Effects of Low Myoplasmic Mg$^{2+}$ on Calcium Binding by Parvalbumin and Calcium Uptake by the Sarcoplasmic Reticulum in Frog Skeletal Muscle

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ABSTRACT The effects of low intracellular free Mg$^{2+}$ on the myoplasmic calcium removal properties of skeletal muscle were studied in voltage-clamped frog skeletal muscle fibers by analyzing the changes in intracellular calcium and magnesium due to membrane depolarization under various conditions of internal free [Mg$^{2+}$]. Batches of fibers were internally equilibrated with cut end solutions containing two calcium indicators, antipyrylazo III (AP III) and fura-2, and different concentrations of free Mg$^{2+}$ (25 μM–1 mM) obtained by adding appropriate total amounts of ATP and magnesium to the solutions. Changes in AP III absorbance were used to monitor [Ca$^{2+}$] and [Mg$^{2+}$] transients, whereas fura-2 fluorescence was mostly used to monitor resting [Ca$^{2+}$]. Shortly after applying an internal solution containing <60 μM free Mg$^{2+}$ to the cut ends of depolarized fibers most of the fibers exhibited spontaneous repetitive movements, suggesting that free internal Mg$^{2+}$ might affect the activity of the sarcoplasmic reticulum (SR) calcium channels at rest. The spontaneous contractions generally subsided. In polarized fibers the maximal amplitude of the calcium transient elicited by a depolarizing pulse was about the same whatever the internal [Mg$^{2+}$], but its decay after the end of the pulse was slower in low [Mg$^{2+}$]. In low [Mg$^{2+}$] (<0.14 mM), the mean rate constant of decay obtained from fitting a single exponential plus a constant to the decay of the calcium transients was ~30% of its value in the control fibers (1 mM internal [Mg$^{2+}$]). A model characterizing the main calcium removal properties of a frog skeletal muscle fiber, including the SR pump and the Ca-Mg sites on parvalbumin, was fitted to the decay of the calcium transients. Results of the fits show that in low internal [Mg$^{2+}$] the slowing of the decay of the calcium transient can be well predicted by both a decreased rate of SR calcium uptake and an expected decreased resting magnesium occupancy of parvalbumin leading to a reduced contribution of parvalbumin to the overall rate of calcium removal. These results are thus consistent with the known properties of parvalbumin as a Ca-Mg buffer and furthermore suggest that in an intact portion of a muscle fiber, the activity of the SR calcium pump can be affected by the level of free Mg$^{2+}$.

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INTRODUCTION

In skeletal muscle, the release of calcium from the sarcoplasmic reticulum (SR) upon membrane depolarization induces a transient rise in free myoplasmic Ca\(^{2+}\) which favors the transient binding of calcium to a series of myoplasmic calcium-binding sites before its eventual reuptake back into the SR. Several earlier studies done on skinned fibers (Stephenson, 1981; Herrmann-Frank, 1989; Lamb and Stephenson, 1991) and SR vesicles or isolated SR calcium channels (Meissner, 1984; Smith, Coronado, and Meissner, 1985, 1986; Meissner, Darling, and Eveleth, 1986; Lai, Erickson, Rousseau, Liu, and Meissner, 1988; Moutin and Dupont, 1988) pointed out a possible role of intracellular free Mg\(^{2+}\) in regulating SR calcium release. On the other hand, the activity of the SR Ca\(^{2+}\)-ATPase depends on the Mg\(^{2+}\) concentration (Martonosi and Beeler, 1983; Bishop and Al-Shawi, 1988), and furthermore, the calcium buffering capability of parvalbumin (Parv) is also expected to be modified by the free [Mg\(^{2+}\)] (Gillis, Thomason, Lefevre, and Kretsinger, 1982).

Using the double Vaseline-gap voltage-clamp technique on cut fibers internally equilibrated with a solution containing calcium indicators, it is possible, from the analysis of the free calcium transient due to membrane depolarization, to calculate the rate of calcium release from the SR in an intact portion of a muscle fiber provided that the calcium removal capability of the fiber can be characterized (Melzer, Rios, and Schneider, 1984, 1987). By modifying the free [Mg\(^{2+}\)] in the internal solution this method can be used to test the efficiency of internal [Mg\(^{2+}\)] as a modulator of the physiologically induced SR calcium release. However, considering the possible [Mg\(^{2+}\)] dependence of both the SR Ca-ATPase and the resting calcium occupancy of Parv (above), a necessary first step was to study the effects of varying the internal free [Mg\(^{2+}\)] on the intrinsic calcium removal properties of the fibers.

This paper describes the main features of the changes in free Ca\(^{2+}\) and Mg\(^{2+}\) that are observed upon membrane depolarization in different conditions of internal [Mg\(^{2+}\)]. Analysis of the decay of the calcium transients after membrane repolarization (when calcium release is turned off) was used to characterize the removal of myoplasmic free Ca\(^{2+}\) in these different conditions. The results agree with the known Ca-Mg binding properties of Parv and suggest that lowering the free intracellular Mg\(^{2+}\) concentration from 1 mM to <0.14 mM strongly reduces the calcium uptake by the SR calcium pump. The effects of low myoplasmic [Mg\(^{2+}\)] on SR calcium release are considered in the following paper (Jacquemond and Schneider, 1992).

METHODS

The methods of fiber preparation, the optical setup, and the data acquisition and analysis have been described in detail previously. In brief, single muscle fibers from the semitendinosus muscle of cold adapted frogs (Rana pipiens, Northern variety) were dissected, cut at both ends, and mounted in a double Vaseline-gap chamber, all in a relaxing solution (Kovacs, Rios, and Schneider, 1983). Fibers were stretched to 3.8–4.1 μm per sarcomere to avoid movement and were notched just beyond the walls in both end pools. After forming Vaseline seals and positioning covering pieces (Kovacs et al., 1983) the solutions in the middle and end pools were changed to those used for the experiments. Fibers were voltage clamped and current and voltage were monitored using standard circuits (Kovacs et al., 1983). The records used for all figures and for calculating all mean values in this and the following paper (Jacquemond and
were obtained 72 ± 4 min after applying the internal solution to the fiber and at least 10 min after voltage clamping the fiber at a holding potential of −100 mV. Experiments were carried out at a holding potential of −100 mV and at 8–12°C.

The “internal” solution applied to the cut ends of the fibers contained (mM): 102.5 Cs+ glutamate, 4.5 Na+ Tris-maleate buffer, 13.2 Cs+ Tris-maleate buffer, 0.1 EGTA, 5 creatine phosphate (Na+ salt), 1 AP III, 0.05 fura-2, 6 glucose, and MgCl2 and ATP to produce various levels of free Mg2+. Assuming the apparent dissociation constant for Mg-ATP to be 0.1 mM (Fabiato and Fabiato, 1975), the following total concentrations of Mg2+ and ATP (Na+ salt) were included in the end pool solution to produce the indicated [Mg2+]: 5.5 MgCl2 and 5 ATP ([Mg2+] = 1 mM), 3 MgCl2 and 5 ATP (134 μM), 3 MgCl2 and 8 ATP (58 μM), and 3 MgCl2 and 15 ATP (25 μM). The calculated Mg-ATP concentrations in these solutions were 4.5, 2.9, 2.9, and 3.0 mM, respectively, in the high to low Mg2+ solutions, and the respective calculated free ATP concentrations were 0.5, 2.1, 5.1, and 12 mM. The “external” solution applied to the intact portion of the fiber in the middle pool contained (mM): 125 TEA CH3SO3, 2 CaCl2, 5 Cs-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer and 10−7 g/ml tetrodotoxin. All solutions were adjusted to pH 7.0 at room temperature. Fura-2 was purchased from Molecular Probes, Inc. (Eugene, OR), and AP III was from ICN Biochemicals, Inc. (Cleveland, OH). All other reagents were of analytical grade.

Optical absorbance and fluorescence measurements were carried out using the apparatus described by Klein, Simon, Szucs, and Schneider (1988) with modifications to allow the absorbance signal at 590 nm to be monitored simultaneously with the three other optical signals that were already recorded simultaneously by the original apparatus (Klein et al., 1988). To monitor the 590-nm signal an additional dichroic mirror (reflecting at 590 nm; Omega Optical Inc., Brattleboro, VT) was placed in the light path and the light reflected by the additional dichroic mirror was passed through a 590-nm interference filter (10-nm width at half peak; Omega Optical Inc.) and monitored by a photodiode/amplifier circuit with track and hold identical to those previously used for monitoring absorbance signals at 700 and 850 nm (Klein et al., 1988).

Recording of optical signals and calculations of the AP III component at 700 nm, the fura-2 saturation, and the [Ca2+] signals were carried out as previously described (Kovacs et al., 1983; Klein et al., 1988). The AP III component of the absorbance signal at 700 nm was calculated by correcting the total signal for the intrinsic component (recorded at 850 nm) and was then used to calculate calcium transients Δ[Ca2+] as previously described (Klein et al., 1988). The AP III apparent dissociation constant for calcium was calculated for each [Mg2+] using 5.6 mM for the Mg AP III dissociation constant (below). Simultaneously recorded fura-2 fluorescence signals (380 nm excitation, 510 nm emission) and AP III calcium transients were used to calibrate the fura-2 signals in each fiber so as to be consistent with the Δ[Ca2+] determined from AP III (Klein et al., 1988). The fura-2 fluorescence signals in the resting fiber were then used with this calibration to calculate resting [Ca2+].

The AP III component of the absorbance signal at 590 nm was calculated by scaling the intrinsic absorbance signal recorded at 850 nm by 1.55 and subtracting it from the total absorbance change at 590 nm. With this scale factor the total signal recorded at 590 nm was fully removed in two fibers studied without AP III in the internal solution, indicating that the scale factor was appropriate for removing intrinsic components from the 590-nm absorbance signal. The AP III absorbance signal at 590 nm remaining after correcting for the intrinsic component was used to calculate the change in [Mg2+] (Irving, Maylie, Sizto, and Chandler, 1989) using the values of 5.6 mM for the dissociation constant and −1.46 × 104 M−1 cm−1 for the extinction coefficient change for the magnesium–AP III complex as obtained in solution calibrations of the magnesium binding to AP III (Klein, M. G., V. Jacquemond, and M. F. Schneider, unpublished observations).
The calcium binding and transport properties of each fiber were characterized by fitting a
specific binding and transport model (Bruin, Rios, and Schneider, 1988) to the decay of
$\Delta[Ca^{2+}]$ starting 15 ms after pulses of 30, 60, and 120 ms to $-20$ mV using the general
procedure for simultaneous fitting of multiple $[Ca^{2+}]$ transients developed by Melzer, Rios, and
Schneider (1986). In brief, during each pulse and for the first 14 ms after each pulse the
measured $\Delta[Ca^{2+}]$ was used to calculate the calcium occupancy of all myoplasmic calcium
binding sites in the model. Thereafter, the subsequent decay of $\Delta[Ca^{2+}]$ predicted by the model
was calculated based on the properties of all binding sites and transport systems in the model.
The value of one or more parameters in the binding and transport system model was adjusted
to produce a best fit of the predicted $\Delta[Ca^{2+}]$ to the measured decay of $\Delta[Ca^{2+}]$ from 15 to 315
ms after the pulse. This analysis assumes that release has fully halted by the start of the interval
beginning 15 ms after each pulse over which $\Delta[Ca^{2+}]$ is calculated.

Details regarding most of the specific myoplasmic calcium binding sites, both rapidly and
slowly equilibrating, used in the present analysis of calcium removal were as described by Klein,
Simon, and Schneider (1990). In all of our calculations we assumed the calcium-specific binding
sites on thin filament troponin C to be present at 250 nM (cf. Baylor, Chandler, and Marshall,
1983; all concentrations referred to myoplasmic water) and to have on and off rate constants of
$1.3 \times 10^8 M^{-1} s^{-1}$ and $10^8 s^{-1}$. These rate constants for calcium binding to troponin C give a
dissociation constant of 7.7 $\mu$M, which is relatively large compared with that reported from
other studies (cf. Baylor et al., 1983, for tabulation). We use the higher value so that our
removal model can reproduce the close to exponential decline of $\Delta[Ca^{2+}]$ after most calcium
transients (Melzer, Rios, and Schneider, 1986, 1987; present results). Use of considerably lower
values results in appreciable deviations from simple exponential decline in the $\Delta[Ca^{2+}]$
predicted by the removal model due to saturation of troponin C.

In most calculations $Ca^{2+}$ and $Mg^{2+}$ binding to myoplasmic Parv were assumed to occur with
on rate constants of $1.6 \times 10^8$ and $4 \times 10^4 M^{-1} s^{-1}$, respectively (Baylor et al., 1983; Bruin,
Rios, and Stefani, 1988) and off rate constants of 1.5 and 8.0 $s^{-1}$ (values similar to those
obtained from fits to the decay of $\Delta[Ca^{2+}]$ under control conditions by Klein et al., 1990) and
the concentration [Parv] of Parv binding sites was assumed to be 1 mM. In most cases [Mg$^{2+}$] in
the fiber was assumed to be equal to the value calculated for the solution applied to the cut
ends of the fiber and that value of [Mg$^{2+}$] together with the measured resting $[Ca^{2+}]$ was used to
calculate the calcium and magnesium occupancy of Parv in the resting fiber. The calcium
binding sites on the SR calcium pump were assumed to be present at 200 nM (cf. Baylor et al.,
1983), to be in instantaneous equilibrium with the myoplasmic $[Ca^{2+}]$, and in most cases to
have a dissociation constant of 1 $\mu$M. The rate of calcium transport across the SR membrane
was generally assumed to be proportional to the fractional occupancy of the SR pump sites by
calcium but in a few analyses the transport rate was assumed to be proportional to the second
or fourth power of the fractional occupancy.

In many cases the value of the maximum pump rate $V_{max}$ was the only calcium removal
system parameter that was adjusted as described by Melzer et al. (1986) to produce a best
(least-squares) fit to the decay of $\Delta[Ca^{2+}]$ from 15 to 315 ms after the pulses. In other cases
other removal system parameters were adjusted to produce the best fit. Since the removal
model contains many more parameters than can be simultaneously adjusted, combinations of
wide ranges of values of several parameters were explored systematically by simply setting the
values of most of the parameters to each of various desired combinations of values and then
adjusting the values of only one or two other parameters (e.g., $V_{max}$ or $V_{max}$ and [Parv]) to
produce a best fit at the set values of all the other parameters.

Throughout this and the following paper (Jacquemond and Schneider, 1992) average values
are given as the mean ± SEM. Statistical significance was determined using a two-tailed $t$ test
assuming significance for $P \leq 0.05$. 

RESULTS

Low [Mg$^{2+}$] Internal Solution Induces Spontaneous Mechanical "Oscillations" of the Fibers

Soon after applying an internal solution containing 25 or 58 μM free magnesium (see Methods) at the cut ends, 19 of 29 fibers surprisingly displayed a repetitive spontaneous contractile activity having periods of seconds or tens of seconds, even though the fibers were not electrically polarized at the time of the experiment. This activity was observed in the central pool of the chamber as slow longitudinal oscillatory movements of the fiber which usually spontaneously stopped after a few minutes. However, in some cases (four fibers) this spontaneous activity kept going for a much longer period of time, preventing any decent measurement of the changes in intracellular calcium and magnesium from the changes in absorbance of AP III. The portions of the fiber in the end pools were probably responsible for these mechanical oscillations since the fiber stretch was set by measuring the sarcomere length only in the middle pool and so was certainly less near the cut ends. Although we did not carry out experiments to precisely determine the origin of this activity, it could be due to spontaneous openings of the SR calcium channels in the presence of low internal free magnesium. Similar slow mechanical oscillations have been observed with skinned fibers bathed in low [Mg$^{2+}$] solution (Herrmann-Frank, 1989).

Effects of Low Internal [Mg$^{2+}$] on Calcium and Magnesium Transients Elicited by Voltage-Clamp Depolarizing Pulses

Fig. 1 shows intracellular [Ca$^{2+}$] and [Mg$^{2+}$] changes induced by depolarizations of increasing duration to the same potential in a control fiber (left panel, 1 mM internal [Mg$^{2+}$]) and in a fiber internally equilibrated with a low [Mg$^{2+}$] containing solution (right panel, 25 μM [Mg$^{2+}$]). Both fibers were polarized at a holding potential of −100 mV before the pulses. The records presented in Fig. 1, as well as those presented in all other figures and used for calculation of all mean values in both this and the following paper were obtained with the first few depolarizing pulses applied to each fiber. Comparison of the two sets of traces in Fig. 1 shows that (a) in presence of low [Mg$^{2+}$] the decay of the calcium transients after the end of the pulses appeared much slower than in control (top row in Fig. 1), as also indicated by the prolonged saturation of fura-2 after the depolarizations in low [Mg$^{2+}$] (second row), and (b) an increase in [Mg$^{2+}$] in response to the depolarizations (Irving et al., 1989) was clearly present late in the control records but could hardly be detected in low internal [Mg$^{2+}$] (third row).

The results in Fig. 1 were typical of the differences in [Ca$^{2+}$] and [Mg$^{2+}$] transients in fibers equilibrated with normal and low internal [Mg$^{2+}$]. Fig. 2 presents the mean values (±1 SEM) of the peak amplitude of the calcium transients (upper plot) and of the magnesium transients (lower plot) elicited by a 120-ms pulse to −20 mV in batches of fibers internally equilibrated with different concentrations of free Mg$^{2+}$. The "peak" Δ[Mg$^{2+}$] was determined as the mean value of Δ[Mg$^{2+}$] from 271 to 320 ms after the pulse, i.e., during the last 50 ms of a 450-ms record that started 10 ms before the 120-ms pulse. The results show that the peak amplitude of Δ[Ca$^{2+}$] for the 120-ms pulse to −20 mV did not appear to depend on the internal [Mg$^{2+}$], whereas
the peak amplitude of the simultaneously recorded $\Delta[Mg^{2+}]$ was reduced in the presence of low $[Mg^{2+}]$ in a $[Mg^{2+}]$-dependent manner and was essentially eliminated in 25 $\mu$M internal $[Mg^{2+}]$ solution. The mean ± SEM values of $\Delta[Ca^{2+}]$ were 6.4 ± 1.6 $\mu$M ($n=23$) in 1 mM $[Mg^{2+}]$ (control) and 7.8 ± 1.8 $\mu$M ($n=12$) in 25 $\mu$M internal $[Mg^{2+}]$, which are not significantly different ($P > 0.5$). For the same fibers the mean values of $\Delta[Mg^{2+}]$ were 80 ± 10 $\mu$M ($n=23$) in control and −2 ± 12 $\mu$M ($n=11$) in 25 $\mu$M internal $[Mg^{2+}]$, which are significantly different ($P < 0.001$). The

![Figure 1](image)

**Figure 1.** Changes in free calcium and magnesium due to depolarizing pulses of increasing duration in a control fiber (1 mM internal $[Mg^{2+}]$, left panel) and in a fiber internally equilibrated with a low (25 $\mu$M) $[Mg^{2+}]$ solution (right panel). First and third rows, respectively: $\Delta[Ca^{2+}]$ and $\Delta[Mg^{2+}]$ measured from AP III; second row: percentage of fura-2 saturation; fourth row: membrane potential. Left panel, fiber 826, AP III concentration 545–548 $\mu$M, resting $[Ca^{2+}]$ 39–46 nM, fura-2 $K_D$ 135 nM, sarcomere length 4.0 $\mu$m, temperature 12°C, records taken 63 min after applying the internal solution at the ends. Right panel, fiber 718, AP III concentration 414–436 $\mu$M, resting $[Ca^{2+}]$ 201–188 nM, fura-2 $K_D$ 187 nM, sarcomere length 4.1 $\mu$m, temperature 10°C, records taken 77 min after applying the internal solution at the ends.

The mean value of $\Delta[Mg^{2+}]$ in 25 $\mu$M internal $[Mg^{2+}]$ was not significantly different from 0 ($P > 0.9$). The difference in $n$ for $\Delta[Ca^{2+}]$ and $\Delta[Mg^{2+}]$ occurred because the 590-nm AP III signal was not monitored in one fiber in 25 $\mu$M internal $[Mg^{2+}]$.

AP III exhibits a similar change in absorbance spectrum for increases in $[Mg^{2+}]$ and increases in pH (Baylor, Chandler, and Marshall, 1982; Baylor, Quinta-Ferreira, and Hui, 1985). Thus the 590-nm signal recorded both here from control fibers and in the experiments of Irving et al. (1989) and attributed to a rise in $[Mg^{2+}]$ might...
contain some component due to a change in pH. To minimize the possibility of a change in pH, 5 of the 23 control fibers used for Fig. 1 were studied using an internal solution identical to the control solution except that 85 mM Cs PIPES replaced the cesium glutamate of the normal 1 mM [Mg\textsuperscript{2+}] internal solution. The mean value of Δ[Mg\textsuperscript{2+}] in these fibers was 97 ± 30 μM (n = 5), whereas the mean Δ[Mg\textsuperscript{2+}] was 75 ± 10 μM in the other 18 fibers studied using normal cesium glutamate-containing 1 mM [Mg\textsuperscript{2+}] internal solution. Since these values are not significantly different (P > 0.2) despite the increased pH buffering capacity of the PIPES internal solution, it seems unlikely that a pH change made a major contribution to the 590-nm AP III signal in control fibers that we attribute to a rise in myoplasmic [Mg\textsuperscript{2+}]. The mean Δ[Ca\textsuperscript{2+}] was not significantly different (P > 0.5) in the two conditions, being 8.2 ± 3.0 in PIPES (n = 5) and 5.8 ± 1.8 (n = 18) in the standard internal solution. We did not use PIPES in any fibers in 25 μM [Mg\textsuperscript{2+}] solution and thus did not test for possible pH contributions to the 590-nm signal in that condition. However, in order for the observed lack of a 590-nm signal during depolarization in 25 μM internal [Mg\textsuperscript{2+}] solution (Fig. 2) to be an artifact due to a pH change, it would require the unlikely possibility that a pH signal appeared in 25 μM internal [Mg\textsuperscript{2+}] and that this pH signal was just opposite to the 590-nm Δ[Mg\textsuperscript{2+}] signal. The more likely interpretation would seem to be that the elevated ATP in the 25 μM internal [Mg\textsuperscript{2+}] solution held [Mg\textsuperscript{2+}] at a constant low level.

Within each condition of internal [Mg\textsuperscript{2+}] there was considerable variability in the peak amplitude of Δ[Ca\textsuperscript{2+}] for the 120-ms pulse to −20 mV in the fibers for Fig. 2.
Considering both the range of Δ[Ca\(^{2+}\)] within each condition and the lack of effect of internal [Mg\(^{2+}\)] on the mean Δ[Ca\(^{2+}\)] (above), each pair of control and low [Mg\(^{2+}\)] fibers chosen for use in a figure involving a 120-ms pulse to -20 mV in this and the following paper (Jacquemond and Schneider, 1992) was selected to have similar peak Δ[Ca\(^{2+}\)] and to be otherwise generally consistent with the average results from all fibers in control and low internal [Mg\(^{2+}\)] conditions.

Fig. 3 presents the dependence of the mean value of resting [Ca\(^{2+}\)] on the internal [Mg\(^{2+}\)] for the same fibers as in Fig. 2 except for two fibers in 25 μM internal [Mg\(^{2+}\)] in which the fura-2 signal was not monitored. Although there was some scatter in the data, the resting [Ca\(^{2+}\)] was apparently elevated in low internal [Mg\(^{2+}\)]. However, much of the elevated mean resting [Ca\(^{2+}\)] in 25 μM internal [Mg\(^{2+}\)] was due to one fiber in which resting [Ca\(^{2+}\)] was 851 nM. Including this fiber the mean ± SEM resting [Ca\(^{2+}\)] was 216 ± 77 nM (n = 10), whereas omitting this fiber the mean value would be 145 ± 47 nM (n = 9), which is not quite significantly greater than the mean value of 86 ± 10 nM for resting [Ca\(^{2+}\)] in the 23 control fibers (0.1 > P > 0.05). The mean ± SEM values of the fura-2 dissociation constant \(K_D\) determined in control fibers and in fibers exposed to 25 μM internal [Mg\(^{2+}\)] were, respectively, 110 ± 8 nM (n = 23) and 88 ± 13 nM (n = 10), which are not significantly different (P > 0.1). The value of \(K_D\) determined in each fiber was used to calculate the resting [Ca\(^{2+}\)] in that fiber. The mean values of the % saturation of fura-2 in the resting fibers were 42 ± 2% (n = 23) in control and 62 ± 5% (n = 10) in 25 μM internal [Mg\(^{2+}\)]. Omitting the one fiber in 25 μM [Mg\(^{2+}\)] with unusually high resting [Ca\(^{2+}\)] the mean fura-2 % saturation would be 59 ± 5% (n = 9), which is still significantly different from the mean value in control (P < 0.01) and indicates that resting [Ca\(^{2+}\)] was in fact elevated in the 25 μM internal [Mg\(^{2+}\)] solution compared with control.

**Low Internal [Mg\(^{2+}\)] Slows the Decay of [Ca\(^{2+}\)] after Depolarizing Pulses**

The decay of the calcium transient after the end of a depolarizing pulse can be well fitted by a single exponential plus a constant (Melzer et al., 1986). This is illustrated in Fig. 4, which shows such exponential fits superimposed on the decay of the corresponding calcium transients for 30-, 60-, and 120-ms pulses to -20 mV in a
control fiber (left panel) and in a different fiber equilibrated with low internal [Mg\textsuperscript{2+}] (right panel). In all cases the fit interval began 20 ms after the end of the depolarizing pulse. The fits were good in both control and low internal [Mg\textsuperscript{2+}]. Thus the parameter values from the fits provide an empirical characterization of the decay of Δ[Ca\textsuperscript{2+}] for each internal [Mg\textsuperscript{2+}]. It should be noted that the “final” level (constant) of the single exponential plus constant fits to the decay of Δ[Ca\textsuperscript{2+}] in Fig. 4 was not in fact maintained. During the 0.5- to 1-min interval between pulse applications, Δ[Ca\textsuperscript{2+}] slowly decayed from the final level back to about the resting level of [Ca\textsuperscript{2+}] in the fiber before the pulse (present results and Klein, Kovacs, Simon, and Schneider, 1991).

The top panel of Fig. 5 presents the mean values of the rate constant of decay of the calcium transient for three pulse durations as obtained from single exponential plus constant fits similar to those in Fig. 4 in different conditions of internal [Mg\textsuperscript{2+}]. It clearly shows that the rate constant of decay is much smaller in the presence of low internal [Mg\textsuperscript{2+}] than in control (1 mM internal [Mg\textsuperscript{2+}]). For instance, in the presence of 25 μM internal [Mg\textsuperscript{2+}], the value of the mean rate constant of decay for a 30-, 60-, and 120-ms pulse to -20 mV was, respectively, 29.2, 30.0, and 31.2% of its value in the presence of 1 mM internal [Mg\textsuperscript{2+}]. The bottom panel of Fig. 5 presents the mean values of the final level (constant), obtained from the same fits. The mean final level was much higher in the presence of low Mg\textsuperscript{2+} than in control. For instance, with 25 μM internal [Mg\textsuperscript{2+}] it was, respectively, 418, 673, and 535% of its value in 1 mM internal [Mg\textsuperscript{2+}] for the 30-, 60-, and 120-ms pulses to -20 mV.
The effects of low internal [Mg\(^{2+}\)] on the magnesium transient, on the resting [Ca\(^{2+}\)], and on the decay of the calcium transient are all qualitatively consistent with expected effects of magnesium on the properties of the systems involved in the removal of free calcium from the myoplasm. In 1 mM internal [Mg\(^{2+}\)] the Mg\(^{2+}\) transient arises from calcium for magnesium exchange on Parv during the calcium transient. In low internal [Mg\(^{2+}\)] the resting level of magnesium occupancy of the Parv sites would be greatly reduced, reducing Mg dissociation from Mg Parv during the calcium transient and thus reducing the Mg\(^{2+}\) transient. Resting [Ca\(^{2+}\)] could be elevated if the SR calcium pump activity were depressed in low internal [Mg\(^{2+}\)] due to loss of Mg\(^{2+}\) activation (Martonosi and Beeler, 1983; Bishop and Al-Shawi, 1988). The decay of Δ[Ca\(^{2+}\)] in low internal [Mg\(^{2+}\)] could be slowed both by a decrease in pump activity and by a decreased contribution of Parv to the removal of Ca\(^{2+}\) from the myoplasmic solution.

**Analysis of the Effects of Low Internal [Mg\(^{2+}\)] on the Decay of Δ[Ca\(^{2+}\)] Using a Model for Myoplasmic Calcium Binding and Transport**

The exponential fits to the decay of Δ[Ca\(^{2+}\)] characterize the marked slowing of decline of Δ[Ca\(^{2+}\)] after pulses in low internal [Mg\(^{2+}\)] but do not provide any insight into the mechanisms underlying the slowed decline. To investigate possible mechanisms, the decline of Δ[Ca\(^{2+}\)] after the pulses was fit using a calcium binding and removal model that represents the components expected to be present in the fiber.
The rapidly equilibrating calcium binding sites were assumed to consist of the calcium-specific sites on troponin C and of the cytoplasmic sites on the SR calcium pump, which were generally assigned set properties (see Methods). The slowly equilibrating sites were assumed to consist of the Parv calcium/magnesium sites. The return of calcium to the SR lumen was represented by the SR calcium pump, which was generally assumed to cycle at a rate proportional to the fractional occupancy of the pump sites by calcium.

The top row of Fig. 6 presents the same sets of calcium transients in control and in low internal [Mg\(^{2+}\)] as shown in Fig. 4, but with the decays of Δ[Ca\(^{2+}\)] starting 15 ms after the end of each pulse fit with the removal model with a set concentration of 1 mM for the Parv sites and with the Parv calcium and magnesium on and off rate constants set (values in Fig. 6 legend). Only the maximum pump rate \(V_{\text{max}}\) at full calcium occupancy of all pump sites was adjusted to give the best fit of the model to the experimental records. In the control fiber the fit very closely followed the decline of Δ[Ca\(^{2+}\)] after all the pulses (Fig. 6 A). For the low internal [Mg\(^{2+}\)] fiber (Fig. 6 B) the removal model did predict a slowed decay of Δ[Ca\(^{2+}\)] after the pulses as observed experimentally. However, the predicted time course of decline of Δ[Ca\(^{2+}\)] for the low internal [Mg\(^{2+}\)] fiber exhibited less curvature than the decay of the actual transients. This discrepancy between the observed and predicted decays was observed systematically in most fibers in low internal [Mg\(^{2+}\)], whereas the fits were consistently good in all control fibers.

The slowed decline of Δ[Ca\(^{2+}\)] predicted by the model for the low internal [Mg\(^{2+}\)] fiber (Fig. 6 B) was produced by decreased contributions of both Parv and the SR pump. The reduced Parv contribution was due to its lower resting occupancy by magnesium and higher occupancy by calcium at the lower resting [Mg\(^{2+}\)], even though the Parv properties were assumed to be the same as in control. The calculated mean values of resting Mg-Parv and Ca-Parv, in percentage of the total Parv site concentration, were, respectively, 34 ± 2 and 58 ± 2% (1 mM [Mg\(^{2+}\)], \(n = 23\)), 8 ± 1 and 79 ± 2% (154 μM [Mg\(^{2+}\)], \(n = 5\)), 4 ± 2 and 92 ± 2% (58 μM [Mg\(^{2+}\)], \(n = 2\)), and 1.0 ± 0.1 and 91 ± 1% (25 μM internal [Mg\(^{2+}\)], \(n = 12\)). In addition to the decreased Parv contribution, the slowed decline of Δ[Ca\(^{2+}\)] also required a reduced pump \(V_{\text{max}}\) in the model. In 23 fibers in control conditions the mean \(V_{\text{max}}\) was 3,352 ± 355 μM s\(^{-1}\), whereas for all 19 fibers in low internal [Mg\(^{2+}\)] the mean \(V_{\text{max}}\) was 1,362 ± 153 μM s\(^{-1}\).

The failure of the removal model with standard Parv parameter values to closely reproduce the decay of Δ[Ca\(^{2+}\)] after the pulses in low internal [Mg\(^{2+}\)] did not appear to be due to an improper choice of SR pump properties. In two fibers in low internal [Mg\(^{2+}\)] (those in Figs. 1 and 6) the effective calcium dissociation constant \(K_p\) for the SR calcium pump sites when oriented toward the myoplasm and the pump power as well as [Parv] were set at all possible combinations of \(K_p = 0.2, 0.5,\) or 1 μM, pump power = 1, 2, or 4, and [Parv] = 1, 2, or 5 mM, and the pump \(V_{\text{max}}\) was adjusted to produce a best fit to the decay of Δ[Ca\(^{2+}\)] after the pulses. There was almost no change in the goodness of fit at any given [Parv] for any of the combinations of SR pump parameter values that we tested. We therefore kept \(K_p\) at 1 μM and the pump power at 1 for the remaining analyses.
Figure 6. Theoretical decay of the calcium transients predicted by models for calcium binding and removal in a control fiber (A, C, and E, 1 mM internal [Mg^{2+}]) and in a fiber internally equilibrated with low [Mg^{2+}] (B, D, and F, 25 μM [Mg^{2+}]). The theoretical curves (smooth) are shown superimposed on the actual decay of the calcium transients (more noisy). (A and B) Fits in which only the maximum SR calcium pump rate $V_{\text{max}}$ was adjusted (3,323 and 1,007 μM s$^{-1}$ for A and B, respectively) to reproduce the decay of Δ[Ca^{2+}] after the pulses. [Parv] was set at 1 mM, the on rate constants for Ca^{2+} and Mg^{2+} binding to Parv were set at $1.6 \times 10^8$ and $4 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively, and the off rate constants were set at 1.5 and 8.0 s$^{-1}$. (C and D) Fits in which the Parv parameters as well as $V_{\text{max}}$ were adjusted to reproduce the decay of Δ[Ca^{2+}]. In D the decay of Δ[Ca^{2+}] was well reproduced with an SR pump $V_{\text{max}}$ of $685$ μM s$^{-1}$, [Parv] = 2 mM, Parv on rate constants for Ca^{2+} and Mg^{2+} of $1.6 \times 10^8$ and $1.6 \times 10^3$ M$^{-1}$ s$^{-1}$ and off rate constants of 2.0 and 8.0 s$^{-1}$, respectively. In control (C), the predicted decays were obtained with a fitted pump $V_{\text{max}}$ of $1,113$ μM s$^{-1}$ and with the Parv properties set at the average values obtained from fits to all fibers in low internal [Mg^{2+}] ([Parv] = $2.4$ mM, on rate constants for Ca^{2+} and Mg^{2+} of $1.6 \times 10^8$ and $2.8 \times 10^5$ M$^{-1}$ s$^{-1}$, and off rate constants of 2.2 and 8.1 s$^{-1}$, respectively). (E and F) Same Parv parameters as in A and B but with an extra calcium binding site ($K_D = 1$ μM) added to the removal model to account for the discrepancy between the predicted and the actual decay of Δ[Ca^{2+}] in the presence of low internal [Mg^{2+}] (B traces). In F, the decay of Δ[Ca^{2+}] was well reproduced with an SR pump $V_{\text{max}}$ of $889$ μM s$^{-1}$ and 100 μM of the extra site with on and off rate constants of $1 \times 10^6$ M$^{-1}$ s$^{-1}$ and 1 s$^{-1}$. In control (E), the predicted decays were obtained with a fitted pump $V_{\text{max}}$ of $3,293$ μM s$^{-1}$ after
Analysis of the Decay of $\Delta[Ca^{2+}]$ Using Alternative Parv Parameter Values

To improve the fit of the model to the decay of $\Delta[Ca^{2+}]$ in low internal [Mg$^{2+}$], various changes were made in the Parv parameter values used for the fit. In the first set of alternative fits, the Parv concentration as well as the pump $V_{\text{max}}$ were both allowed to vary in the fit. In such cases much better fits to the decay of $\Delta[Ca^{2+}]$ in low internal [Mg$^{2+}$] were obtained than with $[\text{Parv}]$ set equal to 1 mM, but with unreasonably high values of $[\text{Parv}]$ in the 10–20-mM range. Although this alternative thus seems unlikely to have applied to our experiments, it did indicate that a greater Parv contribution could result in a better fit of the removal model to the decay of $\Delta[Ca^{2+}]$ after pulses in low internal [Mg$^{2+}$].

An alternative means of achieving a greater effective Parv contribution but at a realistic $[\text{Parv}]$ would be to increase the affinity of Parv for Mg$^{2+}$ and/or decrease its affinity for Ca$^{2+}$. To test these possibilities we carried out a systematic examination of fits for multiple combinations of parameter values including increasing the on rate constant for Mg$^{2+}$ binding to Parv to various values from 4 to $32 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ while allowing the off rate constant for Ca$^{2+}$ from Parv to vary from 0.5 to 8 s$^{-1}$ and the off rate constant for Mg$^{2+}$ to vary from 4 to 16 s$^{-1}$. In all cases a fairly systematic examination of parameter value changes to within multiplicative factors of 2 or less for the increments of each of the above parameters plus $[\text{Parv}]$ within the indicated ranges were investigated. The value of the pump $V_{\text{max}}$ or the values of both the pump $V_{\text{max}}$ and $[\text{Parv}]$ were adjusted to produce a best fit with the values of the other parameters held constant at the selected levels.

Fits with the alternative Parv parameters are presented in the second row of Fig. 6, using the same experimental $\Delta[Ca^{2+}]$ records as in the top row. In low internal [Mg$^{2+}$] (Fig. 6D) the model with alternative Parv parameters very accurately reproduced the decay of $\Delta[Ca^{2+}]$ so that the superimposed experimental and calculated $\Delta[Ca^{2+}]$ records in Fig. 6D are barely distinguishable. The fit in Fig. 6D was achieved by increasing $k_{\text{on,Mg,Parv}}$ fourfold to $16 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ and doubling $[\text{Parv}]$ to 2 mM while holding $k_{\text{off,Mg,Parv}}$ constant at 8 s$^{-1}$ and increasing $k_{\text{off,Cal,Parv}}$ 1.33-fold to 2 s$^{-1}$ (all increases are relative to the “standard” values used in Fig. 6, A and B). The values of $V_{\text{max}}$ and $[\text{Parv}]$ for the best fit were 685 $\mu\text{M} \text{s}^{-1}$ and 2 mM. The results in Fig. 6D were typical of all fibers in low internal [Mg$^{2+}$]. The mean values of the alternative Parv parameter values obtained for fits to decays of 19 fibers in low internal [Mg$^{2+}$] were $k_{\text{on,Mg,Parv}} = 28 \pm 2 \times 10^4 \text{M}^{-1} \text{s}^{-1}$, $k_{\text{off,Mg,Parv}} = 8.1 \pm 1.1 \text{ s}^{-1}$, $k_{\text{off,Cal,Parv}} = 2.2 \pm 0.5 \text{ s}^{-1}$, and $[\text{Parv}] = 2.4 \pm 0.3 \text{ mM}$. In every fiber in low internal [Mg$^{2+}$] the fit with the alternative Parv parameters was much better than the fit obtained using the standard values. For the 19 fibers in low internal [Mg$^{2+}$] the mean...
value of the standard error of the fit was 0.30 ± 0.06 using the standard Parv parameter values, but only 0.12 ± 0.03 using the alternative Parv parameters. The mean value of the SR pump $V_{\text{max}}$ obtained in low internal $[\text{Mg}^2+]$ with the alternative Parv parameter values was 549 ± 72 \(\mu\text{M s}^{-1}\), again indicating a marked suppression of the pump in low internal $[\text{Mg}^2+]$.

Alternative Parv parameter values could not be determined from fits to the decay of $\Delta[\text{Ca}^{2+}]$ in control fibers since the fits were already almost perfect with the standard values (Fig. 6 A). The fits to the decay of $\Delta[\text{Ca}^{2+}]$ after the pulses in the control fiber shown in Fig. 6 C were obtained by setting the Parv parameters to the mean values obtained in low internal $[\text{Mg}^2+]$ (above) and only allowing the pump $V_{\text{max}}$ to vary to produce the fit. The resulting best fit shown in Fig. 6 C clearly fails to reproduce the observed $\Delta[\text{Ca}^{2+}]$, as was the case for all fibers in control solution when fitted with the Parv values determined in low internal $[\text{Mg}^2+]$. Since the same alternative Parv values were not applicable to fibers in both control and low internal $[\text{Mg}^2+]$, whereas Parv should be the same in both conditions, the alternative Parv properties do not appear to provide an explanation for the decay of $\Delta[\text{Ca}^{2+}]$ after pulses in 1 mM and 25 \(\mu\text{M internal}[\text{Mg}^2+]\).

**Analysis of the Decay of $\Delta[\text{Ca}^{2+}]$ Using Higher Resting $[\text{Mg}^2+]$**

Another way in which a greater Parv contribution could have occurred but been overlooked in our model would be if we underestimated the $[\text{Mg}^2+]$ in the fibers exposed to low $[\text{Mg}^2+]$ internal solution. To test this possibility, we recalculated $\Delta[\text{Ca}^{2+}]$ and redid the removal analysis for each of the 12 fibers in 25 \(\mu\text{M internal}[\text{Mg}^2+]\) but with the apparent value of free $\text{Mg}^{2+}$ $[\text{Mg}^2+]_{\text{app}}$ assumed to be considerably higher than 25 \(\mu\text{M}\). For each fiber we repeated the process at several assumed $[\text{Mg}^2+]_{\text{app}}$ values until the removal model with standard values for all parameters except $[\text{Parv}]$ gave a best fit to the decays of $\Delta[\text{Ca}^{2+}]$ with the fit value of $[\text{Parv}]$ equal to the “standard” value of 1 mM. This value of $[\text{Mg}^2+]_{\text{app}}$ was selected for each fiber and resulted in a better fit of the removal model to the observed decay of $\Delta[\text{Ca}^{2+}]$ in each fiber exposed to 25 \(\mu\text{M internal}[\text{Mg}^2+]\) solution than obtained using 25 \(\mu\text{M}[\text{Mg}^2+]\) (fits not shown). The mean value of $[\text{Mg}^2+]_{\text{app}}$ determined in this way in the 12 fibers exposed at their cut ends to the 25 \(\mu\text{M}[\text{Mg}^2+]\) internal solution was 490 ± 62 \(\mu\text{M}\), which would indicate very little equilibration of the end pool $[\text{Mg}^2+]$ with the fiber interior. However, the low internal $[\text{Mg}^2+]$ solution clearly did have a marked effect on the fiber, slowing the decay of $\Delta[\text{Ca}^{2+}]$ after the pulses and eliminating the $[\text{Mg}^2+]$ transient (above). Furthermore, the fits with the higher assumed $[\text{Mg}^2+]_{\text{app}}$ gave a mean pump $V_{\text{max}}$ of only 658 ± 118 \(\mu\text{M s}^{-1}\) in the 25 \(\mu\text{M internal}[\text{Mg}^2+]\) solution compared with the mean value of 3,352 \(\mu\text{M s}^{-1}\) from fits using the same Parv properties in control fibers (above). Since both the data and the modeling indicate a marked effect of applying the 25 \(\mu\text{M internal}[\text{Mg}^2+]\) solution to the cut ends of the fibers, it seems unlikely that the 25 \(\mu\text{M}[\text{Mg}^2+]\) solution could have only caused $[\text{Mg}^2+]$ in the fibers to fall from 1 mM to 490 \(\mu\text{M}\).

**Analysis of the Decay of $\Delta[\text{Ca}^{2+}]$ Using an Extra Calcium Binding Site**

In another attempt to improve the fit of the calcium removal model to the decay of $\Delta[\text{Ca}^{2+}]$ in low internal $[\text{Mg}^2+]$, an additional calcium binding site $X$ was introduced
into the model. The additional site was assumed to be present at 50–200 μM, to have a dissociation constant of 1 μM, and to have an off rate constant of 0.5–5 s⁻¹. Fits with the revised model are presented in the third row of Fig. 6, using the same experimental Δ[Ca²⁺] records as in the top two rows. The revised model was able to quite accurately reproduce the decay of Δ[Ca²⁺] in low internal [Mg²⁺] (Fig. 6 F). These fits were typical of all fibers in low internal [Mg²⁺]. For all 19 fibers in low internal [Mg²⁺] the mean value of the standard error of the fit was 0.14 ± 0.03 using the extra site, again considerably better than the mean of 0.30 ± 0.06 for the standard Parv parameters. The mean values of the properties of the extra site obtained for fits to decays of 19 fibers in low internal [Mg²⁺] were [X] = 158 ± 11 μM and kₐₖ = 2.84 ± 0.19 s⁻¹.

As was the case for the alternative Parv parameters (above), the properties of X could not be determined from fits to the decay of Δ[Ca²⁺] in control fibers since the fits were already almost perfect without including X (Fig. 6 A). The fits to the decay of Δ[Ca²⁺] after the pulses in the control fiber shown in Fig. 6 F were obtained by including X with the mean properties obtained in low internal [Mg²⁺]. In this particular fiber the fit appeared slightly better than that obtained without X (Fig. 6 A), but over all control fibers the fit was about the same or slightly better without X than including X (with the mean properties determined in low internal [Mg²⁺]). The mean value of the standard error of the fits obtained in the 23 control fibers was 0.17 ± 0.06 without the extra site and 0.20 ± 0.07 with the extra site, which are not significantly different (P > 0.5). Thus, the removal model including site X was consistent with the decay of Δ[Ca²⁺] after pulses in both control and low [Mg²⁺] conditions.

From the fits including the extra site the mean pump Vmax was 3,684 ± 417 μM s⁻¹ for the 23 control fibers and 1,219 ± 162 μM s⁻¹ for all 19 fibers in a low internal [Mg²⁺], again indicating a marked suppression of the pump Vmax in low internal [Mg²⁺] as was found in the fits without the extra site (above). Thus the conclusion that the SR pump activity was markedly suppressed in low internal [Mg²⁺] is independent of the assumptions concerning presence or absence of the extra site X.

The results of the fit of the removal model to the decay of Δ[Ca²⁺] provide a basis for estimating the rate of SR calcium pumping in the resting fibers. Using the results from the analysis with the extra calcium binding site, the rate of calcium transport Vrest in the resting fibers was calculated as Vmax [Ca²⁺]rest/(Kp + [Ca²⁺]rest), where Kp is the dissociation constant (1 μM) assumed for the SR calcium pump. The mean value of Vrest in the 23 control fibers was 273 ± 43 μM s⁻¹, whereas the mean value for all 19 fibers in low internal [Mg²⁺] was 108 ± 13 μM s⁻¹. These values indicate that even though [Ca²⁺]rest may have been elevated in low internal [Mg²⁺] solution, the rate of SR calcium transport in the resting fibers was lower in low internal [Mg²⁺] than in control.

Our analysis of the decay of Δ[Ca²⁺] after various pulses demonstrates that the decay of Δ[Ca²⁺] after pulses in low internal [Mg²⁺] could be accounted for by a combination of a decreased Parv contribution and a decreased pump Vmax in low [Mg²⁺] provided that an additional calcium binding site X was introduced into the model. However, another set of alternative interpretations would become available if we dropped one of the basic assumptions underlying the present analyses of the
calcium removal system. Our analyses assumed that calcium release from the SR was completely turned off before the start of the interval, beginning 15 ms after each pulse, over which the decay of $\Delta[Ca^{2+}]$ after the pulse was analyzed. If release were in fact not turned off by that time, $\Delta[Ca^{2+}]$ would decay more slowly than predicted by the removal model, assuming release to equal 0. Thus, a removal model predicting a faster decline of $\Delta[Ca^{2+}]$ than observed could be an accurate representation of the calcium removal properties of the fiber if release were not fully turned off during the observed interval. Although the break in the calcium transient at the end of each pulse in both control and low internal $[Mg^{2+}]$ indicates that a significant amount of release did in fact turn off, we can only assume that the turn off was complete by the start of the removal analysis interval. The possibility of incomplete turn off of release after the pulse was not explored further.

DISCUSSION

This study of the changes in myoplasmic $[Ca^{2+}]$ due to membrane depolarization when the $[Mg^{2+}]$ in the internal solution was buffered to different extents suggests that the rate of removal of free calcium ions from the myoplasmic space was strongly reduced in the presence of low intracellular free $[Mg^{2+}]$.

General Procedure for Studying Effects of Myoplasmic $[Mg^{2+}]$

The procedure used in this study to vary myoplasmic $[Mg^{2+}]$ within the voltage-clamped region of a cut muscle fiber was to apply a solution of buffered $[Mg^{2+}]$ to the cut ends of the fiber and to rely on diffusion from the cut ends to establish the same myoplasmic $[Mg^{2+}]$ in the central (voltage-clamped) region of the fiber. Since fiber equilibration with the cut end solution could be a relatively slow process, we felt that measurements on the same fiber at different $[Mg^{2+}]$ by our procedure would be impractical and unreliable. We therefore resorted to studying different fibers at each $[Mg^{2+}]$, recognizing that a limitation of this approach was that fiber to fiber variation might obscure the effects of internal $[Mg^{2+}]$ under investigation since each fiber was only studied under a single condition and thus could not serve as its own control. By using relatively large numbers of fibers under each condition so as to average out fiber to fiber variations, and by carefully controlling the experiments so that each fiber was studied after a similar time of exposure to internal solution, we have achieved results that appear to reliably indicate systematic effects of $[Mg^{2+}]$ on both calcium removal (this paper) and on calcium release (calcium release calculations on the same fibers as used in the present paper; Jacquemond and Schneider, 1992).

Since we did not reversibly alter $[Mg^{2+}]$ in a given fiber we cannot rule out the possibility that some of the effects reported here and in the accompanying study (Jacquemond and Schneider, 1992) were not directly due to the change in internal $[Mg^{2+}]$ but instead were produced by some other effect secondary to the change in $[Mg^{2+}]$. For example, since properties of cut fibers change with time after cutting (Irving, Maylie, Sizto, and Chandler, 1987), changes in the rate of "run down" of the fibers under conditions of different internal $[Mg^{2+}]$ might have been responsible for some of the observed changes that we attributed to direct effects of altered $[Mg^{2+}]$. Fibers under our control conditions of internal $[Mg^{2+}]$ tend to exhibit slowed decay
of $\Delta[Ca^{2+}]$ after pulses applied relatively late during an experiment, presumably due to run down of the calcium removal systems in the fiber. Thus, the slowing of the decay of $\Delta[Ca^{2+}]$ seen in low internal $[Mg^{2+}]$ in this study might in principle have been produced by accelerated fiber run down under conditions of low internal $[Mg^{2+}]$. In this case the slowing of the decay of $\Delta[Ca^{2+}]$ sometimes observed just before fiber deterioration more than 2 or 3 h after applying control internal solution would have to have occurred ~1 h after applying low $[Mg^{2+}]$ internal solution in the present experiments. However, fiber run down also causes a decline in the peak rate of release of calcium from the SR in response to a given pulse (Klein et al., 1990), whereas we observed an increase rather than a decrease in the peak rate of calcium release in low internal $[Mg^{2+}]$ (Jacquemond and Schneider, 1992; same fibers as in the present study). Since the observed effects of low internal $[Mg^{2+}]$ on calcium removal and on calcium release in the same fibers would require, respectively, that the rate of fiber run down be both increased and decreased in low $[Mg^{2+}]$ if the effects were due to altered run down, we believe that it is unlikely that changes in the rate of fiber run down made a major contribution to the effects attributed to low internal $[Mg^{2+}]$ in this and the following paper. Nonetheless, we cannot rule out the possibility that some of the observed effects were not the direct result of lowered internal $[Mg^{2+}]$.

Buffering Cytoplasmic $[Mg^{2+}]$ with ATP

In the internal solutions used, magnesium buffering was mostly expected to be due to ATP. However, 50–80 min equilibration of the internal solution might not have been sufficient to obtain a steady free $Mg^{2+}$ concentration, identical to the end pool $[Mg^{2+}]$, inside the tested portion of the muscle fiber in the central pool of the chamber. Using a one-dimensional diffusion equation (Crank, 1956) for ATP with a diffusion constant of $1.25 \times 10^{-6}$ cm$^2$ s$^{-1}$ (Kushmerick and Podolsky, 1969) and assuming no binding of ATP to intracellular constituents, we estimated that the fraction of ATP equilibrated in the portion of fiber in the middle pool was ~58–80% of the end pool’s concentration after 60–80 min equilibration, respectively. Thus, our assumption of complete equilibration might have led to an underestimation of the actual free magnesium concentration inside muscle fibers exposed to low internal $[Mg^{2+}]$ solutions at their cut ends. On the other hand, we used a value of 0.1 mM for the apparent dissociation constant ($K_D$) for Mg-ATP (Fabiato and Fabiato, 1975) which might have produced an overestimation of the free $Mg^{2+}$ concentration considering that the affinity might be higher ($K_D = 0.04$ mM according to Godt and Lindley, 1982). Since these two changes are in opposite directions, we felt that we could rely on a $K_D$ of 0.1 mM for Mg-ATP inside the muscle fiber and assume full equilibration with the end pool solution for calculating the free $Mg^{2+}$ concentration in the fiber.

Measuring the change in free $Mg^{2+}$ resulting from membrane depolarization in the different conditions of internal $[Mg^{2+}]$ demonstrated the effectiveness of the present method to lower magnesium inside the muscle fiber: when depolarizing the fiber in control conditions (1 mM free $Mg^{2+}$), a rise in free $Mg^{2+}$ due to calcium for magnesium exchange on Parv was observed to occur (Figs. 1 and 2, and Irving et al., 1989); the reduction of this change, when equilibrating the fibers with a "low $[Mg^{2+}]$"
solution, is thus consistent with a low resting magnesium occupancy of Parv, as expected if the level of free myoplasmic Mg$^{2+}$ in these conditions was actually reduced.

Although there is a qualitative agreement between this reduction in the amplitude of the Mg$^{2+}$ transient and the expected low level of resting free Mg$^{2+}$, the maximal amplitude of the magnesium transient should be quantitatively consistent with the calculated amount of magnesium bound to Parv at rest. In this regard it was surprising to observe little difference between the mean value of the amplitude of the [Mg$^{2+}$] transient when going from 1 to 0.134 mM resting free [Mg$^{2+}$] (see Fig. 2), while the calculated resting Mg occupancy of Parv should correspond to 34 and 8%, respectively, of the total concentration of Parv sites in the fibers. Considering that there was not much difference in the mean values of the maximal amplitude of the [Ca$^{2+}$] transient and of the resting calcium between the two sets of conditions, a reduced [Mg$^{2+}$] transient was already expected in 0.134 mM resting [Mg$^{2+}$]. The uncertainty (high scatter) in the mean value of the amplitude of the Mg$^{2+}$ transient in 0.134 mM internal [Mg$^{2+}$] might be partly responsible for this quantitative inconsistency.

**Fitting the Removal Model to the Decay of the Calcium Transients in the Different Conditions of Internal [Mg$^{2+}$]**

The present results show that, after membrane repolarization, the decay of the calcium transients was much slower in the presence of low myoplasmic [Mg$^{2+}$] than in control conditions, which suggests that the rate of calcium removal from the myoplasm was affected by the free internal [Mg$^{2+}$]. In an attempt to characterize the possible mechanisms involved in this effect, a model taking into account the main processes responsible for calcium binding and removal from the myoplasm (Melzer et al., 1984, 1987) was fitted to the decay of the calcium transients. In the fits relatively wide ranges of values for most of the parameters in the model were explored. After extensive examination of many combinations of properties of both Parv and the SR calcium pump we were unable to find a realistic set of parameter values that could account for the decay of A[Ca$^{2+}$] after the pulses both in control and in low internal [Mg$^{2+}$]. Surprisingly, we found that when an extra calcium binding site, with a $K_D$ of 1 $\mu$M, was added to the model in order to obtain good fits to the decay of the calcium transients for the fibers equilibrated with low internal [Mg$^{2+}$], the same parameters could reproduce the decay of A[Ca$^{2+}$] in control. It could be argued that our "low [Mg$^{2+}$]" conditions actually made a new site available to bind calcium, perhaps by decreasing Mg$^{2+}$ binding to a site that bound Ca$^{2+}$ with a $K_D$ of $\sim$ 1 $\mu$M in the absence of Mg$^{2+}$. However, it seems equally likely that this site bound calcium independently of the [Mg$^{2+}$], but in normal conditions (1 mM [Mg$^{2+}$]) its contribution to the removal of calcium from the myoplasm could not be distinguished from the relatively large contributions of the SR pump and Parv. It is difficult to speculate on the biochemical identity of this site.

**The Rate of Calcium Uptake by the SR Calcium Pump Is Reduced in the Presence of Low Myoplasmic [Mg$^{2+}$]**

Fitting the removal model to the data suggested that, in low internal [Mg$^{2+}$], a decreased availability of the Parv sites to bind calcium was not sufficient to explain
the slow decay of the calcium transients, and that a simultaneous reduced rate of calcium pumping by the SR had to be taken into account. The conclusion that pump activity was suppressed in low internal \([\text{Mg}^{2+}]\) was common to all models used to interpret the decay of \(\Delta[\text{Ca}^{2+}]\). This interpretation is consistent with biochemical studies showing a similar dependence of the SR Ca-ATPase activity of skeletal or cardiac muscle on the free \(\text{Mg}^{2+}\) concentration (Jones, 1979; Bishop and Al-Shawi, 1988). However, the regulatory site is thought to be located on the luminal side of the SR (Bishop and Al-Shawi, 1988), which would indicate that our "low \([\text{Mg}^{2+}]\)" conditions also induce a \(\text{Mg}^{2+}\) depletion from inside the SR, thus resulting in the observed decrease in SR pump activity.

In our experiments, the calculated concentration of \(\text{Mg-ATP}\) in the internal solution varied between 4 mM in the control (1 mM \([\text{Mg}^{2+}]\) solution to 2.9 or 3.0 mM in low internal \([\text{Mg}^{2+}]\) (see Methods), which might in principle also affect the SR calcium pump activity. However, we believe that the observed effect on the SR pump is more likely to be due to the reduced free \(\text{Mg}^{2+}\) than to the reduced \([\text{Mg-ATP}]\) since a significant inhibition of the Ca-stimulated ATPase activity only occurs at a much lower level of \([\text{Mg-ATP}]\) than the ones used in this study (see, for instance, Neet and Green, 1977). The free ATP concentration in our 25 \(\mu\text{M}\) internal \([\text{Mg}^{2+}]\) solution was increased to 12 mM from the level of 0.5 mM in control. If anything, this elevation of free [ATP] in our low internal \([\text{Mg}^{2+}]\) solution would have increased the SR pump activity rather than decreasing it as observed here in low internal \([\text{Mg}^{2+}]\), since free ATP activates the pump at a low affinity site (Weber, 1971; Martonosi and Beeler, 1983).

In conclusion, our analysis of the decay of \(\Delta[\text{Ca}^{2+}]\) after various pulses demonstrates that the decay of \(\Delta[\text{Ca}^{2+}]\) after pulses in low internal \([\text{Mg}^{2+}]\) could be accounted for by a combination of a decreased \(P_{\text{av}}\) contribution and a decreased pump \(V_{\text{max}}\) in low \([\text{Mg}^{2+}]\), provided that an additional calcium binding site \(X\) was introduced into the model.

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