

# Ca<sup>2+</sup> Dependence of Stimulated <sup>45</sup>Ca Efflux in Skinned Muscle Fibers

ELIZABETH W. STEPHENSON

From the Laboratory of Physical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

**ABSTRACT** Stimulation of sarcoplasmic reticulum Ca release by Mg reduction or caffeine was studied *in situ*, to characterize further the Ca<sup>2+</sup> dependence observed previously with stimulation by Cl ion. <sup>45</sup>Ca efflux and isometric force were measured simultaneously at 19°C in frog skeletal muscle fibers skinned by microdissection; EGTA was added to chelate myofilament space Ca either before or after the stimulus. Both Mg<sup>2+</sup> reduction (20 or 110 μM to 4 μM) and caffeine (5 mM) induced large force responses and <sup>45</sup>Ca release, which were inhibited by pretreatment with 5 mM EGTA. In the case of Mg reduction, residual efflux stimulation was undetectable, and <sup>45</sup>Ca efflux in EGTA at 4 μM Mg<sup>2+</sup> was not significantly increased. Residual caffeine stimulation at 20 μM Mg<sup>2+</sup> was substantial and was reduced further in increased EGTA (10 mM); at 600 μM Mg<sup>2+</sup>, residual stimulation in 5 mM EGTA was undetectable. Caffeine appears to initiate a small Ca<sup>2+</sup>-insensitive efflux that produces a large Ca<sup>2+</sup>-dependent efflux. Additional experiments suggested that caffeine also inhibited influx. The results suggest that stimulated efflux is mediated mainly or entirely by a channel controlled by an intrinsic Ca<sup>2+</sup> receptor, which responds to local [Ca<sup>2+</sup>] in or near the channel. Receptor affinity for Ca<sup>2+</sup> probably is influenced by Mg<sup>2+</sup>, but inhibition is weak unless local [Ca<sup>2+</sup>] is very low.

## INTRODUCTION

The central step of excitation-contraction coupling in skeletal muscle is Ca release from the sarcoplasmic reticulum (SR) to the myofilament space (MFS), but the mechanism of this key process is still poorly understood. The experiments described here extend the characterization of stimulated Ca efflux from the SR by analysis of <sup>45</sup>Ca movement in skinned muscle fibers, a preparation in which removal of the outer membrane facilitates more direct observation of the internal membrane system *in situ* (see Stephenson [1978 *a*] and its references).

Skinned fibers can be stimulated to release <sup>45</sup>Ca by bath Ca<sup>2+</sup> itself (Ford and Podolsky, 1972 *b*) and by replacement of a relatively impermeant bathing anion by Cl ion (Stephenson and Podolsky, 1977 *b*; Stephenson, 1978 *a*). The Cl stimulus presumably acts by changing the potential difference across some region of the internal membranes, either transverse tubules or SR, and may

correspond to a step in physiological activation (Costantin and Podolsky, 1967; also see Endo [1977] and Stephenson and Podolsky [1978]). The  $\text{Ca}^{2+}$  stimulus acts by an unknown mechanism, and its physiological significance has been unclear (Endo, 1977); however, the response to  $\text{Ca}^{2+}$  must reflect some property of the SR membrane. A clue to this  $\text{Ca}^{2+}$ -sensitive property came from the observation that Cl stimulation of  $^{45}\text{Ca}$  efflux, under conditions that prevent stimulation by bath Ca, is undetectable when MFS Ca is chelated by EGTA (Stephenson, 1978 *a*). Three possible mechanisms that may be physiologically relevant were suggested for the  $\text{Ca}^{2+}$ -dependence of Cl-induced release. (*a*) If Cl depolarizes the transverse tubules (T tubules),  $\text{Ca}^{2+}$  might be required for signal transmission at the T-SR junction. (*b*) Stimulated Ca efflux in general might utilize channels that are regulated by  $\text{Ca}^{2+}$  at a site accessible to MFS EGTA. (*c*) Secondary stimulation by released  $\text{Ca}^{2+}$  might constitute a major part of the response.

These possible mechanisms are not mutually exclusive. However, with stimuli that act directly on the SR, mechanism *a* would be excluded and the closely related mechanisms *b* and *c* could be evaluated. Therefore, the influence of EGTA on  $^{45}\text{Ca}$  efflux was studied after two stimuli that are unlikely to act on the T tubules, Mg reduction and caffeine. Ca release after Mg reduction has been inferred in skinned (or split) fibers (Stephenson and Podolsky, 1977 *a*; Endo, 1977), and release by caffeine has been observed or studied (often by indirect methods) in intact muscle fibers, skinned (or split) fibers, and fragmented SR membrane vesicles (see Endo [1977]). Because the stimulation mechanisms are not fully understood, these agents were employed empirically, but the experiments helped to clarify the basis of their action.

The results verified directly that Mg reduction and caffeine stimulate Ca release *in situ* and showed that both responses are highly  $\text{Ca}^{2+}$  dependent. The properties of the  $^{45}\text{Ca}$  efflux under different conditions suggested that a common channel (or carrier) that is regulated or gated by a  $\text{Ca}^{2+}$  receptor may mediate stimulated Ca efflux in general; this channel is either coupled in series with a  $\text{Ca}^{2+}$ -independent pathway or part of the same pathway.

Preliminary reports of portions of this work have been made (Stephenson, 1976; Stephenson, 1979).

#### METHODS

Fiber preparation, and mounting, bathing solutions, and tracer procedures have been described in detail previously (Stephenson, 1978 *a*). In brief, the semitendinosus muscle of the southern frog (*Rana berlandieri*) was isolated and suspended in a low-Cl Ringer's solution containing (mM): sucrose, 217; KCl, 2.5;  $\text{CaCl}_2$ , 1.8;  $\text{NaH}_2\text{PO}_4$  +  $\text{Na}_2\text{HPO}_4$ , 3.1. From a bundle of fibers transferred to cold paraffin oil, single fibers were isolated from tendon to tendon, and segments were skinned by microdissection just before use. The skinned segments, usually 2–2.5 mm long and 110–150  $\mu\text{m}$  in diameter, were tied with monofilament thread to small stainless steel rods, one attached to a leaf-spring photodiode force transducer; this permitted continuous measurement of isometric force and transfer of the segment between bathing solutions, which were maintained at 19°C in the wells of a spring-mounted thermoregulated chamber. The bathing solutions (pH 7.00) contained 120 mM potassium propionate,

10 mM imidazole, 5 mM Na<sub>2</sub>ATP, and either 1 mM MgSO<sub>4</sub> ("standard") or 0.25, 3, or 5 mM MgSO<sub>4</sub>. Additional constituents are indicated below. In solutions with 10 mM EGTA, potassium propionate was reduced to 110 mM. Caffeine was obtained from Eastman Kodak Co. (Rochester, N. Y.). The [Mg<sup>2+</sup>] levels calculated from binding by solution ATP and EGTA, as described previously (Stephenson and Podolsky, 1977 *a*), with an apparent ATP binding constant  $1.24 \times 10^4 \text{ M}^{-1}$  at pH 7.00 (Sillén and Martell, 1964), are about 20  $\mu\text{M}$  ("standard") or 4, 110, or 600  $\mu\text{M}$ , respectively. These values are only approximate and relative; for example, the appropriate absolute constant is uncertain, and the values would be decreased by the smaller amounts of binding to other solution constituents and fiber sites, and increased by Donnan charge effects in the MFS. It should be noted that fiber and bath concentrations in general can differ due to lack of instantaneous diffusional equilibration (see Stephenson [1981]), as discussed explicitly with respect to Ca<sup>2+</sup> and EGTA, and stated concentrations refer to simple solution values.

Fiber segments were loaded for 40 s in <sup>45</sup>CaEGTA buffer solution, 0.375 mM CaEGTA with 0.5 mM total EGTA (pCa 6.2, Sillén and Martell, 1964), prepared with high specific activity <sup>45</sup>CaCl<sub>2</sub> (New England Nuclear, Boston, Mass.); final activity was ~15–30  $\mu\text{C}/\text{ml}$ . The fibers were then rinsed twice in 0.1 mM EGTA solutions (for 10 s plus 20 or 90 s), once in 0.01 mM EGTA solution (for 10–15 s), transferred through a series of measured washout solutions, and finally extracted in measured solution with 0.05% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) + 5 mM EGTA, which removes the remaining <sup>45</sup>Ca (Stephenson, 1978 *a*). The amount of <sup>45</sup>Ca lost into each solution was expressed as a fraction of the total initial <sup>45</sup>Ca in the segment after rinsing, which is the sum of the <sup>45</sup>Ca lost to the washout and extraction solutions. The fraction remaining in the fiber at the end of each wash was obtained by sequentially back-adding to the fraction remaining at the end. The fraction lost into each wash was expressed as a flux by dividing by the time actually spent in the wash (i.e., transfer times were not included). The flux divided by the mean fraction in the fiber during that interval gives a first-order rate coefficient for <sup>45</sup>Ca efflux. The calculations were modified slightly in the case of fibers pretreated with high EGTA (for 5–6 s) before application of the stimulus: to make the stimulated losses more directly comparable, the data were normalized to total <sup>45</sup>Ca at the time of application of the stimulus rather than total <sup>45</sup>Ca after rinsing. This modification has only a small effect on the calculated fractional efflux, and no effect on the rate coefficient.

Results are expressed as the mean  $\pm$  SEM, with the number of determinations in parentheses. The significance of the difference between means or between paired values (from segments from the same fiber) was evaluated with Student's *t* tests;  $P < 0.05$  was considered significant.

## RESULTS

### <sup>45</sup>Ca Loss after Mg Reduction

Reduction of the free Mg<sup>2+</sup> bathing skinned fibers induces transient force responses, presumably due to Ca release from the SR (Stephenson and Podolsky, 1977 *a*; Endo, 1977). The force-generating system also is sensitive to low Mg<sup>2+</sup> and MgATP (Donaldson and Kerrick, 1975; Godt, 1974), but the present results immediately verified that Mg reduction stimulated Ca release.

<sup>45</sup>Ca movement after Mg reduction was studied in segments that had been loaded in <sup>45</sup>CaEGTA buffer solution and rinsed in dilute EGTA solutions

containing  $20 \mu\text{M Mg}^{2+}$  and then exposed to a series of solutions containing  $4 \mu\text{M Mg}^{2+}$  ( $0.25 \text{ mM Mg}$  total). A typical force trace and the mean time-course of tracer loss for a series of fibers are shown in Fig. 1. Mg reduction induced a large force response (*top*) with a relatively slow time-course preceded

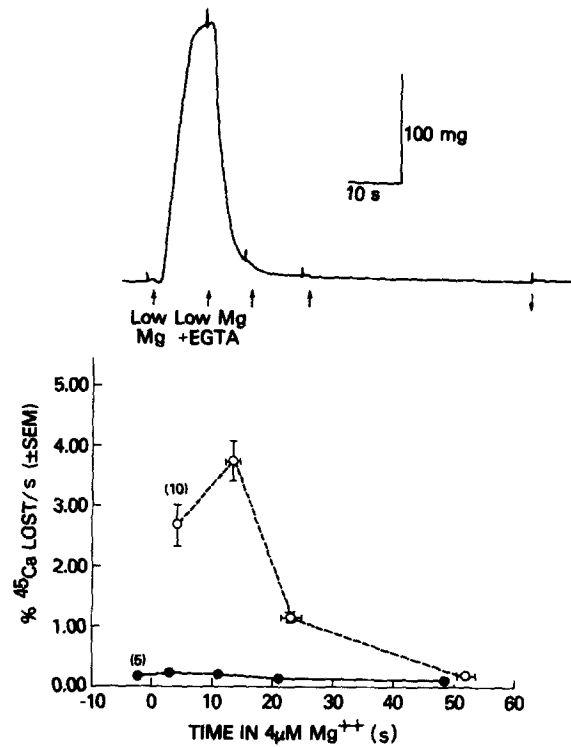


FIGURE 1. The effect of  $\text{Mg}^{2+}$  reduction ( $20 \mu\text{M}$  to  $4 \mu\text{M}$ ) on isometric force (*top*; representative trace) and mean  $^{45}\text{Ca}$  efflux (*bottom*) at  $19^\circ\text{C}$ . Fibers were loaded with  $^{45}\text{Ca}$  and rinsed in dilute EGTA solutions at  $20 \mu\text{M Mg}^{2+}$  (not shown) before stimulation. Upward arrows: transfer into subsequent solutions; downward arrow: removal from last solution before extraction in Triton X-100. (○) Percent  $^{45}\text{Ca}$  lost/s ( $\pm \text{SEM}$ ) during interrupted response, with no EGTA in the first wash and  $5 \text{ mM EGTA}$  in subsequent washes. (●) Percent  $^{45}\text{Ca}$  lost/s ( $\pm \text{SEM}$ ) during pretreated response, with  $5 \text{ mM EGTA}$  present before  $\text{Mg}^{2+}$  reduction at time zero. The efflux point for the pretreatment wash is located at the midpoint of the wash interval (excluding transfer time) in this and subsequent figures, to reflect the duration of this wash. See text for details.

by a small but distinct delay; the time to peak force was rather variable. The mean delay under these conditions was  $1.4 \pm 0.5 \text{ s}$  (9) (excluding one value that differed by 2.5 SD). These responses were interrupted by application of  $5 \text{ mM EGTA}$  near the peak of force development, to minimize the reaccumulation of released tracer from the MFS and maximize the amount collected. After  $\sim 1 \text{ min}$  in the washout solutions, the remaining tracer was extracted in

Triton X-100 EGTA solution. In matching experiments, the same protocol was followed, except the fibers were pretreated with 5 mM EGTA (at 20  $\mu M$   $Mg^{2+}$ ), which was maintained throughout the Mg reduction. No force development was observed under these conditions. Tracer efflux for each protocol, expressed as the percent initial  $^{45}Ca$  lost per second after Mg reduction, is plotted against time (Fig. 1, *graph*). During the interrupted response (*upper curve of graph*), stimulation of  $^{45}Ca$  efflux was substantial; however, the initial efflux from fiber to bath in the absence of EGTA, like the rate of force rise, was rather small in view of the large  $^{45}Ca$  loss over the whole response (see Fig. 3). The total initial efflux from the SR includes MFS-bound components (indicated by the force) as well as  $^{45}Ca$  appearing in the bath (see below). The *lower curve* of the graph in Fig. 1 shows efflux from pretreated fibers, in which the stimulus was applied in the presence of EGTA. When EGTA maintained very low MFS [ $Ca^{2+}$ ], efflux and  $^{45}Ca$  loss did not differ from that in control fibers (see Figs. 3 and 5). (The slight efflux increase in the first 4  $\mu M$   $Mg^{2+}$  wash above the pretreatment wash is associated with the experimental protocol per se [Stephenson, 1978 a]). Thus, stimulation of  $^{45}Ca$  efflux by Mg reduction was inhibited completely by EGTA, within the sensitivity of the method.

Because EGTA was completely inhibitory, stimulation in the interrupted response presumably ceased after EGTA application, and the total initial SR efflux can be calculated using the pretreated response as a control. The initial  $^{45}Ca$  efflux from SR to MFS, given approximately by a total stimulated release of 51.1% (60.4–9.4%) (Fig. 3) restricted to the 8.2-s interval between stimulation and EGTA application, was 5.3%  $s^{-1}$ , about twice the initial flux to the bath (Fig. 1).

The time-course of the stimulation was altered when Mg was reduced after pretreatment with a higher  $Mg^{2+}$  concentration; in five segments from the same fibers studied with the simpler protocol,  $Mg^{2+}$  in the final dilute EGTA rinse (10 s duration) was increased from 20 to 110  $\mu M$  (3 mM Mg, 5 mM ATP). This change produced a striking increase in the delay before detectable force developed, as seen in the representative force trace in Fig. 2 (*top*). The mean delay before a detectable rise in force was  $23.3 \pm 4.3$  s (5), 10 times the mean delay when  $Mg^{2+}$  was reduced from 20 to 4  $\mu M$ . The increase in delay between paired segments was  $19.9 \pm 3.7$  s (5) (including the deviant value mentioned above). To measure efflux during the variable long delay, the duration of the initial wash in low Mg was adjusted to end just as force production was detected, and a second EGTA-free wash was added to correspond to the initial low-Mg wash illustrated in Fig. 1. The mean time-course of tracer efflux (Fig. 2, *graph*) was normalized to the time of application of the second EGTA-free wash, just as the force was rising, but fractional efflux was calculated from the time of Mg reduction as before. Force rose steeply during transfer to the second wash, so efflux evidently had already increased, but the average rate observed during the force delay was only  $0.09 \pm 0.01\%$   $s^{-1}$  (less than control rates in EGTA). The delay in force development thus appeared to be based upon a genuine delay in efflux stimulation. The length of the delay, relative to diffusion times in the MFS, suggested that the time required for  $Mg^{2+}$  to fall to a critical value at the relevant membrane

sites was influenced by a sequestered Mg source, probably the SR (see Discussion). Once the response began, the "initial" rate measured in the second wash was significantly higher than that shown in Fig. 1.

Total  $^{45}\text{Ca}$  loss during these responses is summarized in Fig. 3, with a

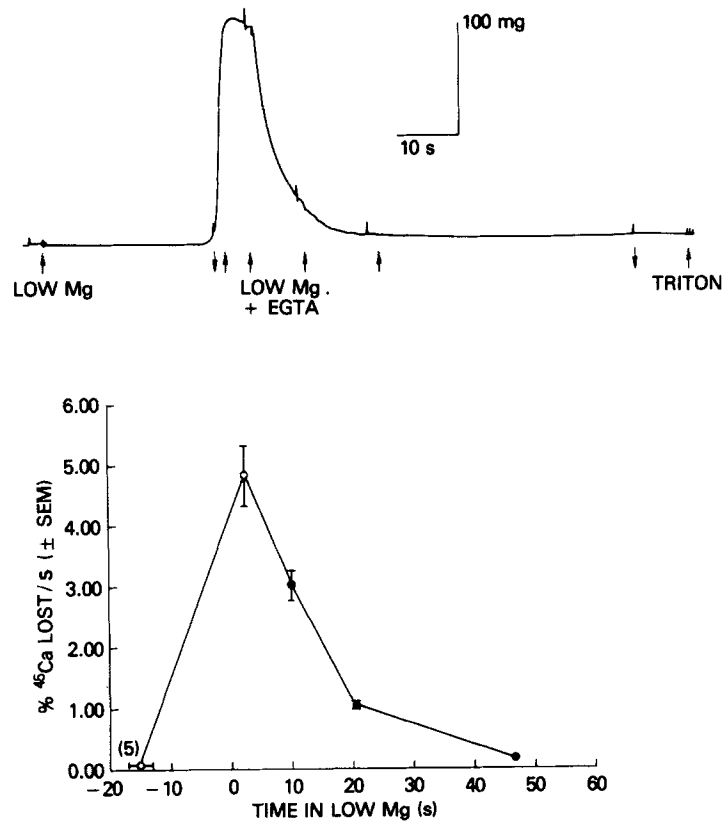


FIGURE 2. The effect of modified  $\text{Mg}^{2+}$  reduction ( $110\ \mu\text{M}$  to  $4\ \mu\text{M}$ ) on isometric force (*top*; representative trace) and mean  $^{45}\text{Ca}$  efflux during interrupted response (*bottom*).  $\text{Mg}^{2+}$  was increased from  $20\ \mu\text{M}$  to  $110\ \mu\text{M}$  during the final dilute EGTA rinse before stimulation. To measure  $^{45}\text{Ca}$  efflux during the long variable force delay, an extra wash was added (note SEM bar on time axis), and the fiber was transferred to a second wash when force was detected; zero time is referred to this transfer, not to  $\text{Mg}^{2+}$  reduction. (○)  $4\ \mu\text{M}\ \text{Mg}^{2+}$ , no EGTA. (●)  $4\ \mu\text{M}\ \text{Mg}^{2+}$ ,  $5\ \text{mM}\ \text{EGTA}$ .

control value for comparison. During interrupted responses, with the stimulus applied before EGTA, 60% of the fiber  $^{45}\text{Ca}$  was lost in about 1 min, whether  $\text{Mg}^{2+}$  was reduced from  $20\ \mu\text{M}$  or from  $110\ \mu\text{M}$  (where the loss during the additional delay period was a small fraction of the total). When the fibers were pretreated with EGTA before Mg reduction, tracer loss was reduced to

<10% and did not differ from that in control fibers kept in  $20 \mu M Mg^{2+}$ . This result indicated, first, that stimulation by Mg reduction was highly  $Ca^{2+}$  dependent and, second, that  $^{45}Ca$  efflux in the presence of EGTA was not detectably increased at  $4 \mu M Mg^{2+}$ , at least not within 1 min.

Because an increase in  $Ca^{2+}$  permeability in low  $Mg^{2+}$  could provide a mechanism for the stimulatory effect of Mg reduction,  $^{45}Ca$  efflux was followed for a longer time in  $4$  or  $20 \mu M Mg^{2+}$  in an additional group of pretreated

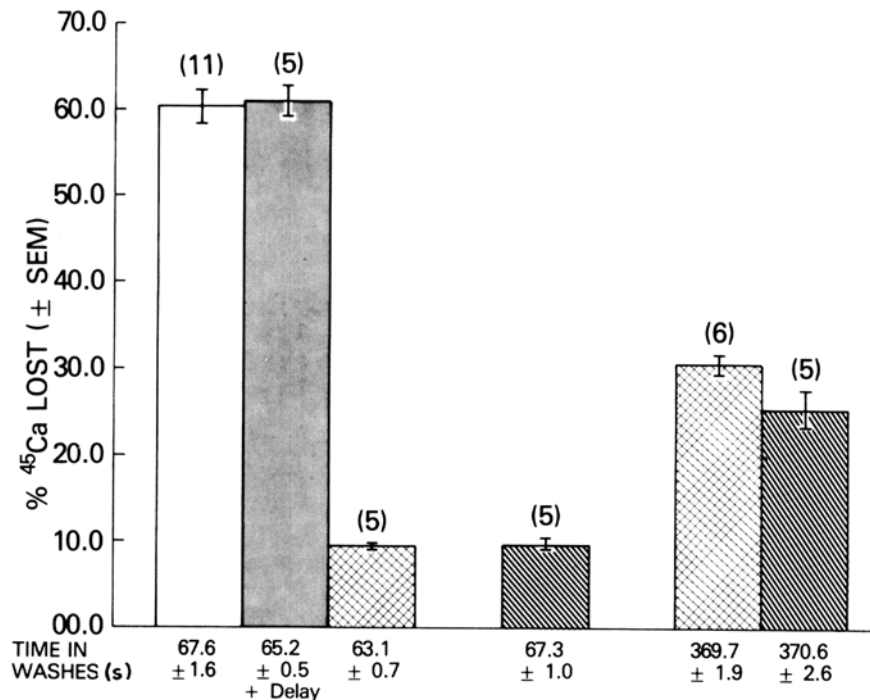


FIGURE 3. Comparison of total percent  $^{45}Ca$  lost ( $\pm$ SEM) after  $Mg^{2+}$  reduction in the absence and presence of EGTA, with control values for comparison. Mean wash times are shown beneath each bar. (open bar)  $Mg^{2+}$  reduction ( $20 \mu M$  to  $4 \mu M$ ), interrupted response; (stippled bar) modified  $Mg^{2+}$  reduction ( $110 \mu M$  to  $4 \mu M$ ), interrupted response; (crosshatched bar)  $Mg^{2+}$  reduction ( $20 \mu M$  to  $4 \mu M$ ), pretreated with  $5 mM$  EGTA; (diagonally striped bar) control EGTA ( $20 \mu M Mg^{2+}$ ,  $5 mM$  EGTA).

fibers. After more than 6 min, the total  $^{45}Ca$  loss in  $4 \mu M Mg^{2+}$  (Fig. 3) was a little larger but not significantly different, even when segments from the same fiber treated with  $4$  or  $20 \mu M Mg^{2+}$  were compared (five pairs). This result extended previous observations over the range  $20 \mu M$  to  $1.3 mM Mg^{2+}$  (Stephenson, 1978 a) that indicate that the passive permeability of the SR membrane to Ca is not very sensitive to free  $Mg^{2+}$ .

A simple increase in Ca permeability did not provide an adequate explanation of the effect of Mg reduction. However, Ca uptake from MFS to SR

in skinned fibers appears to be highly sensitive to  $Mg^{2+}$  changes in this range (Stephenson and Podolsky, 1977 *a*). The known  $Mg^{2+}$  dependence of the isolated active Ca transport system, together with the time-course of stimulation, suggested that the mechanism depended on local  $Ca^{2+}$  accumulation near the SR membrane (see Discussion).

*Preliminary Studies with Various Caffeine Concentrations Applied with EGTA*

Caffeine is known to stimulate Ca loss from the SR in preparations ranging from intact muscle to fragmented vesicles (see Endo [1977]). The effect of caffeine on  $^{45}Ca$  efflux was examined initially at 20  $\mu M$   $Mg^{2+}$  with 5 mM EGTA and caffeine applied simultaneously. The time-course of tracer loss was followed for 3 min in 1, 5, or 25 mM caffeine.

The results of this study are shown in Fig. 4, where the fraction of  $^{45}Ca$  remaining in the fiber is plotted on a semilog scale against the time in caffeine. The effect of 1 mM caffeine was small but distinct; the rate coefficient for tracer loss between 70 and 190 s,  $1.3 \pm 0.1 \times 10^{-3} s^{-1}$  (4), was significantly larger than the control value in the same group of fibers at the same  $Mg^{2+}$  and EGTA concentrations,  $0.8 \pm 0.1 \times 10^{-3} s^{-1}$  (3) (also see Stephenson [1978 *a*]). The curves for 5 and 25 mM caffeine were markedly biphasic, with most of the tracer loss in the first 20 s. At these caffeine concentrations, especially 25 mM, small, brief force transients were observed upon caffeine application, in spite of the presence of 5 mM EGTA in the solution. These transients indicated that caffeine acted before EGTA had equilibrated sufficiently to maintain MFS  $Ca^{2+}$  below  $10^{-7}$  M. The stimulated  $^{45}Ca$  release was graded with caffeine concentration, but the difference between 5 and 25 mM caffeine occurred in the first 20–40 s; the rate coefficients for tracer loss between 70 and 190 s,  $1.8 \pm 0.1 \times 10^{-3} s^{-1}$  (4) and  $1.9 \pm 0.1 \times 10^{-3} s^{-1}$  (4), respectively, were not significantly different (but exceeded that in 1 mM caffeine).

These results confirmed that caffeine stimulated Ca release, in a concentration dependent way, in the presence of EGTA. However, the small initial force transients observed at high caffeine concentrations and the similarity of the rate coefficients for later loss, together with the inhibitory effect of EGTA described below (also see Weber [1968] and Endo [1977]), suggested that the larger effect of 25 mM caffeine was due in part to more rapid inward diffusion, with longer action before MFS EGTA rose substantially. Caffeine at 5 mM caused loss of about half the tissue tracer in the first minute, providing an adequate stimulus for use in further studies. The results also emphasized that simultaneous application of EGTA complicates the interpretation of  $^{45}Ca$  loss; either caffeine application before EGTA or preequilibration with EGTA before caffeine provides a clearer set of conditions for analysis of the stimulated  $^{45}Ca$  efflux.

*$^{45}Ca$  Efflux in Caffeine at 20  $\mu M$   $Mg^{2+}$*

Fibers maintained in 20  $\mu M$   $Mg^{2+}$  were loaded with  $^{45}Ca$ , rinsed, and stimulated by 5 mM caffeine either before or after the application of 5 mM EGTA. When EGTA application preceded caffeine, no force responses were



observed. When caffeine preceded EGTA application, the force rose immediately and rapidly to high (apparently maximal) values, falling promptly when EGTA was applied near the force peak. A typical force trace from an interrupted response and the mean time-course of  $^{45}Ca$  efflux from a series of fibers are shown in Fig. 5. The time-course of  $^{45}Ca$  efflux, expressed as the percent initial  $^{45}Ca$  lost per second after caffeine application, is shown for both

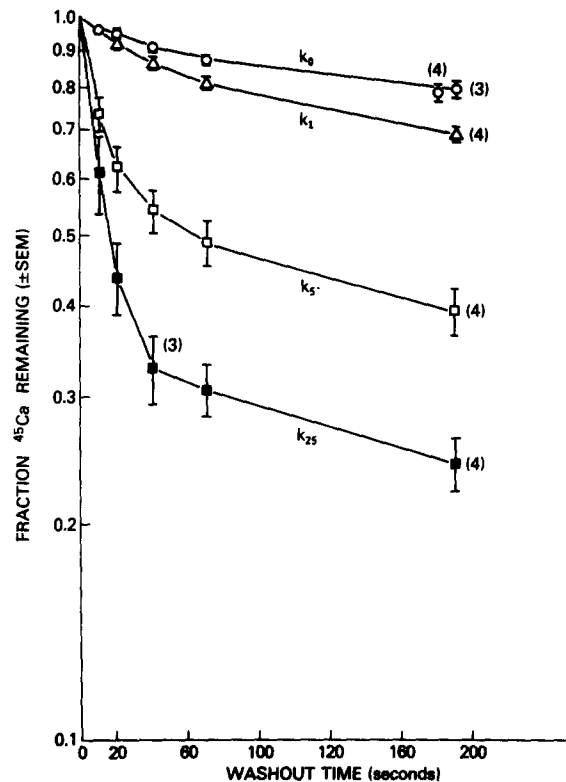


FIGURE 4. The effect of 1–25 mM caffeine + 5 mM EGTA on the mean  $^{45}Ca$  remaining with time. Caffeine and EGTA were applied simultaneously;  $20 \mu M Mg^{2+}$ ,  $19^{\circ}C$ . From upper curve down: O, control;  $\Delta$ , 1 mM caffeine;  $\square$ , 5 mM caffeine;  $\blacksquare$ , 25 mM caffeine.

interrupted and pretreated responses, together with controls for the series ( $20 \mu M Mg^{2+}$ , 5 mM EGTA). When caffeine preceded EGTA application (*upper curve*), the initial rate was very high and fell off steeply. Because the EGTA wash applied at the force peak was chelating myofilament-bound  $^{45}Ca$  released previously, as indicated by the developed force, the initial rate of  $^{45}Ca$  movement from SR to MFS was even higher (see below). When caffeine followed EGTA pretreatment (*middle curve*), the initial  $^{45}Ca$  efflux was reduced

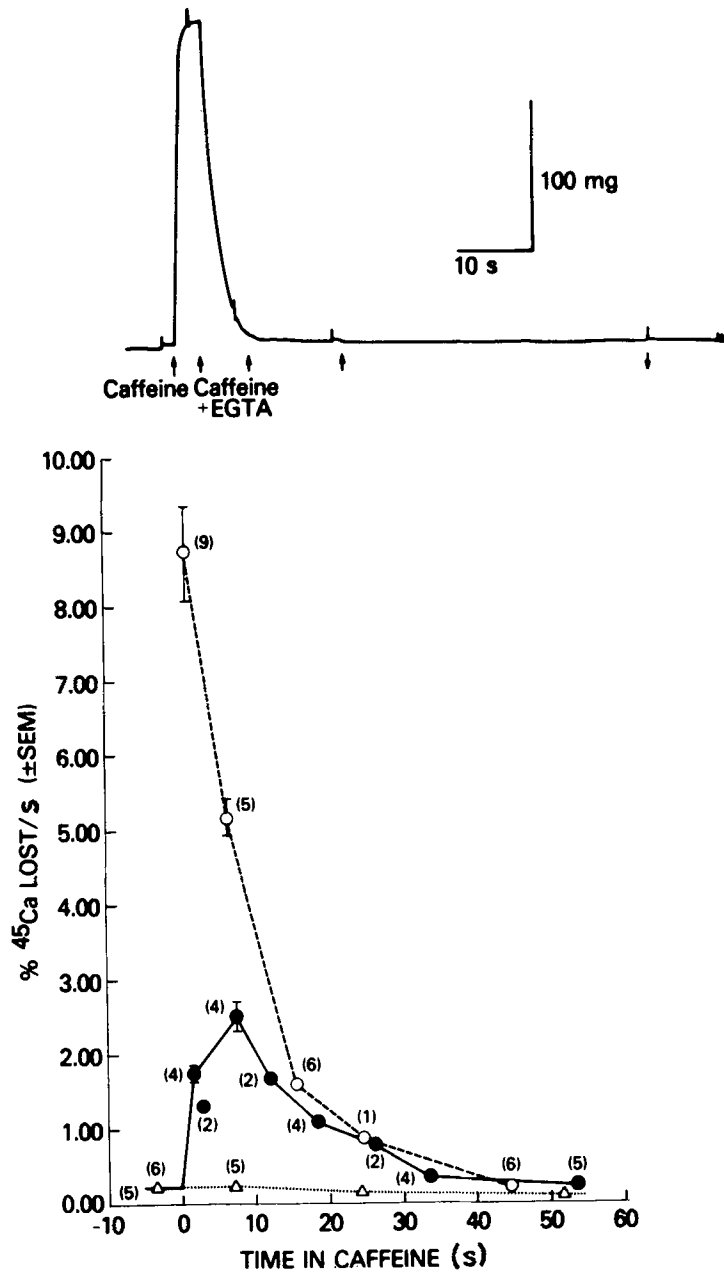


FIGURE 5. The effect of 5 mM caffeine at  $20 \mu\text{M Mg}^{2+}$  on isometric force (*top*; representative trace) and mean  $^{45}\text{Ca}$  efflux (*bottom*). ( $\circ$ )  $^{45}\text{Ca}$  efflux during interrupted response, with no EGTA in the first wash, 5 mM EGTA in subsequent washes; ( $\bullet$ )  $^{45}\text{Ca}$  efflux during pretreated response, with 5 mM EGTA present before caffeine application at time zero; ( $\triangle$ ) control  $^{45}\text{Ca}$  efflux (5 mM EGTA,  $20 \mu\text{M Mg}^{2+}$ ). The initial point on the upper curve includes values pooled from Fig. 7.

to 18% of the initial rate in caffeine alone; the maximum rate was reached during the second caffeine wash, and this was <30% of the initial rate in caffeine alone.

Pretreatment with 5 mM EGTA clearly inhibited stimulation of <sup>45</sup>Ca efflux by caffeine strongly but incompletely. The total <sup>45</sup>Ca loss during washout was  $68.0 \pm 0.9\%$  (6) in the interrupted response, compared with  $41.9 \pm 1.5\%$  (6) in these pretreated fibers (also see Fig. 9), ~60% larger. However, the overall comparison underestimates the inhibitory effect of EGTA on the initial large caffeine stimulation. The initial efflux to the bath in the absence of EGTA was five times the initial rate in caffeine in the presence of EGTA, even with no account taken of the MFS-bound <sup>45</sup>Ca in the interrupted response.

The additional component of initial release from the SR can be assessed with the assumption that the behavior of the SR in the interrupted response after EGTA has entered the MFS resembles that in the pretreated response. The subsequent efflux from the SR is then estimated from the rate coefficients during the pretreated response and the mean <sup>45</sup>Ca contents during the interrupted response. Subtraction of this component from the total efflux to the bath leaves the component of <sup>45</sup>Ca loss to the bath in EGTA due to <sup>45</sup>Ca actually released from the SR before EGTA was applied. The extra initial loss amounted to 27% of fiber <sup>45</sup>Ca during the first two EGTA washes in the interrupted response (the rate coefficient during the final wash was similar to that in the pretreated response). The total initial release from the SR by this calculation was 44% of fiber <sup>45</sup>Ca, the sum of the extra component and the initial loss to the bath,  $17.1 \pm 1.2\%$  (8). This total loss was eight times the initial loss from fiber to bath in the EGTA-pretreated fibers,  $5.6 \pm 0.5\%$  (4); the initial rate (see below) was seven times as large. However, the efflux in the pretreated fibers continued to rise transiently; this interesting effect is described further below.

The total initial loss in the absence of EGTA took place within an average wash period of 2.1 s, with a subsequent transfer interval of ~1.5 s, so the initial rate of caffeine-stimulated <sup>45</sup>Ca efflux from the SR was  $12.2\% \text{ s}^{-1}$ , equivalent to  $\sim 200 \mu\text{M Ca liter}^{-1} \text{ s}^{-1}$  (Stephenson, 1978 *a*). A similar initial rate of  $12.6\% \text{ s}^{-1}$  is estimated from the initial wash interval alone by adding the Ca required for the developed force, which would be  $\sim 150 \mu\text{M liter}^{-1}$  (equivalent to 9.4% tracer), to the 17.1% that appeared in the bath.

The time-course of the pretreated response suggested that the effects of caffeine and EGTA might interact in a complex way. If <sup>45</sup>Ca efflux in the pretreated response were simply reduced by the Ca dependence of the stimulated efflux pathway, one might expect efflux to decline with time as in the interrupted response, but for the data shown in Fig. 5 the rate coefficient for the second caffeine wash,  $29.0 \pm 1.3 \times 10^{-3} \text{ s}^{-1}$  (4) was significantly higher than that for the first caffeine wash,  $18.2 \pm 1.1 \times 10^{-3} \text{ s}^{-1}$  (4). One possible explanation for the continued increase, consistent with the incompleteness of inhibition, was that 5 mM EGTA did not adequately control free Ca<sup>2+</sup> at the sensitive membrane sites when the rate of Ca movement across the SR membrane was high. If this were the case, higher [EGTA] would further reduce the stimulation of efflux and perhaps alter the time-course.

This possibility was examined in additional experiments that compared efflux in 10 and 5 mM EGTA in pretreated stimulated fibers. The results are summarized in Fig. 6, where the rate coefficients for stimulated  $^{45}\text{Ca}$  loss are plotted against time, together with control values in 10 mM EGTA. Both curves showed the delayed maximum, but the curves crossed, suggesting that higher EGTA had a dual effect. When segments from the same fiber were compared (four to five pairs), the first two rate coefficients after caffeine were significantly lower in 10 mM EGTA than in 5 mM EGTA (the second point only by a one-tailed  $t$  test), whereas the last two rate coefficients were slightly but significantly higher. In the control studies in 10 mM EGTA (run concurrently), total  $^{45}\text{Ca}$  loss during the first minute was  $13.9 \pm 0.4\%$  (5), significantly

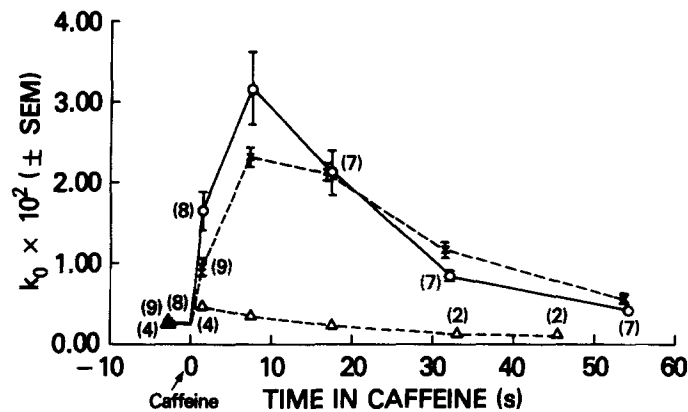


FIGURE 6. The effect of increased EGTA on pretreated responses in 5 mM caffeine at  $20 \mu\text{M Mg}^{2+}$ . The rate coefficients for  $^{45}\text{Ca}$  efflux,  $k_0$  ( $\text{s}^{-1}$ ), are plotted against time in caffeine. (○) 5 mM EGTA, 5 mM caffeine; (×) 10 mM EGTA, 5 mM caffeine; (△) 10 mM EGTA, control.

larger than control loss in 5 mM EGTA,  $9.6 \pm 0.4\%$  (9) (a pooled value from two earlier studies; also see Stephenson [1978 *a*]). This increase was confined to the early (minor) component of unstimulated efflux;  $^{45}\text{Ca}$  loss in 10 mM EGTA over 3 min was  $23.3 \pm 2.6\%$  (5), compared with  $20.8 \pm 2.2\%$  (4) in 5 mM EGTA (Stephenson, 1978 *a*). However, it partially offset the effect of 10 mM EGTA on the stimulated efflux, so the observed ratios of the early rate coefficients in 10 and 5 mM EGTA, 0.60 in the first caffeine wash and 0.80 in the second caffeine wash, gave a minimum estimate of the additional inhibition.

These results showed that inhibition of the stimulated Ca efflux was graded with EGTA concentration, implying that the Ca regulation of this pathway is graded. In addition, the limited ability of 5 mM EGTA (and perhaps 10 mM EGTA) to control free  $\text{Ca}^{2+}$  near the membrane regulatory sites was in marked contrast to the ability of EGTA to buffer at the troponin Ca-binding

sites in the MFS, even at lower concentrations. The continued increase in efflux (delayed maximum) may be related to this limited buffering ability in conjunction with a stimulated Ca efflux (see Discussion).

#### *Effect of Caffeine on Ca Reaccumulation by the SR*

The residual stimulation of  $^{45}Ca$  efflux by caffeine in the presence of EGTA (Figs. 4–6) indicated a direct effect on efflux (also see Endo [1977]). On the other hand, studies on SR vesicles suggest that caffeine acts on the active transport mechanism for Ca influx (Weber, 1968). This effect was examined by delaying EGTA application to permit Ca reaccumulation from the MFS. Segments were exposed to EGTA-free caffeine solution ( $20 \mu M Mg^{2+}$ ) for 40–50 s before 5 mM EGTA was applied, and the amount of  $^{45}Ca$  loss and efflux were compared with that in the interrupted response. A representative force trace and the mean time-course of efflux from four fibers are shown in Fig. 7. Unlike fibers giving “completed” responses to stimulation by Cl ion or Mg reduction (Stephenson and Podolsky, 1977 *a* and 1977 *b*), the fibers did not relax spontaneously in caffeine but maintained high levels of force until EGTA was applied. The slow decline in the force plateau was due at least in part to mechanical deterioration of the preparations, which tolerated maintained caffeine contractures poorly; small breaks in the myofibrils were usually detectable. The high initial  $^{45}Ca$  efflux declined more rapidly than in the interrupted responses (compare Fig. 5), and total tracer loss,  $60.9 \pm 1.1\%$  (5), was significantly less than in the interrupted response. Because 44% of the fiber  $^{45}Ca$  left the SR during the initial EGTA-free wash and transfer in both types of protocol (see above), the subsequent loss in the “completed” response was ~65% of that in the interrupted response. The longer exposure to EGTA during the interrupted response had a dual effect, but some reaccumulation of  $^{45}Ca$  in the absence of EGTA was suggested.

Influx in caffeine-treated fibers was assessed more specifically by resolving the net  $^{45}Ca$  flux in the absence of EGTA, about 15 s after caffeine application, into estimated efflux and influx components. Influx was calculated by subtracting the measured net flux to the bath from an estimated unidirectional efflux, given by the product of the SR [ $^{45}Ca$ ] and an “uninhibited” efflux rate coefficient,  $k_o$  (in the absence of EGTA). The net flux at this time was  $1.32 \pm 0.23\% s^{-1}$  (4) (Fig. 6), corresponding to  $0.0211 \text{ mM Ca liter}^{-1} s^{-1}$ . The mean amount in the tissue was  $58.6 \pm 1.1\%$  (4), corresponding to  $0.938 \text{ mM liter}^{-1}$ , but an appreciable part of this Ca obviously was bound in the MFS; assuming that at least  $0.150 \text{ mM liter}^{-1}$  was in the MFS, the SR content would be  $\leq 0.788 \text{ mM liter}^{-1}$ . To estimate  $k_o$  at this time, the corresponding efflux rate coefficient in EGTA was scaled up, assuming the initial efflux in the absence of EGTA approximates the unidirectional efflux (see Discussion). Increasing the efflux rate coefficient in the interrupted response by a factor of 7, the ratio of total initial SR efflux in the absence of EGTA to initial efflux in the presence of EGTA (see above), gives  $k_o = 0.232 s^{-1}$ , efflux =  $0.183 \text{ mmol liter}^{-1} s^{-1}$ , and influx =  $0.162 \text{ mmol liter}^{-1} s^{-1}$ . This estimate is <12% of the influx that is predicted from MFS  $[Ca^{2+}] > 10^{-6} \text{ M}$  with the minimum rate constant

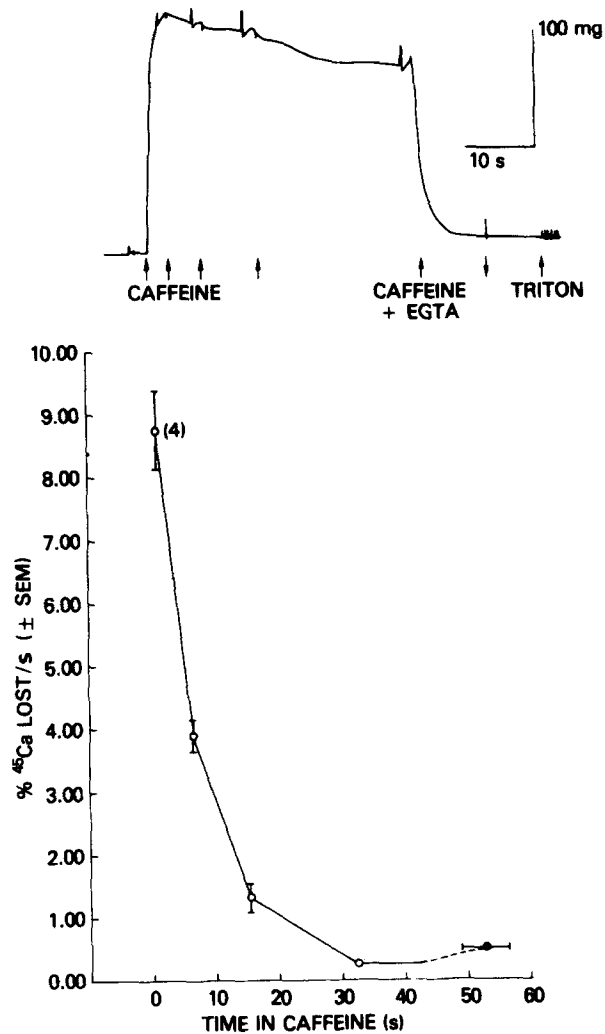


FIGURE 7. The effect of 5 mM caffeine at  $20 \mu\text{M Mg}^{2+}$  with delayed application of EGTA ("completed" response). A representative force trace is shown in the upper part, and mean  $^{45}\text{Ca}$  efflux is shown in the lower part. Fibers withstood the maintained caffeine contracture poorly (see text for details).

$1.4 \times 10^3 \text{ s}^{-1}$  estimated from  $^{45}\text{Ca}$  uptake in unstimulated fibers (Ford and Podolsky, 1972 a), suggesting that caffeine inhibited influx.

#### $^{45}\text{Ca}$ Efflux in Caffeine at $600 \mu\text{M Mg}^{2+}$

Increased  $\text{Mg}^{2+}$  reduces caffeine-stimulated Ca release in fragmented SR vesicles (Weber, 1968; Fairhurst and Hasselbach, 1970) and in skinned (split) muscle fibers (see Endo [1977]). Because  $20 \mu\text{M}$  free  $\text{Mg}^{2+}$  is probably far below the level in intact fibers, it was pertinent to examine the effect of higher

$Mg^{2+}$  on caffeine stimulation of  $^{45}Ca$  efflux in the absence and presence of EGTA.

For these experiments, fibers were loaded with  $^{45}Ca$  and rinsed in the usual way at  $20 \mu M Mg^{2+}$ , but the final EGTA rinse (0.01 mM) and the caffeine solutions contained  $600 \mu M Mg^{2+}$  (5 mM Mg and ATP). The effect of 5 mM caffeine was reduced, but the experiments formed two distinct groups. In earlier studies (winter frogs), stimulation of  $^{45}Ca$  loss in interrupted responses (six segments) was so small that it did not differ significantly from pretreated responses in which  $^{45}Ca$  loss was at control levels. (These data are included in Fig. 9.) Two additional segments were allowed to remain in caffeine without EGTA for about 40 s; these developed minute tension oscillations, with larger transient peaks during the transfers (through air) between washes, but increased  $^{45}Ca$  efflux was not detectable. The tension records suggested that MFS [ $Ca^{2+}$ ] was close to the force threshold.

In later studies (spring frogs), substantial force responses and stimulation of  $^{45}Ca$  efflux were observed consistently.<sup>1</sup> These results are summarized separately in Fig. 8, where a representative force trace is shown (*top*) and mean  $^{45}Ca$  efflux is plotted against time in caffeine (*bottom*) for interrupted responses, pretreated responses, and control conditions ( $600 \mu M Mg^{2+}$ , 5 mM EGTA). Segments used for pretreated responses were all from the same fibers used for interrupted responses. (Additional points are shown for the initial efflux in caffeine at  $20 \mu M Mg^{2+}$  from two fibers tested during the earlier studies at  $600 \mu M Mg^{2+}$ , verifying that responsiveness to caffeine itself had not decreased.) In the fibers stimulated at  $600 \mu M Mg^{2+}$ ,  $^{45}Ca$  efflux early in the interrupted response (*upper curve*) was smaller than in caffeine at  $20 \mu M Mg^{2+}$  (compare Fig. 5), and the initial efflux was lower than efflux during the first EGTA wash. During the pretreated response,  $^{45}Ca$  efflux was the same as control efflux (*lower curves*), indicating no detectable residual stimulation in these responsive fibers.

The total initial efflux from the SR in the interrupted response, including the MFS-bound component, can be estimated as before on the assumption that after EGTA has entered the MFS, the behavior of the SR in the interrupted response resembles that in the pretreated response. The initial loss to the bath in the absence of EGTA,  $7.54 \pm 1.3\%$  of fiber  $^{45}Ca$  (5) took place within an average wash period of 2.9 s, with a subsequent transfer time of  $\sim 1.5$  s. The additional  $^{45}Ca$  released to the MFS and appearing later was calculated (as for  $20 \mu M Mg^{2+}$ ) from the subsequent loss and the mean  $^{45}Ca$  remaining in the interrupted response, and the appropriate rate coefficient in the pretreated response. The extra loss, 37.1% of fiber  $^{45}Ca$ , and the initial loss to the bath gave a total initial loss of 44.6% and a resulting initial efflux from the SR of  $10.1\% s^{-1}$ . The initial rate is 83% of that estimated at  $20 \mu M Mg^{2+}$ , much more similar than the  $^{45}Ca$  efflux curves. The smaller difference is

<sup>1</sup> A fresh set of caffeine solutions was used for this series; however, two segments tested gave force responses to the remaining  $600 \mu M Mg^{2+}$  caffeine solution used in the earlier studies. This check was made because caffeine precipitates that could have been difficult to resolubilize were often seen after thawing frozen aliquots of working solution (containing ATP), so a slightly lower caffeine concentration was possible.

attributable to differences in the early time-course; the calculated rates are averaged over the initial wash period and the subsequent transfer time (which approaches the duration of the short initial wash). At  $600 \mu\text{M Mg}^{2+}$ , the force, and presumably the efflux, were still rising during transfer; at  $20 \mu\text{M Mg}^{2+}$ , the total initial rate estimated from the wash interval alone was at least as

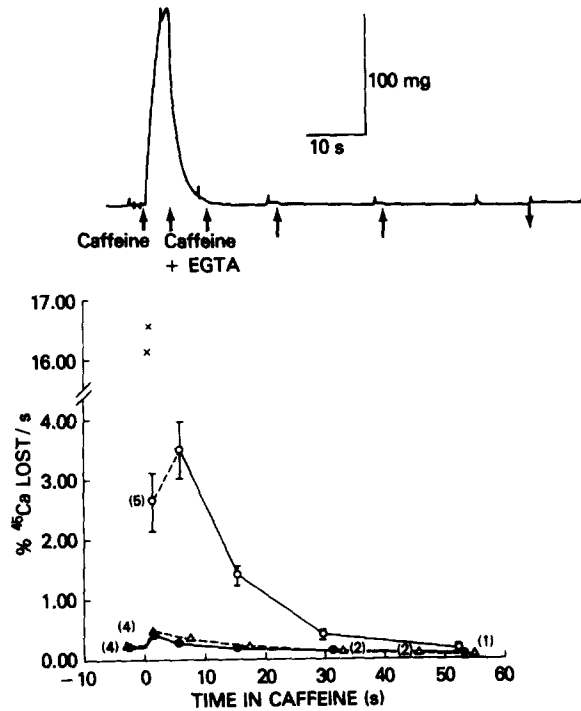


FIGURE 8. The effect of 5 mM caffeine at  $600 \mu\text{M Mg}^{2+}$  in the responsive group of fibers (see text for details) on isometric force (*top*; representative trace) and mean  $^{45}\text{Ca}$  efflux (*bottom*). (○)  $^{45}\text{Ca}$  efflux during the interrupted response, with no EGTA in the first wash, 5 mM EGTA in subsequent washes; (●)  $^{45}\text{Ca}$  efflux during pretreated response, with 5 mM EGTA present before caffeine application at time zero; (△) control  $^{45}\text{Ca}$  efflux (5 mM EGTA,  $600 \mu\text{M Mg}^{2+}$ ); (×) initial  $^{45}\text{Ca}$  efflux during two interrupted responses at  $20 \mu\text{M Mg}^{2+}$  (see text for details).

large as the average rate (see above), probably associated with rapid propagation of  $\text{Ca}^{2+}$  stimulation at low  $\text{Mg}^{2+}$  (Ford and Podolsky, 1972 *b*). After the first wash, stimulation can continue in 5 mM EGTA at  $20 \mu\text{M Mg}^{2+}$  (Fig. 5) but not  $600 \mu\text{M Mg}^{2+}$  (Fig. 8).

The results of both groups of experiments at  $600 \mu\text{M Mg}^{2+}$  are summarized in Fig. 9, which shows the total  $^{45}\text{Ca}$  lost during the interrupted, pretreated, and control protocols and compares them with the results at  $20 \mu\text{M Mg}^{2+}$ .



During the interrupted response, <sup>45</sup>Ca release at the higher Mg<sup>2+</sup> concentration was either not significantly above the control loss, or ~50% of fiber <sup>45</sup>Ca; the stimulated loss (above control) was 67% of that at lower Mg<sup>2+</sup>. During the pretreated response, stimulation of <sup>45</sup>Ca release at the higher Mg<sup>2+</sup> concentration was undetectable in both groups of experiments. At the lower Mg<sup>2+</sup>, <sup>45</sup>Ca release during the pretreated response (pooled data from Figs. 5 and 6) was ~46% of fiber tracer, with the increase above control loss 60% of that in the interrupted response. It should be noted that EGTA pretreatment had a much larger proportional effect at the higher Mg<sup>2+</sup> concentration, i.e., EGTA and Mg<sup>2+</sup> were synergistic; if the increase above control loss had been 60% (or

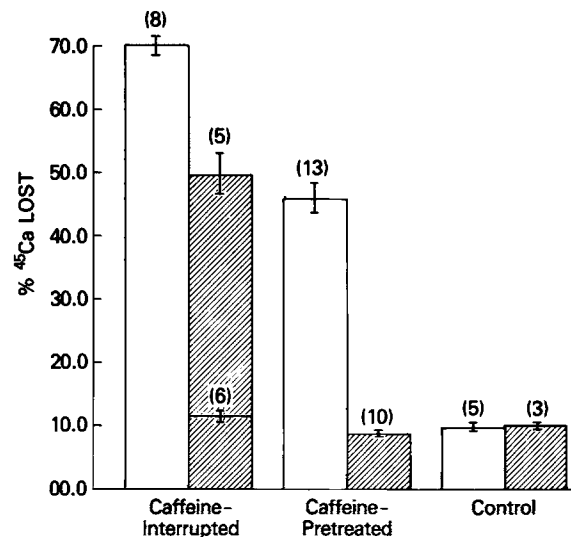


FIGURE 9. Comparison of the total percent <sup>45</sup>Ca lost ( $\pm$ SEM) at 20  $\mu$ M Mg<sup>2+</sup> (open bars) or 600  $\mu$ M Mg<sup>2+</sup> (striped bars) during interrupted and pretreated responses to 5 mM caffeine; control loss is shown for comparison. At 600  $\mu$ M Mg<sup>2+</sup>, the interrupted responses formed two distinct groups, which are shown separately, whereas the pretreated response was the same for both groups (see text for details).

even 10%) of that in the effective interrupted response, then residual stimulation in the pretreated response would have been readily detectable.

These results showed that the Ca<sup>2+</sup> dependence of the stimulated efflux pathway was not restricted to low Mg<sup>2+</sup> conditions but was actually more apparent at equimolar Mg and ATP. In addition, Mg<sup>2+</sup> appeared to have a direct inhibitory effect on caffeine-stimulated efflux.

#### DISCUSSION

##### *Ca<sup>2+</sup> Dependence as a Property of SR Ca Efflux*

The Ca<sup>2+</sup> dependence of stimulation by Mg<sup>2+</sup> reduction and caffeine is of particular interest, because these agents appear to act on the SR side of the

excitation-contraction coupling pathway. In intact fibers, caffeine can stimulate Ca release under conditions that inhibit stimulation via the T tubules: sustained depolarization (see Lüttgau and Oetliker [1968]), T tubule disruption by glycerol osmotic shock (Howell, 1969), or increased sarcomere length (Frank and Winegrad, 1976). Caffeine also stimulates  $^{45}\text{Ca}$  release from isolated SR vesicles (Weber and Herz, 1968; Fairhurst and Hasselbach, 1970). In the case of  $\text{Mg}^{2+}$  reduction, the steady-state Ca content of SR vesicles in the presence of ATP is a positive function of medium  $[\text{Mg}^{2+}]$  (Carvalho and Leo, 1967), implying net loss when  $[\text{Mg}^{2+}]$  is lowered. The present results are consistent with this site of action; for example, the long delays after reduction from a higher  $[\text{Mg}^{2+}]$  would be difficult to explain by a primary effect on the T tubules (see below). In addition, stimulation of  $^{45}\text{Ca}$  release by  $\text{Mg}^{2+}$  reduction or caffeine is not inhibited in skinned fibers stretched to twice slack length (Stephenson, 1978 *b*), whereas the increased  $^{45}\text{Ca}$  efflux (Frank and Winegrad, 1976) and aequorin-monitored  $\text{Ca}^{2+}$  signal (Blinks et al., 1978) from electrically stimulated intact fibers are inhibited at long sarcomere lengths.

An SR locus for these  $\text{Ca}^{2+}$  dependent stimuli implies a Ca efflux pathway directly regulated by  $\text{Ca}^{2+}$ . It does not exclude a  $\text{Ca}^{2+}$  requirement for signal transmission at the T-SR junction, which could underly the  $\text{Ca}^{2+}$  dependence of Cl-stimulated  $^{45}\text{Ca}$  efflux (Stephenson, 1978 *a*). The Cl-stimulated efflux, like physiological stimulation (see above), is inhibited in stretched skinned fibers (Stephenson, 1978 *b*), consistent with the possibility that stretch interferes with T-SR coupling and Cl acts by depolarization of the T tubules (Stephenson, 1978 *b*).

#### *Mechanism of Stimulation by Mg Reduction*

When Mg reduction preceded EGTA application, total  $^{45}\text{Ca}$  release (above control) was nearly as large as with caffeine stimulation at  $20\ \mu\text{M}\ \text{Mg}^{2+}$  (Figs. 3 and 7), but the average initial flux from SR to MFS,  $5.3\% \text{ s}^{-1}$ , was substantially slower. This net flux still greatly exceeds resting efflux in EGTA, indicating direct or indirect stimulation of efflux (see Stephenson [1978 *a*]). A direct increase in simple permeability could not be demonstrated; at the low MFS  $[\text{Ca}^{2+}]$  in 5 mM EGTA, reduction to  $4\ \mu\text{M}\ \text{Mg}^{2+}$  had no detectable effect on  $^{45}\text{Ca}$  efflux during the first minute and no significant effect even over 6 min (Figs. 1 and 3). Thus, most or all of the stimulation was indirect.

The most plausible mechanism for the efflux stimulation is a rise in local  $[\text{Ca}^{2+}]$  near the SR, due to inhibition of active Ca influx.  $\text{Ca}^{2+}$  is an adequate stimulus of net  $^{45}\text{Ca}$  release (Ford and Podolsky, 1972 *b*; also see Endo [1977]), and  $[\text{Mg}^{2+}]$  reduction is likely to promote its local concentration and facilitate its action. The rate-limiting step in active Ca transport by SR vesicles is  $\text{Mg}^{2+}$  dependent (see MacLennan and Holland [1975] and Tada et al. [1978]), and increasing  $[\text{Mg}^{2+}]$  bathing skinned fibers increases net Ca uptake from MFS to SR (Stephenson and Podolsky, 1977 *a*). Both the rate of active Ca transport in SR vesicles (Weber, 1971) and the delay in force response by skinned fibers to  $[\text{Mg}^{2+}]$  reduction (Stephenson and Podolsky,

1977 *a*) decrease with increased Ca loading of the SR. Facilitation by low [Mg<sup>2+</sup>] of Ca<sup>2+</sup> stimulation is consistent with the ability of low (Mg<sup>2+</sup>) to reduce the bath [Ca<sup>2+</sup>] required for net release in split fibers (see Endo [1977]), and is suggested by the competitive effect of Mg<sup>2+</sup> on Ca<sup>2+</sup> stimulation of <sup>45</sup>Ca efflux in SR vesicles (Katz et al., 1977).

The present observations support operation of these mechanisms. Local MFS [Ca<sup>2+</sup>] near the SR presumably is determined by efflux, active influx, and diffusion; as local [Mg<sup>2+</sup>] falls, decreasing influx would increase this [Ca<sup>2+</sup>], even with little or no increase in efflux. The force pattern and initial <sup>45</sup>Ca efflux after [Mg<sup>2+</sup>] reduction from 20 to 4 μM are consistent with a secondary rise in local [Ca<sup>2+</sup>]. The long delays after reduction from 110 to 4 μM Mg<sup>2+</sup> suggest that a substantial sequestered Mg source is close to the sensitive sites; the time required for 90% diffusional equilibration of MFS [Mg<sup>2+</sup>] (Hill, 1928) is <0.1 the observed delay before effective stimulation. The SR luminal and membrane Mg constitutes such as source, close to the active transport system (see Tada et al. [1978]) and to a possible Mg<sup>2+</sup>-sensitive site on the Ca<sup>2+</sup>-regulated efflux channel. Two observations are consistent with facilitation by low [Mg<sup>2+</sup>] of the effectiveness of local [Ca<sup>2+</sup>]: total <sup>45</sup>Ca release is large (Fig. 3), twice that observed with stimulation by 0.1 mM bath Ca at 20 μM Mg<sup>2+</sup> (Ford and Podolsky, 1972 *b*), and inhibition of caffeine stimulation by Mg<sup>2+</sup> and EGTA is synergistic, as discussed below.

#### *Mechanism of Stimulation by Caffeine*

Net Ca release after caffeine treatment has been attributed to direct stimulation of SR efflux or inhibition of active Ca uptake. The present results gave evidence of both processes. In 10 mM EGTA, [Ca<sup>2+</sup>] approaches 10<sup>-10</sup> M; the ability of caffeine to increase <sup>45</sup>Ca efflux under these initial conditions (Fig. 6) implies that caffeine stimulates efflux directly, since initial influx is negligible. Furthermore, caffeine must initiate stimulation at a site that is either sequestered from EGTA or not Ca<sup>2+</sup> sensitive, although this efflux component is small (see below).

In addition, influx appears to be inhibited concurrently in the absence of EGTA (Fig. 7 and Results). When the net outward <sup>45</sup>Ca flux ~15 s after caffeine application was analyzed, using parallel data from interrupted and pretreated responses, the estimated influx component was <12% of influx predicted from <sup>45</sup>Ca uptake rates in unstimulated skinned fibers (Ford and Podolsky, 1972 *a*). This fraction is an upper limit, provided that the efflux rate coefficient was scaled up sufficiently by the ratio of initial flux in the absence or presence of EGTA (see Results); if the initial <sup>45</sup>Ca flux from the SR were the difference between much larger unidirectional fluxes, inhibition could be less. However, the unidirectional efflux predicted by the sum of the uninhibited influx and the net flux, >1.42 mM s<sup>-1</sup>, exceeds the SR Ca content by at least 0.3–0.6 mM s<sup>-1</sup>, requiring recycling of released Ca at improbably high rates (Winegrad, 1968; see Stephenson [1978 *a*]). Therefore, the net <sup>45</sup>Ca flux in the absence of EGTA, both at 15 s and initially, is likely to reflect substantial inhibition of influx.

### *Ca<sup>2+</sup> Dependence of Caffeine Stimulation*

The effects of EGTA pretreatment on caffeine-stimulated <sup>45</sup>Ca efflux reveal both a Ca<sup>2+</sup>-insensitive component (M<sub>1</sub>) and a Ca<sup>2+</sup>-dependent component (M<sub>2</sub>). At 20 μM Mg<sup>2+</sup>, pretreatment with 5 mM EGTA reduced the large initial stimulation substantially but incompletely, and increasing EGTA to 10 mM reduced the early efflux further (Figs. 5, 6, and 9); in the first caffeine wash, the stimulated <sup>45</sup>Ca efflux (above corresponding control rates) was 1.1% s<sup>-1</sup> in 5 mM EGTA and only 0.38% s<sup>-1</sup> in 10 mM EGTA. The additional inhibition suggests that 5 mM EGTA did not buffer adequately near a Ca<sup>2+</sup> receptor for the M<sub>2</sub> component, although initial [Ca<sup>2+</sup>] was below 10<sup>-9</sup> M. The M<sub>1</sub> component behaves like a potent local Ca<sup>2+</sup> source, suggesting that the Ca<sup>2+</sup> receptor for the M<sub>2</sub> component is closely coupled to the caffeine-stimulated pathway. The results are most simply explained by two fluxes in series, e.g., M<sub>2</sub> = αM<sub>1</sub>, where α is a coupling factor between them.

The early time-course of <sup>45</sup>Ca efflux in EGTA may be a manifestation of close coupling. Because EGTA preceded stimulation, the transient delayed maximum (Fig. 6) reflects increased transmembrane flux, not chelation of MFS <sup>45</sup>Ca released initially. Increasing efflux could reflect time-dependent caffeine binding; caffeine stimulation of SR vesicles in the absence of ATP increases over 5–15 min (Fairhurst and Hasselbach, 1970). However, the increase seen here was early and not sustained (Figs. 4–6). A more interesting possibility is that positive feedback at the postulated Ca<sup>2+</sup> receptor further reduces the effectiveness of EGTA buffering, implying that the residual efflux in 10 mM EGTA still contains an M<sub>2</sub> component and overestimates the M<sub>1</sub> component. A small M<sub>1</sub> is consistent with the small effect of caffeine on the later efflux in EGTA (Fig. 4) and with the efflux at higher [Mg<sup>2+</sup>], discussed below.

The resistance to buffering is not due to diffusional limitation on the *average* fiber [EGTA]. With a diffusion coefficient of 4.6 × 10<sup>-6</sup> cm<sup>2</sup> s<sup>-1</sup> (Moisescu and Thieleczek, 1978) and a reaction rate equal to the initial Ca efflux in 10 mM EGTA (neglecting the slow back reaction), free EGTA at the axis should be 4.97 mM when a fiber of radius 60 μm is stimulated in 5 mM EGTA (Hill, 1928). Consistent with high MFS [EGTA], force development was never seen in pretreated fibers. However, the rate of Ca<sup>2+</sup> binding to EGTA is relatively slow (Smith et al., 1977), and the calculation above does not apply to local reaction conditions as Ca<sup>2+</sup> emerges from discrete sites of unknown number and distribution. Present information is insufficient for a detailed analysis of the diffusion-reaction kinetics in this geometry.

The M<sub>2</sub> component and its Ca<sup>2+</sup> receptor could be intrinsic to a channel mediating the M<sub>1</sub> component or in a separate channel. A simple operational model emphasizing the close coupling between the two components consists of a single channel gated by an intrinsic Ca<sup>2+</sup> receptor, which could also mediate stimulation by local MFS Ca<sup>2+</sup> after Mg<sup>2+</sup> reduction. In the simplest case of the schematic common channel (CH2) shown in Fig. 10, two receptor sites are postulated to control a channel gate. A caffeine receptor (R<sub>1</sub>) is located at the luminal end to indicate inaccessibility to MFS EGTA or Ca<sup>2+</sup>

insensitivity. A  $Ca^{2+}$  receptor ( $R_2$ ) is located within the MFS end of the channel to indicate accessibility to MFS  $Ca^{2+}$  and EGTA but more favorable exposure to channel  $Ca^{2+}$ ; it would have to be sequestered from luminal  $Ca^{2+}$  before stimulation. Activation of  $R_1$  would shift the gate and increase channel  $[Ca^{2+}]$ , which would activate  $R_2$ , widen the gate, and greatly increase efflux. In the absence of EGTA, emerging  $Ca^{2+}$  would bind at sites accessible to the MFS or diffuse into the bath. Moderate EGTA would prevent MFS binding

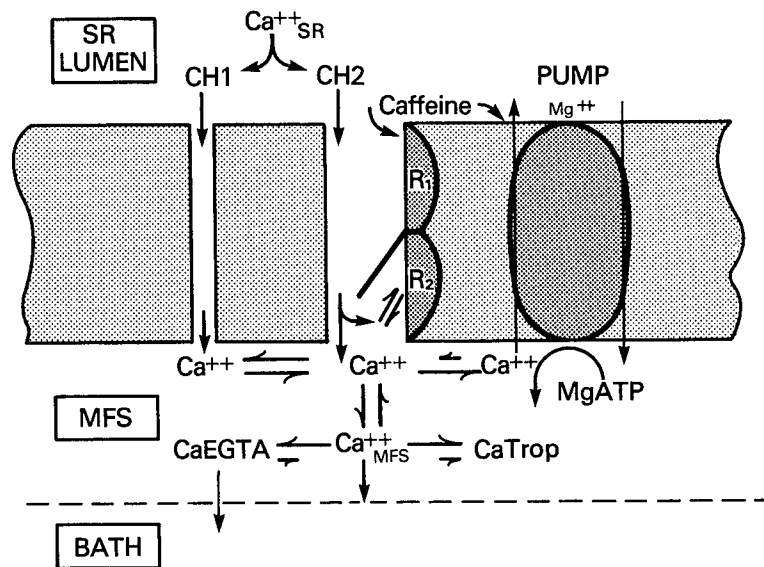


FIGURE 10. Schematic representation of a hypothetical common channel for stimulated efflux ( $CH2$ ) gated by a caffeine receptor ( $R_1$ ) and a Ca receptor ( $R_2$ ). Interactions with a leak channel ( $CH1$ ), an active Ca transport unit ( $PUMP$ ), and MFS reactions are indicated. The two receptors may be located in morphologically separate channels; the common channel emphasizes the strong series coupling between them. (See text for details.)

and decrease  $[Ca^{2+}]$  near the SR, but the efficacy of EGTA buffering at  $R_2$  would be reduced by the channel  $Ca^{2+}$  source. (In a dual channel model,  $[Ca^{2+}]$  would remain above threshold for some finite distance along the SR surface from the caffeine channel, even in EGTA.) When conditions permitted sufficient  $Ca^{2+}$  binding,  $R_2$  alone could open the channel.  $Mg^{2+}$  reduction would activate  $R_2$  as local  $[Mg^{2+}]$  fell and  $[Ca^{2+}]$  rose, due to decreased pump influx and perhaps slightly increased leak flux ( $CH1$ ). The effective affinity of  $R_2$  for  $Ca^{2+}$  probably would increase as well.

#### *Influence of $Mg^{2+}$ on Caffeine Stimulation*

The combined effects of  $Mg^{2+}$  and EGTA were much greater than the sum of the separate effects (Fig. 9), and the apparent  $Ca^{2+}$  dependence of efflux was enhanced. This synergistic inhibition indicates that the  $Mg^{2+}$  effect depends

on local  $[Ca^{2+}]$  near the SR. In the absence of EGTA, stimulation was reduced only modestly at  $600 \mu M Mg^{2+}$  in the responsive group of fibers, so the maximum effect on either  $M_1$  or  $M_2$  efflux component was small. The total stimulated  $^{45}Ca$  loss (above control) during the interrupted response was 67% of that in  $20 \mu M Mg^{2+}$ , but in  $20 \mu M Mg^{2+}$  a residual  $M_2$  component persists in 5 mM EGTA; the average *initial* efflux from the SR was calculated to be 83% of that in  $20 \mu M Mg^{2+}$ . No residual stimulation was detectable after pretreatment with 5 mM EGTA (Figs. 8 and 9), so  $Mg^{2+}$  directly inhibited some component of efflux.

The large differential effect of  $Mg^{2+}$  in the presence or absence of EGTA suggests, first, that the  $M_1$  component of efflux is extremely small and, second, that  $Mg^{2+}$  influences the  $M_2$  component, since  $M_1$  (by definition) is independent of EGTA. The efflux in EGTA sets an upper limit on the initial  $M_1$  component in  $600 \mu M Mg^{2+}$ , possibly  $0.2\% s^{-1}$  that might have been undetected. This  $M_1$  component must be at least 67–83% of  $M_1$  in  $20 \mu M Mg^{2+}$ , unless the coupling to  $M_2$  had *increased*, which is improbable; apparently the  $M_1$  component in  $20 \mu M Mg^{2+}$  was  $\leq 0.24$ – $0.30 s^{-1}$ . Because stimulated  $^{45}Ca$  efflux (above control) in 10 mM EGTA was initially  $0.38\% s^{-1}$  and rose to  $1.78\% s^{-1}$ , an  $M_2$  component is possible in  $20 \mu M Mg^{2+}$  even in 10 mM EGTA at  $19^\circ C$ . A small  $M_1$  component in  $20 \mu M Mg^{2+}$  is also consistent with the relatively small caffeine effect in 5 mM EGTA after the first minute (Fig. 4), when more effective buffering of the slower efflux would be expected.

The large effect of  $Mg^{2+}$  on the  $M_2$  component in 5 mM EGTA is likely to reflect reduced coupling to  $M_1$  when the  $[Mg^{2+}]/[Ca^{2+}]$  ratio is increased 30-fold at abnormally low local  $[Ca^{2+}]$ . Preferential  $Ca^{2+}$  binding would be expected to decrease under these conditions if  $Mg^{2+}$  can bind at all to  $R_2$ . This interpretation is consistent with the competitive inhibition seen in SR vesicles, where 1.1 mM  $Mg^{2+}$  reduces  $^{45}Ca$  efflux strongly at submicromolar bath  $[Ca^{2+}]$  and weakly at micromolar  $[Ca^{2+}]$  (Katz et al., 1977). If the large effect on  $M_2$  were due solely to a small decrease in  $M_1$ , local  $[Ca^{2+}]$  in  $20 \mu M Mg^{2+}$  plus 5 mM EGTA would have been close to threshold; however, 10 mM EGTA did not prevent stimulation.

This analysis with the proposed operational model has several immediate implications. First, since caffeine initiates a very small  $M_1$  efflux component that is insensitive to MFS  $[Ca^{2+}]$ , facilitation of the  $Ca^{2+}$ -induced response by caffeine (Endo, 1977) actually would consist of efficient stimulation of  $R_2$  by an independent SR  $Ca^{2+}$  source. Second,  $Mg^{2+}$  probably would inhibit the  $M_2$  component directly by influencing  $R_2$ , but the effect would be small at the local  $[Ca^{2+}]$  normally generated by  $M_1$ ; it would become large only at low local  $[Ca^{2+}]$ , in this case due to MFS EGTA. The  $M_2$  component appears to be separable from the direct effect of caffeine, so it is reasonable to infer that a similar  $[Ca^{2+}]-[Mg^{2+}]$  relation would apply with other stimuli, increasing the threshold and control of  $R_2$  at physiological  $[Mg^{2+}]$ .

#### *Relation of the $Ca^{2+}$ -dependent Channel to Other Flux Pathways*

Adjacent sources and sinks would influence local  $[Ca^{2+}]$  at the postulated  $R_2$  receptor, as shown schematically by the "leak" channel (*CHI*) and pump unit

in Fig. 10. CH1 represents Ca<sup>2+</sup>-insensitive efflux pathways besides M<sub>1</sub> (perhaps actually residual flux through CH2). Unstimulated CH1 flux is small, with low Mg<sup>2+</sup> sensitivity over the range 4 μM to 1.3 mM (Fig. 3 and Stephenson, 1978 *a*). Stimulation of CH1 by Cl (Stephenson and Podolsky, 1977 *b*; Stephenson, 1978 *a*) or Mg reduction (Fig. 1) is too small to detect (if present). The M<sub>1</sub> component in caffeine, which could be assigned to CH1, is the first Ca<sup>2+</sup>-insensitive <sup>45</sup>Ca efflux detected, but even this corresponds to 3–4 μM s<sup>-1</sup> at most. Therefore, R<sub>2</sub> stimulation by very small adjacent or intrinsic sources seems to produce almost all the stimulated <sup>45</sup>Ca efflux observed so far in skinned fibers, with no evidence of CH1 stimulation sufficient to cause contraction.

The Mg<sup>2+</sup>-dependent Ca pump is a potentially strong sink for local [Ca<sup>2+</sup>]. This influence appears to be important when skinned fibers are stimulated by bath Ca<sup>2+</sup> (Stephenson and Podolsky, 1977 *a*) or Mg reduction, and would potentiate the Mg<sup>2+</sup> effect on R<sub>2</sub> suggested by the caffeine results. However, the pump may be inhibited during the depolarization process. Analysis of net <sup>45</sup>Ca flux after Cl stimulation raised this possibility; the estimated Ca influx was only 4% of the influx predicted for unstimulated fibers (Stephenson, 1978 *a*). Analysis of MFS Ca<sup>2+</sup> transients (monitored optically) in voltage-clamped cut fibers strongly supported this suggestion; the transfer coefficient for Ca<sup>2+</sup> removal decreases with increasing depolarization (Kovács et al., 1979). Such inhibition would be functionally adaptive; if correct, the hypothesis also suggests a link between stimulated efflux and the Ca pump. Dependence of Ca-induced release on the pump state has been suggested (Ogawa and Ebashi, 1976). One mechanism that could act as a link is the ionophore moiety of the active transport system (see Shamoo and Murphy [1979]); if the postulated R<sub>2</sub> channel utilized the pump ionophore, operation in the efflux mode would preempt influx operation. Inhibition of influx associated with efflux stimulation by caffeine (or Mg reduction) as well as Cl is consistent with this possibility.

#### *Relation to activation of Intact Fibers*

In intact fibers, low caffeine concentrations potentiate subthreshold depolarization; contraction remains graded, but the tension threshold and dependence on membrane potential are shifted, and contraction is prolonged (see Lüttgau and Oetlicker [1968] and Caputo, [1976]). The present results indicate that the potentiation is mediated by a Ca<sup>2+</sup>-dependent pathway, postulated to be controlled by R<sub>2</sub> activation, which is responsible for most of the caffeine-stimulated <sup>45</sup>Ca efflux and can be graded. An important question is whether this represents a new flux pathway or an increase in a preexisting pathway for physiological stimulation. Caffeine does not invoke an R<sub>2</sub> channel, which also appears to mediate net efflux stimulated by bath Ca<sup>2+</sup>, Mg<sup>2+</sup> reduction, and Cl depolarization. Both physiological Ca release (Winegrad, 1968) and caffeine-stimulated release (Weber and Herz, 1968; Fairhurst and Hasselbach, 1970) are associated with the terminal cisternae, and the caffeine receptor and R<sub>2</sub> channel are closely coupled functionally. Therefore, physiological activation of such a channel seems feasible, inasmuch as the diffusion gradient from

junctional or nonjunctional release sites to myofilaments implies higher local  $[Ca^{2+}]$  near an  $R_2$  receptor.

The quantitative contribution of an  $R_2$  channel after normal depolarization needs experimental evaluation. If it predominates, as with  $M_1$  stimulation by caffeine at a physiological  $Mg^{2+}$  level, the distinction between primary  $Ca^{2+}$  regulation and secondary amplification becomes ambiguous; an independent efflux like  $M_1$  would function mainly as a graded  $Ca^{2+}$  source for  $R_2$ . If it is smaller, the  $R_2$  channel might function mainly to conduct stimulation along the SR and concurrently inhibit active transport (see above). In either case, the  $Ca^{2+}$ -dependent efflux would play an important role in physiological activation.

*Received for publication 28 May 1979.*

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