30], repolarization after longer (>120 ms) depolarizations abruptly changed the time derivative of Ca^{2+} transient from positive to negative values in the presence of caffeine. Therefore, it is not surprising that repolarization especially after short depolarization did not turn off caffeine-activated Ca^{2+} release within a few milliseconds. To clarify whether observations obtained from cultured myotubes apply also to adult skeletal muscle, one needs to conduct adult skeletal muscle experiments over a broader time scale. The possibility remains that the behaviour in mammalian and amphibian skeletal muscle cell is different, because RyRs not coupled to the DHPR may exist in amphibian skeletal muscle, as has been suggested by Rios and Pizarro [31]. In addition, it should be noted that even in the absence of caffeine, low $[Mg^{2+}]_i$ perfusion slowed the decay of $[Ca^{2+}]_i$ after repolarization, compatible with the previous report by Jacquemond and Schneider [29], in which lowering $[Mg^{2+}]_i$ from 1 to <0.14 mM slowed the decay of $[Ca^{2+}]_i$ after repolarization in frog skeletal muscle fibers.

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