# Regulation by Magnesium of Intracellular Calcium Movement in Skinned Muscle Fibers

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ABSTRACT The effect of Mg on Ca movement between the sarcoplasmic reticulum (SR) and myofilament space (MFS) was studied in skinned muscle fibers by using isometric force as an indicator of MFS Ca. In Ca-loaded fibers at 20°C, the large force spike induced by Ca in 1 mM Mg (5 mM ATP) was strongly inhibited in 3 mM Mg, and force development was extremely slow. After a brief Ca stimulus in 1 mM Mg, relaxation in Ca-free solution was significantly faster in 3 mM Mg. These changes were due to altered Ca movements, since the effect of 3 mM Mg on steady force in CaEGTA solutions was small. Changes in Mg alone induced force transients apparently due to altered Ca movement. In relaxed fibers, decreasing the Mg to 0.25 mM caused phasic force development. In contracting fibers in Ca solutions, increasing the Mg caused a large transient relaxation. The effects of increased Mg were antagonized by 0.5 mM Cd, an inhibitor of the SR Ca transport system. The results indicate that active Ca uptake by the SR *in situ* is stimulated by Mg, and that it can affect local MFS [$Ca^{++}$] in the presence of a substantial Ca source. These results provide evidence that an increased rate of Ca uptake in 3 mM Mg could account for inhibition of the large force spike associated with Ca-induced Ca release in skinned fibers.

## INTRODUCTION

In skinned fibers from skeletal muscle, calcium ions can cause a transient force spike (Ford and Podolsky, 1970; Endo et al., 1970) due to Ca release from the sarcoplasmic reticulum (SR) to the myofilament space (MFS) (Ford and Podolsky, 1972 *b*). This regenerative effect reflects a Ca-sensitive efflux mechanism in the SR membrane which could be an important feature of excitation-contraction coupling in intact fibers. Excitation of the transverse tubules could be transmitted to the SR by a small Ca signal (Ford and Podolsky, 1972 *b*), or a small amount of Ca released from the SR by some other signal could be required for the large increase in Ca efflux associated with contraction. Ca-induced force spikes were studied at low Mg ion concentration ($<10^{-4}$ M), and millimolar Mg ion prevented a large response (Ford and Podolsky, 1970). The mechanism underlying this inhibition is important both in assessing the role of Ca-induced release in intact fibers, where free Mg is likely to be at least $10^{-4}$ M, and in understanding the regulation of net Ca movement between the intracellular compartments.

Mg inhibition of force spikes in skinned fibers could be related to an increased

rate of Ca reaccumulation by the SR. Mg promotes Ca uptake by vesicles of SR membranes (Carvalho and Leo, 1967) and stimulates the rate-limiting step of the ATPase activity associated with Ca transport (see Yamada and Tonomura, 1972). In the present studies, the effects of changes in Mg on Ca-induced force spikes and on steady force were used to investigate the contribution of Mg-dependent Ca transport to the action of Mg *in situ*. A preliminary account of some of the results has been presented (Stephenson and Podolsky, 1973).

## METHODS

### *Fiber Preparation*

Skinned fibers were prepared and studied by the general methods described by Ford and Podolsky (1972*b*), with the following modifications. Semitendinosus muscles dissected from double-pithed frogs (*Rana pipiens*) were suspended in cold Ringer solution (115.5 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 3.1 mM $NaH_2PO_4$ + $Na_2HPO_4$ buffer, 9 mg/liter *d*-tubocurarine, pH 7.0–7.2). A bundle of fibers was dissected with most of the fibers intact, blotted lightly, placed on a glass slide, and immersed in cold mineral oil. An intact fiber (~50–100 $\mu$m diam) was isolated from tendon to tendon under × 40 magnification; if the sarcolemma has been intact in Ringer solution and is disrupted only in oil, changes in fiber solute composition should be minimized. A fiber with no visible damage was cut into segments about 3 mm long, which were stored on individual cover slips in cold oil. Each segment was skinned with fine stainless steel probes just before use and transferred to the oil-filled mounting well of the experimental chamber.

### *Force Transducer and Experimental Chamber*

The segment was mounted between clamps at 1.05–1.1 slack length, and isometric force measured with the leaf-spring photodiode force transducer described by Hellam and Podolsky (1969) was recorded on a chart recorder (Honeywell Electronik 193, Honeywell Inc., Minneapolis, Minn.) with a span step response time less than 0.5 s. The experimental chambers contained wells in a spring-mounted block, similar in design to the chamber described by Hellam and Podolsky. Some early experiments were carried out at room temperature (19–26°C) in a lucite chamber; most experiments were carried out at 19–20°C or 5°C in a thermoregulated anodized aluminum chamber with a glass bottom to permit microscope visualization of the preparation. Fresh aqueous solutions which had been stored on ice were placed in the wells of the chamber just before each segment was mounted. The surface oil layer was omitted.

### *Bathing Solutions*

The solutions for bathing skinned fibers were similar to those described by Ford and Podolsky (1972*a*). All solutions contained 120 mM K propionate, 5 mM $Na_2ATP$, 10 mM imidazole, and 0.25 mM, 1.0 mM, or 3.0 mM $MgCl_2$. Due to binding to ATP, $[Mg^{++}]$ in these solutions is about 4 $\mu$M, 0.02 mM, or 0.11 mM, respectively (see below). The pH was adjusted to 7.0 ± 0.02 at room temperature. This basic solution contained about $1.8 \times 10^{-5}$ M contaminant Ca (assayed by atomic absorption spectrophotometry), derived partly from the ATP (Sigma grade, low Ca disodium salt, 99–100% pure, Sigma Chemical Co., St. Louis, Mo.). The *total* Ca contamination is about 0.02% of the ATP by weight. Unbuffered Ca solution (no EGTA) contained added 0.1 mM $CaCl_2$; similar solutions with Cd, Mn, or Co were made with addition of the Cl salts. Inorganic salts and propionic acid were reagent grade (Fisher Scientific Co., Pittsburgh, Pa.); imidazole was obtained from Eastman Organic Chemicals, Rochester, N. Y., and EGTA from Geigy Chemical

Corp., Ardsley, N. Y. Buffered Ca solutions were prepared at room temperature with stock solutions of CaEGTA and $K_2$EGTA. The concentrations of $Ca^{++}$ and $Mg^{++}$ were computed with a program for equilibrium binding between two ions and two ligands considering only the major binding agents ATP and EGTA. The binding constants used were: CaEGTA, $5.01 \times 10^6$ (at pH 7.0); MgEGTA, 10; MgATP, $1.25 \times 10^4$; CaATP, $6.97 \times 10^3$ (Sillén and Martell, 1964). The pH of these solutions at 5°C was 7.3, and the CaEGTA binding constant was adjusted to $2.2 \times 10^7$.

*Ca Loading Procedures*

For Ca release experiments, segments were loaded in buffered Ca solution, pCa 6.2, total EGTA 0.5 mM, as described by Ford and Podolsky (1970) and usually only one response per segment was examined. In some experiments, indicated in Results, responses of the same segment under different conditions were compared. These segments were recycled by a procedure intended to give uniform Ca loading for each test: (*a*) the segment was loaded in the buffered Ca solution until force began to develop; (*b*) the SR was partially emptied by initiating Ca-induced Ca release and trapping the released Ca with 0.1 mM EGTA; (*c*) the SR was reloaded for 20 s in buffered Ca solution; (*d*) the segment was exposed to the test condition. When this sequence was repeated, the effects of test solutions were reproducible, but the maximum force developed tended to decrease progressively. Any bias due to progressive changes in the segment was avoided by varying the order of presentation of different test conditions.

## RESULTS

*Effects of Mg on the Ca-Induced Response*

Ford and Podolsky (1970) found that 0.1 mM Ca induces a large force spike in the presence of 1 mM Mg (5 mM ATP) but not 6 mM Mg. The present experiments showed that much lower Mg levels similarly reduced the force spike. In the force records in Fig. 1, segments from the same muscle fiber were preloaded with Ca in CaEGTA buffer solution (a requirement for the Ca-induced force spike at room temperature [Ford and Podolsky, 1970]) and rinsed in dilute EGTA solutions before exposure to solutions containing 0.1 mM Ca. The top trace shows a force spike in Ca solution containing 1 mM Mg. Force rose rapidly to maximum then decreased transiently before stabilizing. The second trace shows the effect of increasing Mg in the last rinse and the Ca solution to 3 mM. The immediate response was reduced to a few percent maximum force and a few seconds' duration, and force rose with a time course of minutes.

The delayed development of full force in the presence of high bath Ca suggested a high rate of Ca transport by the SR. (Force delays in CaEGTA buffer solutions containing 1 mM Mg are associated with $^{45}Ca$ accumulation by the fiber, presumably in the SR [Ford and Podolsky, 1972*a*].) If increased Mg were acting by increasing the rate of active Ca transport, inhibitors of the transport system should antagonize its effect. Addition of Cd (0.5 mM), which inhibits SR Ca uptake and ATPase activity (Carvalho, 1968; MacLennan, 1970), counteracted the effect of 3 mM Mg; force rose rapidly, although not as fast as in the first trace, and remained maximal (Fig. 1*c*). The effect of Cd was not due to a direct action on the myofilaments, as shown in experiments described below. This result was consistent with the idea that the effect of Mg depends on active Ca accumulation by the SR.

Records for all three conditions on segments from the same muscle fiber were obtained from five fibers. The time required for force to rise to the peak value (mean ± SEM) was 6 ± 1 s in 1 mM Mg, 91 ± 8 s in 3 mM Mg, and 27 ± 2 s in 3 mM Mg + 0.5 mM Cd solutions, all containing 0.1 mM Ca.

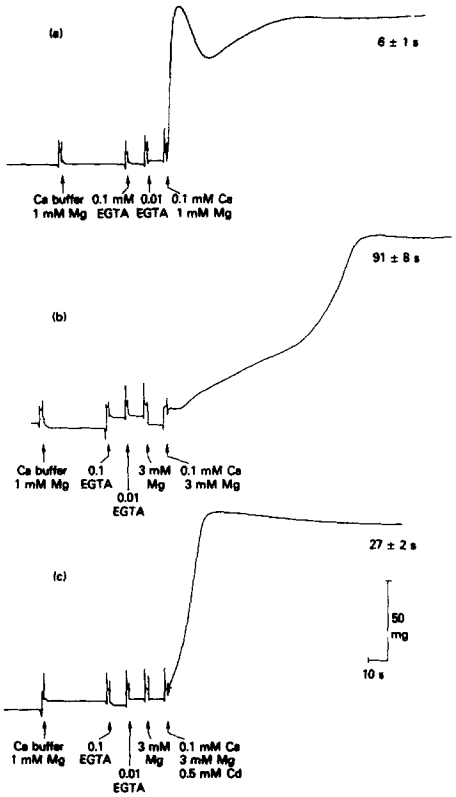


FIGURE 1. The effect of Mg on Ca-induced force responses. Ca-loaded segments from the same fiber were rinsed in dilute EGTA solutions and then exposed to 0.1 mM Ca solution, which contained (*a*) 1 mM Mg, (*b*) 3 mM Mg, (*c*) 3 mM Mg + 0.5 mM Cd. In (*a*), all solutions contained 1 mM Mg. In (*b*) and (*c*), Mg was increased to 3 mM in the final rinse. Temperature, 18–19°C.

*Effect of Mg on Reaccumulation of Released Ca*

Most of the $^{45}Ca$ released early in a force spike in 1 mM Mg is reaccumulated (Ford and Podolsky, 1972*b*). The effect of increasing the Mg immediately after release thus could provide a test of its action on the rate of net Ca uptake by the SR. Ca release from loaded segments was induced by brief exposure to 1 mM Mg, 0.1 mM Ca solution, and the stimulus was then terminated by transfer to solutions containing 1 mM or 3 mM Mg and no added Ca. The initial exposure releases the same amount of Ca from the SR to the MFS in both cases; if Mg has no additional effects, the time course of relaxation should reflect the rates of disappearance of released Ca from the MFS, and so the rates of Ca reaccumula-

tion by the SR. In order to minimize variation, each condition was tested on the same segments, by using the procedure for recycling the segments described in Methods; the time course of force spikes with the recycling procedure was somewhat longer than with the simple procedure for a single test spike per segment used in most other experiments. In the typical records shown in Fig. 2, force fell slowly in 1 mM Mg solution, reaching half-maximum in about 16 s, while relaxation was faster in 3 mM Mg solution, with force half-maximum in about 10 s. The third trace shows that 0.5 mM Cd antagonized the effect of increased Mg in this type of experiment also, and inhibited the relaxation process.

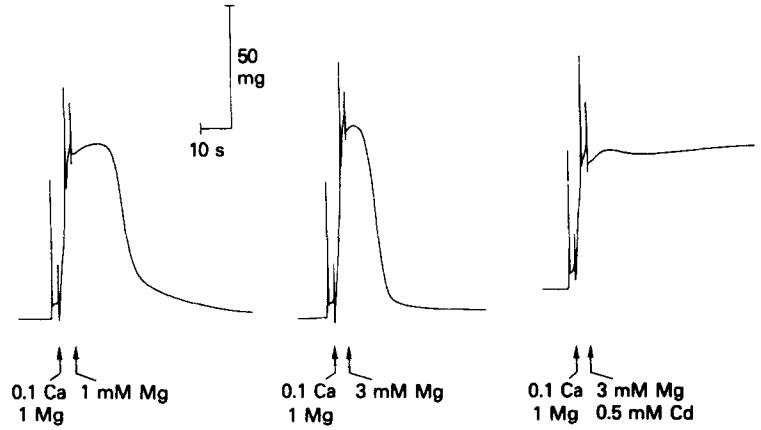


FIGURE 2. The effect of Mg on relaxation from a brief Ca stimulus. Large force spikes were initiated by exposing the Ca-loaded segment to 1 mM Mg, 0.1 mM Ca solution. After 4–5 s, the segments were transferred to Ca-free solution with (*a*) 1 mM Mg, (*b*) 3 mM Mg, (*c*) 3 mM Mg + 0.5 mM Cd. Different conditions were tested on the same fiber segment by the recycling procedure described in Methods. Temperature, 26°C (chamber not thermoregulated).

Force was expressed as a fraction of the maximum force of the segment and averaged for six pairs of records like those in Fig. 2. The mean time course of relaxation, plotted in Fig. 3, was significantly faster in 3 mM Mg than in 1 mM Mg; the time required for force to fall to half-maximum in 3 mM Mg was 0.62 ± 0.02 that in 1 mM Mg. In order to attribute the increased rate of force decay to an increase in the rate of Ca reaccumulation, several factors were considered. First, increased Mg can decrease the steady force at a given Ca ion concentration (Ebashi and Endo, 1968); such a shift would increase the observed relaxation rate even if the time course of Ca disappearance were unchanged. The direct effect of Mg (and of MgATP) on force was evaluated by comparing the steady forces developed by segments exposed sequentially to CaEGTA buffer solutions, total EGTA 5 mM, containing 1 or 3 mM Mg; at 20°C, with a high buffer concentration and heavily loaded SR, the influence of the SR is minimal and MFS ($Ca^{++}$) is well controlled by the bathing solution (as seen in the next section). The mean relative force at pCa 6.2 with 1 mM Mg is shown by the open square in Fig. 3,

placed on the 1 mM Mg relaxation curve. The mean relative force in the same buffer solution with 3 mM Mg is shown by the filled square, placed at the same point in relaxation time. The maximum effect of the higher Mg on steady force clearly was too small to account for the low force at that time on the 3 mM Mg relaxation curve, if the pCa in the MFS had been the same.[1]

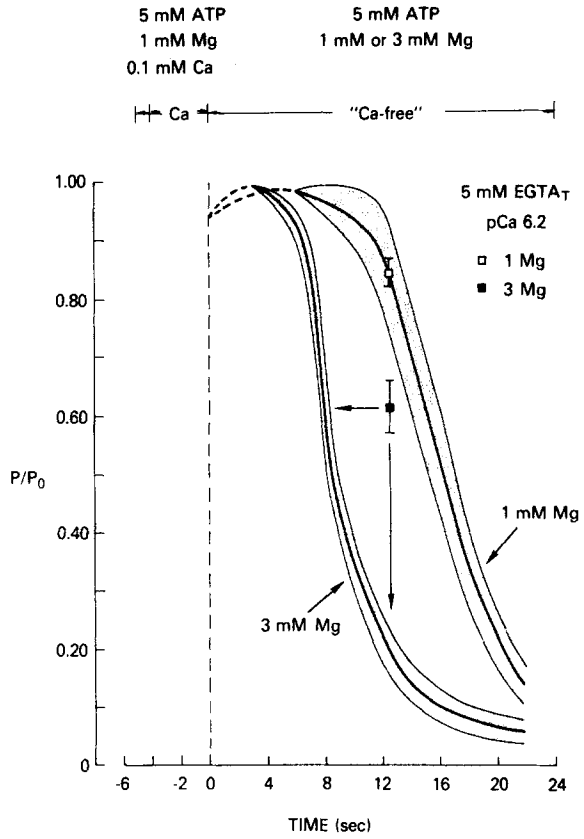


FIGURE 3. The mean time course of relaxation in 1 mM and 3 mM Mg in six segments tested as in Fig. 2. Force was normalized to the maximum force of the segment at seven time points after transfer to Ca-free solutions, and smooth curves were drawn by eye. The shaded areas represent ±1 SEM, determined at each mean point. The squares show the steady forces (± SEM) in CaEGTA buffer solution (pCa 6.2, total EGTA 5 mM) containing 1 mM Mg (open square) or 3 mM Mg (filled square). The steady forces are expressed as a fraction of the maximal force of the segment (in 5 mM CaEGTA); force at pCa 6.2 was measured at alternating Mg levels in the same fibers.

[1] In the values shown for steady force in buffer solutions, the base line used was the final "resting" tension, which increased appreciably after repeated self-controlled steady force measurements at 20°C. This procedure tends to exaggerate decreases in force, particularly at low relative forces, and gives an upper limit for this effect of Mg. Furthermore, since high force levels were maintained for relatively long periods during these measurements, any contribution of ATP depletion within the fiber to the Mg effects on force would be maximized.

The measured steady forces in CaEGTA buffer solutions permit the relaxation curves in Fig. 3 to be compared for [$Ca^{++}$] at a given time. The times required for the force to fall to the value corresponding to pCa 6.2 ($P/P_0$ = 0.84 for 1 mM Mg and 0.61 for 3 mM Mg) gives a direct comparison of the times required for the MFS Ca to fall from the same initial value to the same final value (with slightly more Ca removed from the myofilaments in 3 mM Mg [see Weber and Murray, 1973]). These times were 8 s in 3 mM Mg and 12.5 s in 1 mM Mg, with a ratio of 0.64. The time required in 3 mM Mg solution includes also the time required for the increased Mg to diffuse and act throughout the fiber volume. These results show that the shift of the force-pCa relation due to this change in Mg is a minor factor in the difference between the relaxation curves.

A second possibility considered was that the relaxation rate might be limited by the rate of dissociation of Ca from the myofilaments or of force-generating complexes (Weber and Murray, 1973). In order to rule out this possibility, force spikes were induced in 1 mM Mg, and relaxation in 3 mM Mg was compared with that in 1 mM Mg solution containing 1 mM EGTA. The time required for force to fall to half-maximum in 3 mM Mg was several times longer than in the Ca-chelating solution with low Mg. These results confirmed that the rate of decrease of ($Ca^{++}$) in the MFS was the rate-limiting step that was increased by higher Mg.

*Force Transients Induced by Changes in Mg Concentration*

Changes in Mg alone, in the presence of no added Ca or constant Ca in the bathing medium, caused transient changes in force. When Mg was lowered to 0.25 mM, phasic force developed in the absence of added Ca. The time course and shape of these transients were correlated with the initial Ca content of the SR (Fig. 4). In segments which had been loaded in CaEGTA buffer solution, as for a Ca-induced response, 0.25 mM Mg solution caused an immediate maximum force spike, rapid relaxation, and smaller subsequent peaks (Fig. 4*a*). In segments which contained only endogenous Ca in the SR, 0.25 mM Mg solution caused force oscillations with small or large spikes after a delay of 4–180 s (Fig. 4 *b*, *c*). When segments were pretreated with 3 mM EGTA solutions to lower the endogenous Ca in the SR (Ford and Podolsky, 1972*a*), force spikes appeared only after very long delays, 100–360 s. These results suggested that the ability of the SR to retain Ca was imparied at very low Mg ion concentrations. However, Ca transport was not entirely inhibited, since the periods of zero or low tension indicated that the contaminant Ca of the EGTA-free solution, and probably some of the released Ca, were accumulated. The phasic force pattern suggested that the Ca transport rate was not constant in time or space under these conditions.

When loaded segments were exposed to 0.25 mM Mg solutions containing 0.1 mM Ca, maximum force developed rapidly and remained steady; the secondary dip in tension typical of a Ca-induced force spike in 1 mM Mg was absent. However, a large transient decrease in the steady force was produced by increasing the Mg level. A typical transition from 0.25 mM Mg, 0.1 mM Ca solution to 1 mM Mg, 0.1 mM Ca solution is shown in Fig. 5*a*; force fell steeply after a few seconds' delay and required about 2 min to return to maximum. The

steady maximum forces were indistinguishable. The force trace in Fig. 5*b* made on a segment from the same fiber shows that this negative force transient was nearly abolished by 0.5 mM Cd, the SR inhibitor. The transition from 1 mM Mg, 0.1 mM Ca solution to 3 mM Mg, 0.1 mM Ca solution caused a similar force transient, as shown in Fig. 5*c*. These results indicate that when Mg was in-

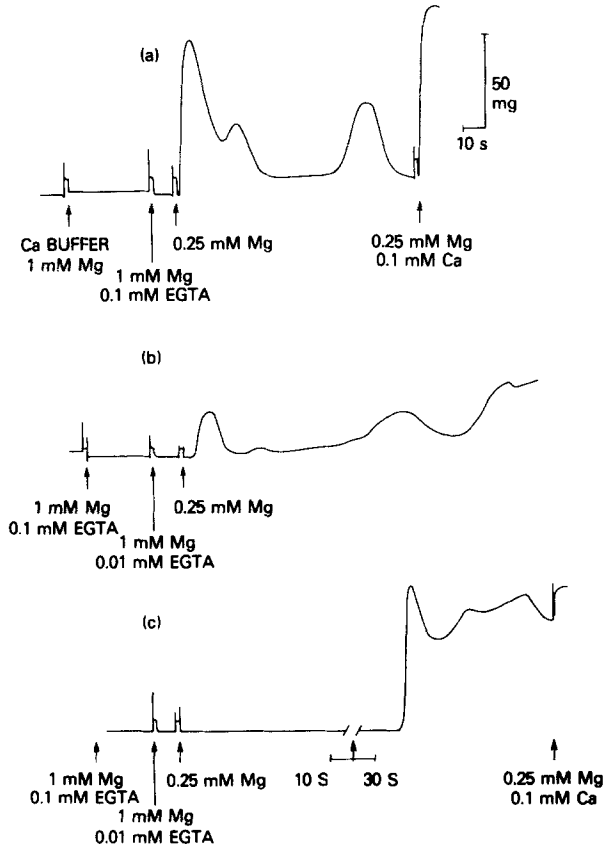


FIGURE 4. Phasic force responses in relaxed fibers after Mg reduction from 1 to 0.25 mM. (*a*) Ca-loaded segment. (*b*, *c*) Segments containing only endogenous Ca. Note the change to a slower time base during (*c*). Temperature, 19°C.

creased, the rate of Ca removal from the MFS by the SR exceeded the rate of Ca movement into the MFS by diffusion (see Discussion).

Force transients were observed even in CaEGTA buffer solutions after changes in Mg at constant pCa (or changes in pCa at constant Mg). At 20°C, the temperature in the preceding experiments, such transients were small or absent, especially at high EGTA concentrations. An example of a small negative transient due to increase in Mg from 1 to 3 mM in a CaEGTA buffer of pCa 6.2, total EGTA 5 mM, is shown in Fig. 6*a*. Note that the steady force decreased about 12% in 3 mM Mg; the negative transient is the *additional* decrease before the new force became steady, which took about 60 s in this case. The small effect when

MFS [$Ca^{++}$] is fixed primarily by the buffer can be compared directly with the large effect of the same Mg changes in unbuffered Ca solution (Fig. 5*c*); since the initial conditions of tension and ATPase activity were similar, the size of the force transient must reflect the influence of SR activity on MFS [$Ca^{++}$] rather than the direct action of $Mg^{++}$ or MgATP on the myofilaments. At 5°C, with a 3

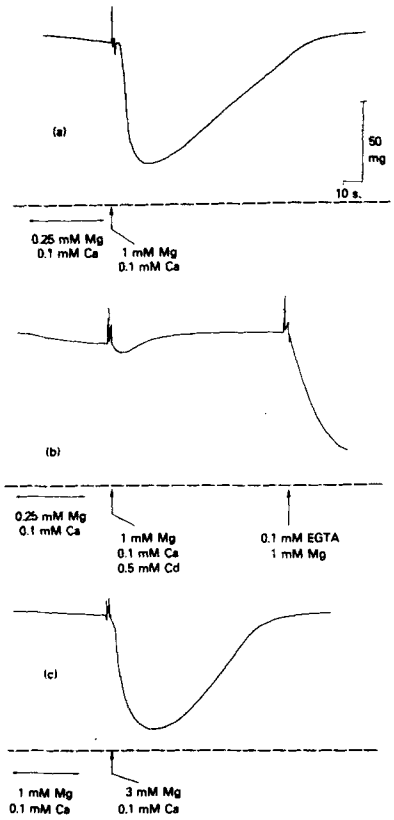


FIGURE 5. Negative force transients after an increase in Mg during continuous exposure to 0.1 mM Ca. All segments had been loaded previously in buffered Ca solution. (*a*) Mg increased from 0.25 mM to 1 mM. (*b*) The same transition in a segment from the same fiber, with 0.5 mM Cd in the 1 mM Mg solution. (*c*) Mg increased from 1 to 3 mM. Temperature, 19–20°C.

mM EGTA buffer system, [$Ca^{++}$] control by the solution was less effective, and increased Mg caused large negative transients, as shown in Fig. 6*b*. Since the steady force in this buffer, pCa 6.4, total EGTA 3 mM, was well below the maximum force, the inverse phenomenon could be observed as well: a transient overshoot in force when the Mg was lowered again, followed by a return to the steady level. The effect of Mg on the steady force was small, and the substantial positive transient must have been due to rapid net Ca loss from the SR. These results were additional evidence that small changes in Mg induced net Ca movements between SR and MFS at rates that could exceed the rate of the EGTA buffering reactions and diffusion from the bath.

*Mechanism of Cd Antagonism*

In three types of experiments described above, the effects of 3 mM Mg were antagonized by Cd. Although Cd is known to be a potent inhibitor of the Ca transport system of the SR, the possibility was considered that Cd could act directly on the myofilaments as a Ca substitute. In order to rule out this

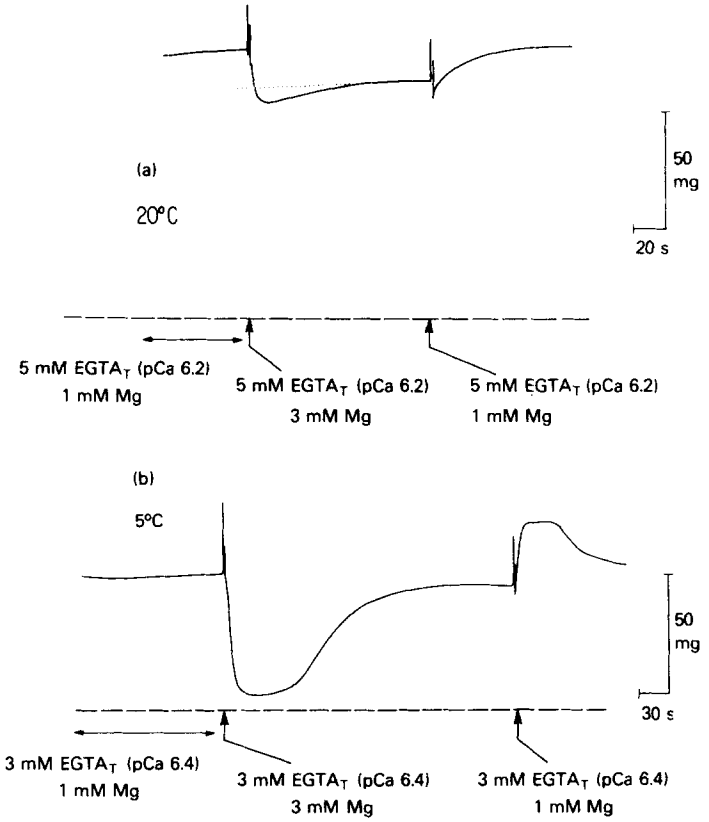


FIGURE 6. Force transients after changes in Mg between 1 and 3 mM in CaEGTA buffer solutions. (*a*) Small negative transient after Mg increase at 19–20°C; pCa 6.2, total EGTA 5 mM. The transient is the undershoot below the interrupted line which shows the new steady force in 3 mM Mg. (*b*) Large negative and positive transients after Mg increase and decrease at 2–5°C; pCa 6.4, total EGTA 3 mM. Note the different time bases; both are slower than in most other records shown.

mechanism, fibers were studied in 0.5 mM $CdCl_2$ solutions with no added Ca. The results of these experiments, summarized in Table I, showed that the time course of force development in Cd depended on the Ca content of the SR. When segments were loaded in CaEGTA buffer solution, rinsed, and exposed to Cd alone, force started to rise immediately or with a brief delay; the mean delay was less than 2 s. However, when SR Ca was depleted by pretreatment with 3 mM EGTA solutions, force development in Cd solution began only after long delays, averaging about 2 min. (In several segments from a different frog batch, delays

exceeded 10 min under these conditions.) The long periods of zero force in Ca-depleted segments were strong evidence that 0.5 mM Cd did not cause force by binding directly to the myofilaments; rapid Cd transport into the SR is extremely unlikely (see Discussion). The data in Table I show also that the rate of force development (indicated by the time between just detectable force and maximum force) was very slow in loaded segments as well as depleted segments, compared to the rapid rise of force in loaded segments exposed to Ca (e.g., Fig. 1). This difference indicated that Cd did not substitute for Ca in initiating rapid Ca release, and might even have slowed release initiated by Ca lost from the SR. The slow rate of force development was not due to interference with Ca binding to the myofilaments, because force rose immediately and rapidly when 0.1 mM Ca was added during the long delays in Cd in Ca-depleted fibers. The long delays after Ca-depletion suggested also that 0.5 mM Cd did not completely

TABLE I

TIME COURSE OF FORCE DEVELOPMENT IN 0.5 mM Cd SOLUTIONS

| Pretreatment* | Time to detectable force | Time to maximum force |
|---|---|---|
| | (s)±*SEM* | (s)±*SEM* |
| CaEGTA buffer (0.5 mM, pCa 6.2) | 1.7± 2.6 (11)‡ | 93.6±30.4 (11) |
| 3 mM EGTA (3–6 min) | 130.0±69.9 (6) | 223.0±70.6 (3) |
| 3 mM EGTA, 0.25 mM Mg (10 min) | 102.0±39.0 (5)§ | 183.0±40.6 (5) |

* Before exposure to Cd, segments were rinsed in sequence with 0.1 mM EGTA, 0.01 mM EGTA, and zero EGTA. Solutions contained 1 mM Mg unless indicated.

‡ Six segments showed no detectable delay.

§ Two additional segments from a different frog batch had delays exceeding 10 min.

block Ca uptake by the SR, since the contaminant Ca ($>10^{-5}$ M total) in the EGTA-free Cd solutions would be likely to cause force if MFS [$Ca^{++}$] had been equal to that of the bath. High forces could be maintained for long periods in Cd solutions, probably due to the contaminant Ca in the bath.

The effects of Cd were reversible. After being rinsed with dilute EGTA solutions, Cd-treated fibers maintained zero force in the CaEGTA buffer solution, indicating Ca accumulation, and gave Ca-induced force spikes. The force rise in Ca-loaded fibers exposed to Cd did not occur with Mn or Co ions; during 30-s exposures no responses were detected in either 0.6 mM $MnCl_2$ (eight trials) or 0.5 mM $CoCl_2$ (four trials).

## DISCUSSION

The main conclusions from these experiments are: (*a*) Mg ion promotes the rate of Ca uptake by the SR *in situ*; (*b*) Ca transport by the SR can control the MFS Ca in the presence of substantial Ca diffusion between MFS and bathing medium; (*c*) the acceleration of Ca uptake by Mg could account for Mg inhibition of the Ca-induced response in skinned fibers.

### *Mg Dependence of Ca Uptake in Skinned Fibers*

Ca accumulation by SR vesicles is Mg dependent in the presence of ATP (Carvalho and Leo, 1967), and the rate-limiting step of the associated ATPase

activity depends on [$Mg^{++}$] and the intravesicular Mg:Ca ratio (see Yamada and Tonomura, 1972). The present results show that Ca transport by the intact SR, in its normal spatial relation to the MFS, is strongly influenced by Mg. First, reaccumulation of internally released Ca is accelerated by increased Mg (Figs. 2, 3). After a brief Ca stimulus at least 0.3–0.5 mM Ca/kg has moved from SR to MFS (Ford and Podolsky, 1972*b*); the observed ratio of times required for force to fall to half-maximum in 3 and 1 mM Mg was 0.62. The difference in Mg has only a small effect on the force generated at a given MFS [$Ca^{++}$] (Fig. 3; also see Kerrick and Donaldson, 1972), and the ratio of times required for force to fall to the levels corresponding to the same pCa, 6.2, was 0.64. Ca transport thus was increased, on the average, by a factor of at least 1.6, the reciprocal of the time ratio. This is a lower limit for the Mg stimulation of Ca transport into the SR because some Ca must leave the MFS by diffusion into the bath, and a step increase in bath Mg cannot affect target sites instantaneously. It should be noted that this analysis of Ca movement based on force measurements agrees well with changes in $^{45}Ca$ movement described in the following paper (Stephenson and Podolsky, 1977).

Second, accumulation of externally supplied Ca is promoted by increased Mg. During steady force in 0.1 mM Ca solution, Ca distribution among the saturated myofilaments, MFS water, SR, and bathing medium is in a steady state. The large negative force transient which follows an increase in Mg indicates Ca removal from the myofilaments, while the slow return to maximum force shows that the bath Ca can saturate the myofilaments at the new Mg level when a new steady state is reached (Fig. 5). The negative transient evidently reflects a rapid fall in MFS [$Ca^{++}$], in the continuous presence of 0.1 mM Ca in the bath, due to an increased rate of SR uptake. At the minimum of the transient, the new rate of SR transport has removed much of the myofilament-bound Ca, and most of the 0.1 mM Ca initially in the MFS water, and maintains the effective MFS [$Ca^{++}$] close to the force threshold. During the large transient seen at 5°C in buffered Ca solutions with low [$Ca^{++}$], $Ca^{++}$ derived from CaEGTA dissociation in the MFS must be accumulated as well (Fig. 6).

Third, retention of SR Ca is decreased by a decrease in Mg. Mg reduction from 1 to 0.25 mM induces a force response which is large and immediate in Ca-loaded segments but is preceded by a delay in segments with only endogenous Ca or depleted Ca (Fig. 4). The delays show that the force response is due to Ca but that some Ca uptake remains; while MFS [$Ca^{++}$] is below the force threshold, contaminant Ca and Ca leaking from the SR must be accumulated. The abrupt patterns of force development suggest that Ca-induced release may be triggered when accumulation is slow and local Ca exceeds some threshold. The effect of the SR Ca level on the time course of release, and the oscillatory nature of the responses, may reflect the inhibition of Ca uptake by accumulated Ca (Weber, 1971) and the dependence of ATPase activity on the intravesicular Mg:Ca ratio (Yamada and Tonomura, 1972) reported in the isolated SR. Mg reduction between higher concentrations also causes Ca release (Fig. 6; also see Ford and Podolsky, 1972*b*).

The antagonistic effect between Cd and Mg is additional evidence that Mg acts to accelerate Ca transport. The ability of Cd to inhibit relaxation from force

spikes (Fig. 2) and to prevent the negative transients in steady force induced by increased Mg (Fig. 5) reveals the major role of Ca uptake by the SR in these processes. Cd is a potent inhibitor of the Ca transport system in the isolated SR (Carvalho, 1968; MacLennan, 1970). In the skinned fiber, the Cd appears to act primarily on Ca transport, rather than directly on the myofilaments. Although Cd binds to isolated troponin (Fuchs, 1971), 0.5 mM Cd solution (with no added Ca) produces no force for several minutes when Ca in the SR has been reduced (Table I). The delay is unlikely to be due to Cd accumulation by the SR because Cd interaction with isolated SR ATPase differs markedly from Ca interaction. The purified ATPase is enzymatically inactivated by 50 $\mu$M Cd (MacLennan, 1970). Furthermore, when the purified solubilized ATPase functions as an ionophore in lipid membranes, Cd transport is negligible (A. E. Shamoo, personal communication), similar to published data for Zn (Shamoo and MacLennan, 1974).

*Diffusion and Transport in Skinned Fibers*

Simple diffusional equilibration of Ca between bath and MFS should be rapid. For example, a cylindrical fiber 100 $\mu$m in diameter should be 90% equilibrated with bath Ca in about 2 s if the diffusion coefficent of $Ca^{++}$ in the MFS itself is about $3.5 \times 10^{-6}$ cm $s^{-1}$, and in about 8 s if Ca diffused only as CaATP with a diffusion coefficient of $1 \times 10^{-6}$ cm $s^{-1}$ (Hill, 1928). The assigned diffusion coefficients are about half the free diffusion coefficients; this is the physical factor for retardation of longitudinal diffusion within skinned fibers (Kushmerick and Podolsky, 1969).

The large deviations of transient force from that predicted by simple Ca diffusion are a measure of the relative influence of SR transport and diffusion within the fiber. The present experiments include three types of initial conditions for Ca diffusion between bath and MFS: an inward gradient, an outward gradient, and zero gradient. An inward gradient occurs when relaxed Ca-loaded fibers are exposed to 0.1 mM Ca, buffered only by ATP (Fig. 1). In 3 mM Mg, 0.1 mM Ca, force remained low for many seconds, indicating average MFS $[Ca^{++}]$ near $10^{-7}$ M and little myofilament-bound Ca, and required 90 s to reach maximum, indicating MFS $[Ca^{++}]$ near $10^{-6}$ M and myofilament-bound Ca near 0.1 mM/kg. The early rate of SR Ca uptake must nearly match the rate of Ca diffusion into the MFS from the bath, delaying equilibration of the MFS. The time course of rise in MFS Ca gives an estimate of an apparent diffusion coefficient under these conditions. If 60 s were required for the MFS Ca content to be 90% equilibrated with the 0.1 mM bath Ca, in a 100-$\mu$m diam fiber, the apparent diffusion coefficient would be $1.4 \times 10^{-7}$ cm $s^{-1}$. It is interesting to note that this is the value obtained from measurements of longitudinal $^{45}Ca$ diffusion in skinned fibers in oil (Kushmerick and Podolsky, 1969).

An outward Ca gradient occurs when Ca release has been induced by a brief Ca stimulus in 1 mM Mg (Figs. 2, 3). If 0.3–0.5 mM Ca/kg is in the MFS, with 0.1 mM/kg bound to the myofilaments, and the MFS water is 0.65 liter/kg fiber, Ca in the MFS water would be 0.3–0.6 mM when the fiber is transferred to "Ca-free" solutions. The rate of net Ca uptake by the SR must be comparable to the rate of outward diffusion along this gradient, since increasing Mg significantly increases

the total rate of disappearance of Ca from the MFS. The ability of SR transport to compete with outward diffusion for *net* Ca movement in these experiments is consistent with the finding that released $^{45}Ca$ is largely reaccumulated during a complete spike in 1 mM Mg, 0.1 mM Ca solution (Ford and Podolsky, 1972*b*).

The initial Ca gradient between bath and MFS is zero when Mg is increased during steady force in 0.1 mM Ca solution (Fig. 5). An inward Ca gradient develops when the rate of Ca movement from MFS to SR exceeds the rate of Ca diffusion from bath to MFS. The negative force transients show that the average MFS $[Ca^{++}]$ falls to about 1 $\mu$M in a few seconds, then to less than 0.5 $\mu$M, and remains below 1 $\mu$M for more than 1 min. Continuous inward Ca diffusion from the 0.1 mM Ca bathing solution evidently has little influence on local MFS $[Ca^{++}]$ within the fiber when Ca uptake by the SR is rapid.

*Mg Inhibition of the Ca-Induced Response*

The response to a 0.1 mM Ca stimulus is much more sensitive to Mg than previously realized. The large force spike which occurs in 1 mM Mg (0.02 mM $Mg^{++}$) is reduced to a few percent and a few seconds' duration as effectively by 3 mM Mg (0.1 mM $Mg^{++}$) as by 6 mM Mg (1.3 mM $Mg^{++}$), and the subsequent force rise is as slow. Since the effect of 3 mM Mg on the force-$[Ca^{++}]$ relation is small, the altered force pattern is more directly attributable to altered MFS $[Ca^{++}]$.

This force pattern indicates net Ca uptake by the SR during the time that net Ca release and a large force spike occur in 1 mM Mg. The increased rate of SR transport in 3 mM Mg could be the primary basis for the altered balance in net Ca movement. Even during a large response in 1 mM Mg, Ca uptake exceeds release within a few seconds (Ford and Podolsky, 1972*b*). In 3 mM Mg, released Ca is reaccumulated more rapidly; if 3 mM Mg acts from the time of stimulation, the duration of high MFS $[Ca^{++}]$ would be reduced and the maximum concentration decreased. The reduction in observed response is likely to be exaggerated in the skinned fiber. First, if the response duration is short compared to the time required for activation to spread, Ca released near the fiber surface could be reaccumulated by the time Ca was released near the fiber axis, and local responses might not fully summate. This could be the case since the duration of a twitch in intact frog muscle at 20°C is less than 10% of the time required for the large force spike in the skinned fiber to reach $P_0$. Second, if rapid reaccumulation decreased the local MFS $[Ca^{++}]$ sufficiently, the spread of activation would be impeded since Ca-induced released from interior SR elements depends on the inward diffusion of Ca. This could be the case since SR uptake competes effectively with Ca diffusion under other conditions, as shown above. Increased Mg then would block Ca-induced release indirectly, by truncation of the Ca stimulus.

There is no evidence that increased Mg directly blocks the Ca-induced release mechanism. The presence of miniature force responses in higher Mg (Fig. 1*b*; also see Podolsky and Hatchett, 1971; Ford and Podolsky, 1972*b*) can be interpreted to mean that a direct block does not occur. The antagonistic effect of Cd on the Mg inhibition (Fig. 1*c*) is consistent with this idea. However, a direct block cannot be excluded clearly by force measurements in the presence of an external

Ca stimulus. With a weak Cl stimulus, Mg does not appear to block the release mechanism directly (Stephenson and Podolsky, 1977).

*Ca Release and Reaccumulation in the Intact Fiber*

While the concentration of free $Mg^{++}$ in the MFS of intact fibers is not known, it is likely to be at least as high as 0.1 mM, the concentration in the 3 mM Mg (5 mM ATP) bathing solutions for skinned fibers. Frog skeletal muscle contains 6–8 mM/kg total Mg, but Gilbert (1960) found that 75–80% does not exchange with external $^{28}Mg$, and estimated exchanging intracellular [Mg] to be about 1.1 mM. This corresponds to about 0.1 mM [$Mg^{++}$] if the main ligand were 2 mM ATP. In rehydrated freeze-dried rat muscle the average $Mg^{++}$ activity measured with an Mg, Ca-sensitive electrode was 0.96 mM (Gunther and Dorn, 1971). These observations suggest that [$Mg^{++}$] is in the range $10^{-4}$ to $10^{-3}$ M.

The behavior of skinned fibers is compatible with this range of values. The rate of Ca uptake by the SR in 1 mM Mg (0.02 mM $Mg^{++}$) is adequate to account for the rate of relaxation in intact fibers (Ford and Podolsky, 1972*a*), and released Ca is reaccumulated at least 60% faster in 3 mM Mg. In 1 mM Mg, the Ca uptake rate may be inadequate to account for the localization of response to intracellularly applied $Ca^{++}$ in intact fibers (Niedergerke, 1955); in 3 mM Mg, however, the immediate response to applied Ca is small, even when the SR is loaded with extra Ca, and the SR uptake rate clearly is adequate to restrict the spread of MFS Ca. In 3 mM Mg, the retardation of Ca diffusion by SR transport suggested by the time required for force development is similar to the retardation of $^{45}Ca$ diffusion in skinned fibers in oil (Kushmerick and Podolsky, 1969), which have the endogenous Mg and Ca content of the intact fiber.

The comparisons above suggest that 0.1 mM $Mg^{++}$ is adequate for SR function similar to that in intact fibers, although their [$Mg^{++}$] could be higher; the precise $Mg^{++}$ sensitivity of the relaxation rate, localization of response, and retardation of diffusion is not known. In the skinned fiber, the increased rate of SR Ca uptake at 0.1 mM $Mg^{++}$ could inhibit the large response to applied Ca by shortening the duration of sequential local responses and by truncating the spread of the Ca stimulus. In the intact fiber, where the entire cross section normally is activated simultaneously, the higher rate of uptake should influence the duration of the response but need not preclude the local operation of Ca-induced Ca release.

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