

Properties of Chloride-Stimulated ^{45}Ca Flux in Skinned Muscle Fibers

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ABSTRACT Isometric force and ^{45}Ca loss from fiber to bath were measured simultaneously in skinned fibers from frog muscle at 19°C. In unstimulated fibers, ^{45}Ca efflux from the sarcoplasmic reticulum (SR) was very slow, with little or no dependence on EGTA (0.1–5 mM) or Mg^{++} (20 μM –1.3 mM). Stimulation by high $[\text{Cl}]$ at 0.11 mM Mg^{++} caused rapid force transients (duration ~ 10 s) and ^{45}Ca release. This response was followed for 55 s, with 5 mM EGTA added to chelate myofilament space (MFS) Ca either (a) after relaxation, (b) near the peak of the force spike, or (c) before or with the stimulus. When EGTA was present during Cl application, stimulation of ^{45}Ca release was undetectable. Analysis of the time-course of tracer loss during the three protocols showed that when EGTA was absent, 16% of the fiber tracer was released from the SR within ~ 3 s, and 70% of the tracer still in the MFS near the peak of the force spike was subsequently reaccumulated. The results suggest that (a) the Cl response is highly Ca-dependent; (b) stimulation increases ^{45}Ca efflux from the SR at least 100–200-fold; and (c) the rate of reaccumulation is much slower than the influx predicted from published data on resting fibers, raising the possibility that depolarization inhibits active Ca transport by the SR.

INTRODUCTION

Excitation of skeletal muscle is known to be coupled to contraction by Ca release from the sarcoplasmic reticulum (SR) to the myofilament space (MFS). However, the genesis and nature of the increased Ca efflux from the SR are uncertain, and the activity of the Ca transport system of the SR during excitation is not known, although this system is capable of high rates of Ca uptake in isolated SR membranes (see Inesi, 1972) and in unstimulated skinned muscle fibers (Ford and Podolsky, 1972a).

Skinned muscle fibers provide a useful preparation of the analysis of Ca release and reaccumulation. These fibers can be stimulated to release Ca by increasing the external $[\text{Cl}]$, which presumably depolarizes the internal membranes (Costantin and Podolsky, 1967; Ford and Podolsky, 1970). The strength of the Cl stimulus, as judged by the size of the response, appears to depend on the gradient between the Cl applied to the myofilament space and the Cl in the lumen of the internal membrane systems (Nakajima and Endo, 1973; Stephenson and Podolsky, 1977b). Recent work has shown that it is possible to measure continuously the ^{45}Ca loss from fiber to bath and the isometric force during the

Cl response (Stephenson and Podolsky, 1977*b*). In those experiments, a weak Cl stimulus in the presence of low $[Mg^{++}]$ induced a large transient release of ^{45}Ca from SR to MFS, followed by reaccumulation of much of the released tracer. Chelation of MFS Ca inhibited the release, which suggested that it was Ca-dependent under those experimental conditions. This interesting property of the stimulated efflux could be related to the ability of Ca itself to stimulate Ca release at low $[Mg^{++}]$ (Ford and Podolsky, 1970, 1972*b*; Endo et al., 1970).

The present experiments were carried out to follow the time-course of ^{45}Ca movement during the Cl response in more detail under a different set of conditions: a stronger Cl stimulus was applied in the presence of 0.11 mM Mg^{++} , which inhibits stimulation by externally applied Ca (Stephenson and Podolsky, 1977*a*). If the Ca-dependence of this response were negligible, the net ^{45}Ca movement could be dissected completely into efflux and influx components by comparison of ^{45}Ca flux in the presence and absence of Ca chelation by EGTA. If EGTA were still strongly inhibitory, as the results showed to be the case, the net ^{45}Ca movement could be dissected only partially, but the Ca-dependent property of release assumes a new significance. As an adjunct to the stimulation experiments, the unstimulated (resting) ^{45}Ca loss was measured at several levels of Mg^{++} and EGTA.

Preliminary reports of this work have been made (Stephenson, 1976, 1977; Stephenson and Podolsky, 1977*c*).

METHODS

Fiber Preparation and Mounting

Single intact fibers from frog semitendinosus muscle were isolated and skinned in paraffin oil as described previously (Stephenson and Podolsky, 1977*a*). Large frogs from Texas and Mexico (*Rana berlandieri*) were used exclusively. The muscles from which the fibers were obtained were suspended in cold Ringer solution of either normal composition, with (mM) NaCl 115.5, KCl 2.5, $CaCl_2$ 1.8, NaH_2PO_4 + Na_2HPO_4 3.1, and *d*-tubocurarine 9 mg/liter, or low Cl composition, with the NaCl substituted by 217 mM sucrose leaving 6.1 mM total Cl (Stephenson and Podolsky, 1977*b*). Fibers for the Cl stimulation experiments and for the later control series referred to in Results were from muscles in the low Cl Ringer solution. All skinned fiber segments were mounted in oil by tying with monofilament silk to fine stainless steel rods, as described previously; one rod was attached to a leaf-spring photodiode force transducer for measurement of isometric force (Stephenson and Podolsky, 1977*a, b*). The mounted fiber was exposed to experimental solutions at 19°C in the wells of a spring-mounted thermoregulated chamber similar to that described previously (Stephenson and Podolsky, 1977*a*) except that the number of wells was increased to permit a larger sequence of washout solutions.

Approximate Cross-Sectional Area and Volume

The width and length of most of the skinned fiber segments used in the Cl stimulation experiments were estimated to the nearest 5–10 μm with the eyepiece micrometer of the dissecting microscope at $\times 40$ magnification. The dimensions were measured both before the fiber was mounted (fiber on cover slip) and after the fiber was mounted, raised, and adjusted to 1.05–1.10 "slack" length. The length was taken as the mean of the length before mounting and the length between ties after mounting. The diameter generally

was taken as the width after mounting; when this width was substantially smaller than the width observed before mounting, the two estimates were averaged, because frog fibers are known to have a variably elliptical cross-section (Blinks, 1965), and in skinned fibers the axes sometimes appear to differ severalfold. The mean diameter estimated for the fiber segments shown in Fig. 5 was $122 \pm 3 \mu\text{m}$ ($n = 24$), and for all measured fiber segments was $122 \pm 2 \mu\text{m}$ ($n = 42$). A circular cross section was assumed for the calculation of cross-sectional area and volume. Segment lengths were usually 2.0–2.5 mm.

Bathing Solutions

The bathing solutions used for skinned fibers have been described previously (Stephenson and Podolsky, 1977*b*). In brief, all solutions contained 120 mM K propionate or KCl, 10 mM imidazole, and 5 mM Na_2ATP , and were adjusted to pH 7.00. The [Mg] was set at 1, 3, or 6 mM with $\text{MgCl}_2 + \text{MgSO}_4$ (giving $[\text{Mg}^{++}]$ about 20 μM , 110 μM , and 1.3 mM). Other constituents are indicated in Results. The buffered ⁴⁵Ca solution used to load the segments with tracer contained 0.375 mM total CaEGTA with 0.5 mM total EGTA (pCa 6.2); it was prepared from high specific activity ⁴⁵CaCl₂ (New England Nuclear, Boston, Mass.) diluted 10-fold with CaCl₂, and had a final activity of about 15–30 $\mu\text{Ci/ml}$.

Tracer Procedures

The methods used for tracer loading and washout were as described previously (Stephenson and Podolsky, 1977*b*), except for modifications in the timing and number of transfers through washout solutions. The skinned fiber segments were exposed to ⁴⁵CaEGTA buffer solution for about 40 s, rinsed in three dilute EGTA solutions, and then transferred through a series of measured washout solutions, described in each case in the Results. After the washout period, the fiber was extracted in measured solution with 0.05% Triton-X100 (Rohm & Haas Co., Philadelphia) + 5 mM EGTA, which removes the remaining ⁴⁵Ca (Stephenson and Podolsky, 1977*b*). The sum of the ⁴⁵Ca lost to the washout and extraction solutions gives the total ⁴⁵Ca in the segment after rinsing, and the amount lost into each solution was expressed as a fraction of the total. The fraction remaining in the segment at the end of each wash was obtained by back-adding sequentially to the fraction remaining at the end. The fraction lost into each wash was expressed as a flux by dividing by the time the segment actually spent in that solution; i.e., transfer times were not included. In the Cl stimulation experiments, the transfer time, ~1.5–2 s, was not negligible with respect to the wash time for the first few washes. However, there was no straightforward way to correct these fluxes for ⁴⁵Ca movement within the fiber during the preceding transfer time, particularly when the solution composition was altered between washes.

The washout method requires that apparatus contamination be small, as noted previously, and the total radioactivity washed from the mounting rods and monofilament silk ties alone in the earlier study was found to be ~3% of the total activity from mounted fiber segments (Stephenson and Podolsky, 1977*b*). Similar blanks were run using the washout protocols of the present control (6 min) and Cl stimulation (1 min) experiments. In nine blank runs, the total activity washed out corresponded to $1.9 \pm 0.1\%$ of the mean total activity of 18 randomly selected muscle segments; blank activity was only $1.2 \pm 0.1\%$ that of those segments from Cl stimulation experiments, which were larger, and $2.9 \pm 0.1\%$ that of those segments from control experiments. The main difference observable between blank protocols was that with 1 min total washout time, 43% of the tracer appeared in the Triton-X100 extraction, whereas after 6 min total washout time only 19% remained to be extracted. Apparatus contamination evidently made a negligible

contribution to the washout solutions in the Cl stimulation experiments, and a small contribution of about 2% total tracer in the control experiments.

The washout and extracting solutions were sampled and assayed by liquid scintillation counting as described previously (Stephenson and Podolsky, 1977*b*).

Total Ca Uptake

For the segments used in the Cl stimulation experiments, the Ca uptake from the ^{45}Ca buffer solution was estimated from the total ^{45}Ca in the segment, the specific activity of the buffer, and the segment volume, calculated as described above. It should be emphasized that the volume estimate, and therefore the normalization, is only approximate. The mean Ca uptake during about 40 s exposure was $1.60 \pm 0.04 \text{ mM} \cdot \text{liter}^{-1}$ fiber volume ($n = 41$). Multiplication of the measured fractional ^{45}Ca loss and efflux values by this calcium content gives the minimum Ca loss and efflux ($\text{mM} \cdot \text{liter}^{-1} \cdot \text{s}^{-1}$) in each case. The total Ca loss and efflux could be up to 50% higher, if the endogenous fiber Ca did not equilibrate to the specific activity of the buffer (see Ford and Podolsky, 1972*a*).

RESULTS

Passive Efflux from Skinned Fibers

Previous work has shown that the net ^{45}Ca release by skinned fibers after stimulation is influenced by reaccumulation as well as efflux from the SR (Ford and Podolsky, 1972*b*; Stephenson and Podolsky, 1977*b*). The Ca uptake is stimulated by Mg^{++} (Stephenson and Podolsky, 1977*a, b*) and minimized by chelation by EGTA of Ca which has moved from the SR to the MFS (Ford and Podolsky, 1972*b*; Stephenson and Podolsky, 1977*b*). To assess the effect of these agents on efflux itself, ^{45}Ca loss from unstimulated fibers was measured as a function of [EGTA] at high and low [Mg^{++}]. Two questions were of particular interest. First, does [Mg^{++}] have a large effect on passive Ca permeability that could account for the Mg inhibition of stimulated net release that has been observed (Ford and Podolsky, 1970; Stephenson and Podolsky, 1977*a, b*; also see Endo, 1977)? Second, can reasonably high [EGTA] be applied during stimulation, to improve the rate of ^{45}Ca chelation and the time resolution of the transient net release, without adverse changes in Ca permeability?

Skinned fiber segments were loaded with ^{45}Ca , rinsed, transferred through a series of inactive washout solutions for a total of 3 or 6 min, and finally extracted in solution with Triton-X100 and 5 mM EGTA (see Methods). The sum of the ^{45}Ca lost to the washout and extraction solutions gives the total ^{45}Ca in the segment at the onset, and the amount lost into each solution can be expressed as a fraction of the total. Tracer loss into EGTA solutions with 6 mM total Mg (1.3 mM Mg^{++}) is shown in Fig. 1. The fraction of ^{45}Ca remaining in the fiber is plotted against the time in either 0.01, 0.1, 1, or 5 mM EGTA. Three main features are apparent. First, tracer loss in EGTA was very slow compared to the release following a Ca or Cl stimulus (Ford and Podolsky, 1972*b*; Stephenson and Podolsky, 1977*b*). In 360 s, only 20% of the initial ^{45}Ca had left the fiber in the highest [EGTA]. Second, fiber ^{45}Ca did not decrease as a single exponential. A faster component appeared during the 1st min which was the same in 1 and 5 mM EGTA and smaller in low EGTA. This initial component, about 10% of the fiber tracer in higher [EGTA], was too small to correspond to total SR Ca, but

much larger than the instrument contamination (see Methods), and its origin is unclear (see Discussion). Third, after the 1st min, the slopes of the washout curves were similar in 0.1–5 mM EGTA. The apparent rate constants at later times, calculated as the fraction lost per minute divided by the mean fraction remaining during that time interval, were not significantly different. In 0.01 mM EGTA, the time-course of tracer loss was slower; total loss was only slightly larger than the instrument contamination (see Methods), and it seems likely that this low concentration of chelator permitted substantial tracer reaccumulation.

Tracer loss into EGTA solutions with 1 mM Mg (20 μ M Mg⁺⁺) is shown in Fig. 2. The results were mainly similar to those in Fig. 1, but the scatter in the data was greater. The initial fast component was larger in high EGTA than in

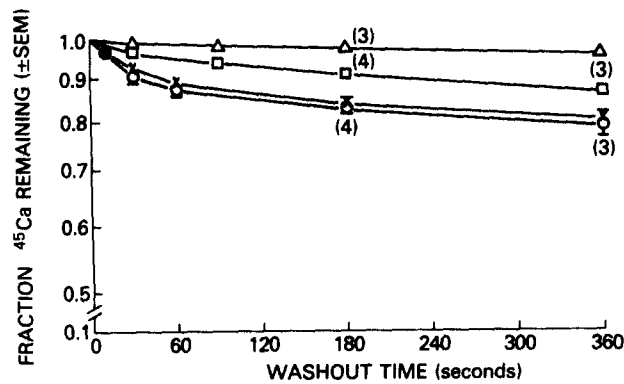


FIGURE 1. The effect of [EGTA] on ⁴⁵Ca loss in the presence of 1.3 mM Mg⁺⁺. Fiber segments were loaded with ⁴⁵Ca, rinsed, and washed out for a total of 180 or 360 s in K propionate solutions containing 6 mM Mg and 5 mM ATP (1.3 mM Mg⁺⁺) and either 0.01 mM EGTA (Δ), 0.1 mM EGTA (□), 1 mM EGTA (×), or 5 mM EGTA (○). Tracer loss into each wash solution was expressed as a fraction of the total ⁴⁵Ca in the segment at the onset (see Methods). The fraction remaining at the end of each wash is plotted against time, with the number of segments shown in parentheses.

0.1 mM EGTA; the curves for 1 and 5 mM EGTA did not differ significantly, but the later loss of tracer was faster than in 0.1 mM EGTA, unlike the results in high Mg. The time-course of tracer loss in 0.1 mM EGTA in low Mg was the same as that in high Mg; the fast component in higher EGTA was also similar. However, the mean ⁴⁵Ca remaining after 3 or 6 min in 1 or 5 mM EGTA was less than in high Mg, although the differences were not statistically significant. To obtain a better estimate of the effect of Mg at higher EGTA, the data for 1 and 5 mM EGTA were pooled for each Mg and compared at each point in time, as shown in Fig. 3. Only the tracer remaining after 6 min in high EGTA differed significantly ($P < 0.05$); the mean difference was 6.4% of the fiber tracer. The apparent rate constants (pooled) at later times were significantly larger in low Mg. These comparisons suggested that the combination of 20 μ M Mg⁺⁺ and high [EGTA] could increase the slow component of passive Ca efflux from the SR, at least after several minutes of exposure. The effect was not very large, possibly a factor of 2, but the slow rates and large scatter preclude precise

quantitation. If the same effect is present initially, it might contribute an additional 1% to passive tracer loss during the 1st min.

Although the passive efflux was complicated by the presence of a small fast component, the overall rate was slow under all conditions and the rate of the slower component(s) was lower yet by a factor of 5–10. A difference in $[Mg^{++}]$ between $20 \mu M$ and 1.3 mM did not cause large changes in passive Ca permeability that could account directly for inhibition of stimulated release (see Discussion). The effects on the slow component of increasing $[EGTA]$ from 0.1 to 5 mM suggested that Ca permeability of the SR was not changed substantially. The size of the small fast component increased up to 1 mM EGTA, but not between 1 and 5 mM EGTA. Therefore, the application of 5 mM EGTA in stimulation experiments appeared to be feasible, provided that suitable controls were used for the effect of EGTA on the small fast component.

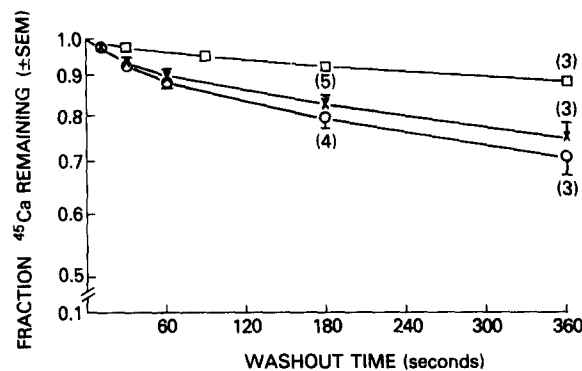


FIGURE 2. The effect of $[EGTA]$ on ^{45}Ca loss in the presence of $20 \mu M$ Mg^{++} . Procedures were the same as in Fig. 1, except that the washout solutions contained 1 mM Mg and 5 mM ATP ($20 \mu M$ Mg^{++}), and either 0.1 mM EGTA (\square), 1 mM EGTA (\times), or 5 mM EGTA (\circ).

Cl-Stimulated Ca Movement in 3 mM Mg (0.11 mM Mg^{++})

Cl-stimulated force responses and ^{45}Ca movement were studied in skinned fibers prepared from muscles which had been soaked in low-Cl Ringer solution (see Methods). With this pretreatment, it was possible to obtain substantial responses at 19°C in 0.11 mM Mg^{++} (Stephenson and Podolsky, 1977b). The segments were loaded in ^{45}Ca -EGTA buffer solution, rinsed in dilute EGTA solutions (all with propionate anion), and then transferred through a series of washout solutions containing Cl as the main anion. The force traces shown in Fig. 4 reflect the rise in MFS $[Ca^{++}]$ induced by Cl, and illustrate the procedures used to analyze ^{45}Ca movement between SR and MFS. In the upper trace, the segment was exposed to a series of Cl solutions during a complete force spike, then to solution with 5 mM EGTA to trap MFS ^{45}Ca , and finally to solution with Triton-X100 and 5 mM EGTA, to extract the tracer remaining in the SR. Force rose and fell rapidly, and relaxation was nearly complete in 10s; EGTA was applied at about 40 s, with a total washout time of about 55 s. The fiber was transferred rapidly through several solutions during the initial 10 s to resolve

net ⁴⁵Ca release during the rising and falling phases of the force transient. In the lower trace, the response was interrupted by EGTA application near the peak of the force spike; the fiber was in the first Cl wash, without EGTA, for <2 s, and the subsequent washes contained EGTA (applied at about 3 s) to trap ⁴⁵Ca which had been released to the MFS and minimize reaccumulation. Total washout time was the same as in the completed response. In a third type of protocol, to be discussed later, EGTA was applied simultaneously with Cl, or a few seconds preceding it.

The size of the Cl responses under these conditions was much more variable than in low Mg experiments, as described previously (Stephenson and Podolsky, 1977*b*), but the force generally was close to its peak value by the end of the first wash. To identify the source of variability, the amount of force developed during the first wash (when EGTA was absent in both complete and interrupted

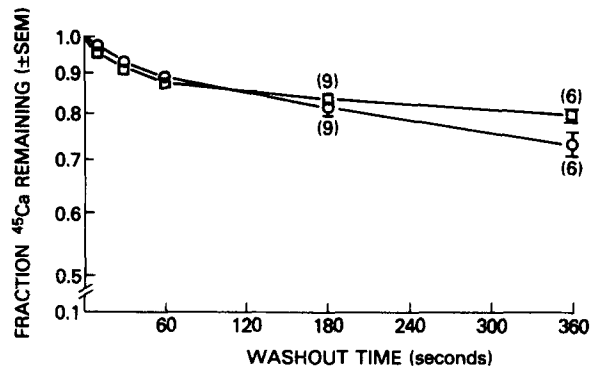


FIGURE 3. The effect of [Mg⁺⁺] on ⁴⁵Ca loss in high EGTA. The data on ⁴⁵Ca remaining at each point in time in 1 and 5 mM EGTA were pooled for 20 μM Mg⁺⁺ (O) and for 1.3 mM Mg⁺⁺ (□), and plotted as in Figs. 1 and 2. The only significant difference was at 360 s.

responses) was compared to the amount of ⁴⁵Ca that diffused out of the fiber during the same time period. Fig. 5 shows that the normalized peak force (see Methods) correlated well with the fractional ⁴⁵Ca loss into the first wash, as one would expect if both depend on the amount of Ca released from the SR to the MFS. This correlation indicates that Ca release itself was the primary variable, rather than force development at a given Ca or ⁴⁵Ca measurement for a given release. It can be seen that the responses of several fiber segments were extremely weak, with small forces and less than 2% ⁴⁵Ca loss; in two of these, ⁴⁵Ca loading appeared to have been lower than in other segments from the same fiber.

The mean time-course of tracer efflux from the fiber throughout the completed and interrupted responses is shown in Fig. 6. The fractional ⁴⁵Ca loss per second is plotted against time for each protocol; for the analysis of stimulated release, the weakest responses shown in Fig. 5, with tracer loss less than 2% during the initial wash, were excluded. With the time resolution of these protocols, it could be seen that the initial rate of net ⁴⁵Ca release was more than 20 times the highest initial rates of passive efflux (Figs. 1 and 2), but fell off very rapidly. In

the absence of EGTA, the rate of net ^{45}Ca loss from the fiber was less than 10% the initial stimulated rate in 10 s and less than 1% after the fiber had relaxed completely. When EGTA was applied at ~ 3 s, near the peak of the force spike,

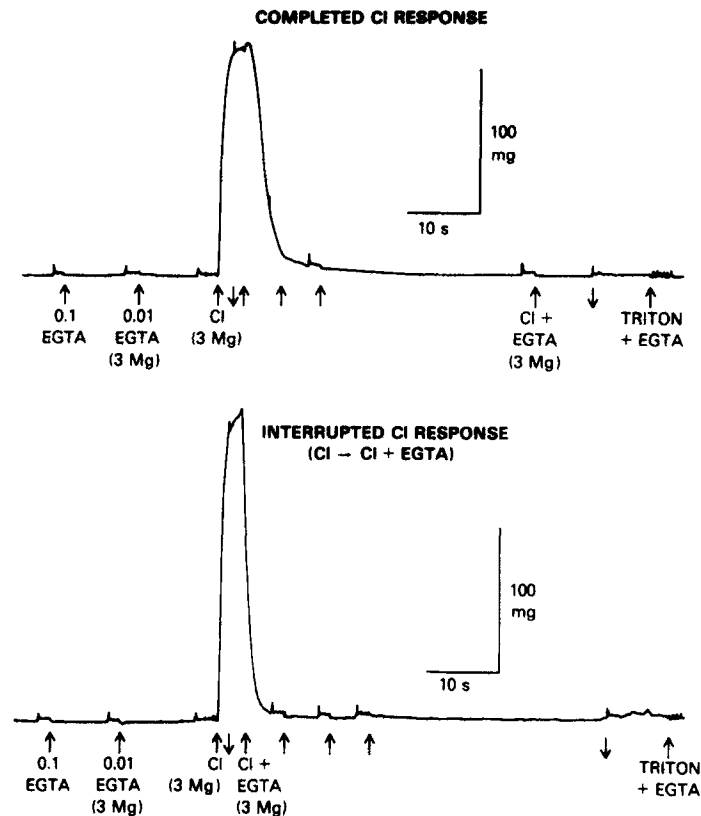


FIGURE 4. Isometric force traces illustrating the time-course of tension and the ^{45}Ca washout protocol with application of a Cl stimulus at 3 mM Mg (0.11 mM Mg^{++}) to segments from fibers with low luminal Cl (see text). After loading with ^{45}Ca (not shown) and rinsing three times, propionate anion was replaced by Cl. Upward arrows indicate transfer into subsequent solutions; downward arrows show removal from the first wash and the last wash before extraction. Upper trace: transfer through a series of Cl solutions, with 5 mM EGTA applied well after relaxation (completed Cl response). Lower trace: transfer through only one Cl solution without EGTA, with 5 mM EGTA applied near the peak of the force spike and maintained in subsequent Cl solutions (interrupted Cl response). Details are given in the text.

^{45}Ca efflux from the fiber remained substantially higher, particularly during the first 20–30 s. (The time protocol was varied to follow the time-course more closely.) The difference in tracer loss between the completed and interrupted responses during this period reflects the ability of the SR to reaccumulate MFS ^{45}Ca when it has not been chelated rapidly by EGTA. However, appropriate EGTA controls must be applied before the tracer efflux during the interrupted

response can be used to evaluate the total amount of ^{45}Ca released to the MFS during the first 3 s of stimulation. This analysis is made in later sections.

The Effect of Cl on Ca Movement in the Presence of EGTA

The response to a weak Cl stimulus at low $[\text{Mg}^{++}]$ appears to be Ca-dependent, in that EGTA inhibits ^{45}Ca release as well as force (Stephenson and Podolsky, 1977*b*). Under these conditions, the Ca-dependence is likely to be related to the

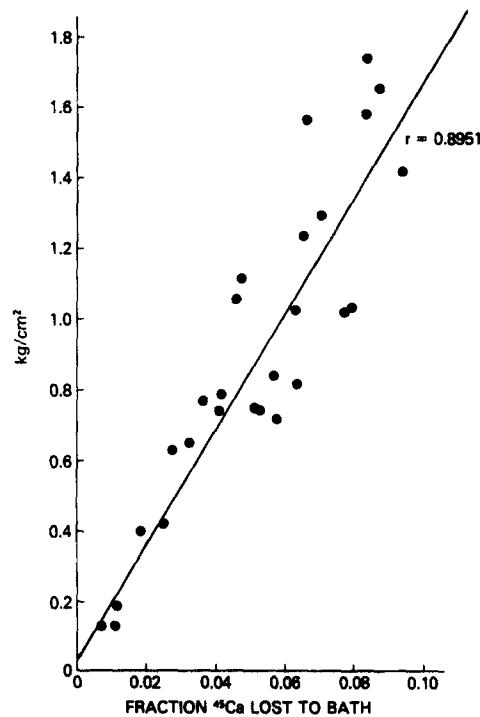


FIGURE 5. Correlation between force development and ^{45}Ca loss during the initial exposure to Cl. The isometric tension at the end of the first Cl wash (normalized by the cross-sectional area as described in Methods) is plotted against the fraction of ^{45}Ca released into the first Cl wash, for each segment. Data are from both completed and interrupted responses. The correlation coefficient, r , is given next to the linear regression line.

known ability of bath Ca to act as a stimulus for net Ca release when $[\text{Mg}^{++}]$ is low (Ford and Podolsky, 1972*b*). Therefore it was important to study the Cl response to a larger stimulus at 0.11 mM Mg^{++} in the presence of EGTA; this $[\text{Mg}^{++}]$ inhibits the force response to bath Ca nearly completely (Stephenson and Podolsky, 1977*a*). If EGTA has little effect on the Cl stimulus proper, it should be possible to dissect the entire time-course of Cl-stimulated ^{45}Ca movement into efflux and influx components. If EGTA does inhibit the Cl-stimulated ^{45}Ca release, then Ca is likely to play an important role in the release mechanism even under conditions that appear to prevent the response to an external Ca stimulus.

The time-course of tracer efflux during Cl application in the presence of 5 mM EGTA is shown in Fig. 7, plotted as in Fig. 6. The open circle during the first wash is the initial net flux in Cl for all completed and interrupted responses; for this comparison, the weakest responses shown in Fig. 5 were included. The solid curve below represents the tracer loss from the fiber when EGTA was applied simultaneously with the Cl (four fibers) or a few seconds preceding it (six fibers). The dashed line represents the passive efflux during the first 60 s in 5 mM EGTA propionate solution, taken from the data in Figs. 1 and 2 (pooled).

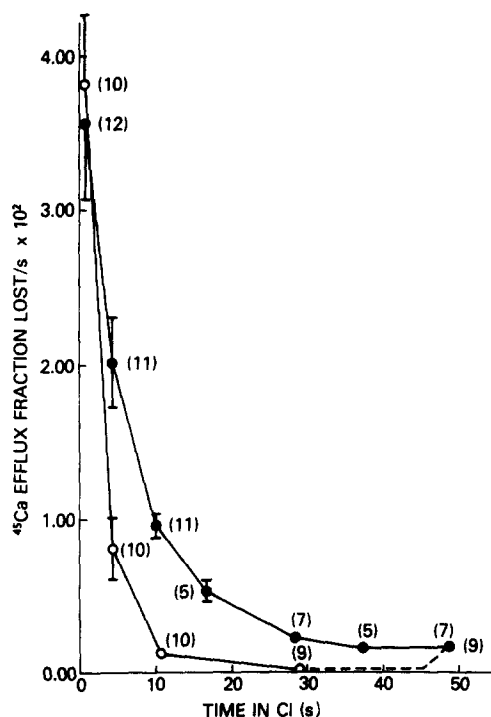


FIGURE 6. The rate of ^{45}Ca loss to the bath during completed Cl responses (○) and interrupted Cl responses (●), defined as in Fig. 4. ^{45}Ca efflux, expressed as the fraction lost/second ($\times 10^2$) \pm SEM is plotted against the exposure time in Cl for responsive segments. The protocol during interrupted responses was varied after 10 s to improve the time resolution of the decline in efflux.

The efflux in Cl-EGTA was the same as the unstimulated efflux, except for the initial few seconds. During the first brief Cl-EGTA wash, efflux appeared to increase by a small but significant amount in fibers that had been preexposed to EGTA in propionate solution. However, this apparent increase was found to be a systematic artifact of the procedure. In subsequent control experiments on identically prepared fibers the same effect was observed when efflux into 3 mM Mg, 5 mM EGTA propionate solution was measured using the same initial time protocol as the fibers that were preexposed to EGTA before Cl. The efflux during the brief propionate wash, corresponding to the first Cl wash, was 2.1 ± 0.0 times ($n = 5$) that in the preceding wash, whereas this ratio was 2.3 ± 0.4 (n

= 6) in the Cl experiments. The increase thus appeared to be associated with the very short duration of the wash, rather than with Cl application in the presence of EGTA.¹ This group of experiments showed that Cl had no detectable effect on ⁴⁵Ca efflux in the presence of 5 mM EGTA, i.e., EGTA inhibited the response to Cl stimulation completely, within the sensitivity of the method.

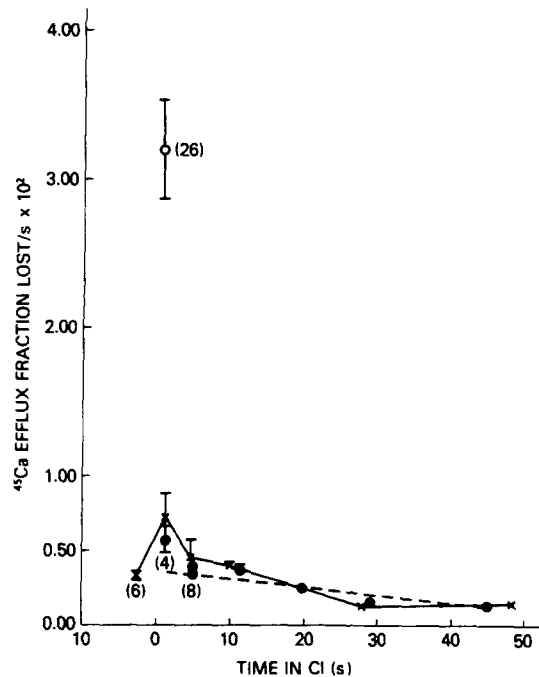


FIGURE 7. The rate of ⁴⁵Ca loss to the bath with EGTA present initially; 5 mM EGTA was applied just preceding the Cl stimulus (×) or simultaneously (⊗). Data are plotted as in Fig. 6. (○) shows the initial ⁴⁵Ca efflux in Cl in the absence of EGTA (pooled mean ± SEM for all data on completed and interrupted responses). The unstimulated ⁴⁵Ca efflux in propionate solution with 5 mM EGTA is shown by the dashed line (●) (mean ± SEM of pooled data shown in Figs. 1 and 2).

Analysis of ⁴⁵Ca Movement during the Interrupted Response

Although the stimulated ⁴⁵Ca release could not be resolved completely by the presence of EGTA throughout, the efflux during the inhibited response could be used as a control for the interrupted response, where EGTA was applied about 3 s after stimulation. With this control, it was possible to evaluate the amount of extra ⁴⁵Ca released by stimulation which was trapped by EGTA and to estimate when this Ca had been released.

¹ In this later series of controls, tracer loss was smaller than in the experiments shown in Fig. 7. Inasmuch as the initial efflux from these fibers was significantly smaller than the initial efflux (into propionate) from the fibers that were preexposed to EGTA before Cl treatment, the difference appears to be due to variation between batches of animals.

This analysis is shown in Fig. 8. The fraction of ^{45}Ca remaining in the fiber after the first wash is plotted against time for the inhibited response in EGTA (upper curve) and the stimulated response in EGTA (lower solid curve). The extra ^{45}Ca released to the MFS by stimulation is given by the total fractional tracer loss after the first wash in the stimulated fibers, 0.185, minus the total loss in the inhibited (control) fibers, 0.081. This difference, 0.104 of the fiber tracer,

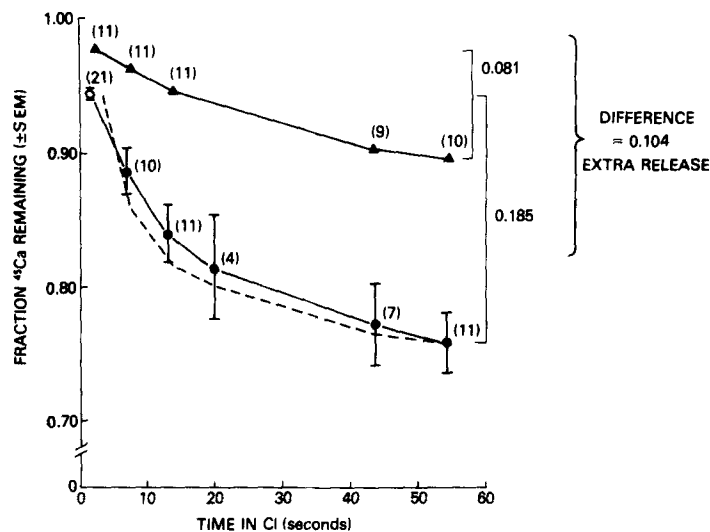


FIGURE 8. Analysis of the extra ^{45}Ca released by Cl stimulation during the interrupted response (EGTA applied at ~ 3 s). The inhibited response observed when EGTA was present initially (Fig. 7) is used as a control (see text). The fraction of ^{45}Ca remaining in the segment at the end of each wash (\pm SEM) is plotted against the time in Cl solution for the interrupted response (\bullet) and the inhibited response (\blacktriangle). The first point for the interrupted response (\circ), obtained in the absence of EGTA, is the pooled mean from completed and interrupted responses. Braces indicate the ^{45}Ca released to the bath subsequent to the first wash for each protocol; the curved bracket indicates the difference in subsequent release between the two protocols, which is the extra ^{45}Ca released by effective stimulation. The dashed line is a theoretical curve calculated on the assumption that this extra ^{45}Ca is in the MFS at the start of the second wash, and the time-course of ^{45}Ca loss in the interrupted response is the sum of the control loss and the outward diffusion of the extra ^{45}Ca as $^{45}\text{CaEGTA}$ (see text for details).

represents the portion of stimulated Ca release which did not diffuse into the bath during the first Cl wash.

In view of the inhibitory effect of EGTA on stimulated release, it seemed likely that this Ca had already been released when EGTA was applied. To check this point, the observed time-course of tracer loss in the interrupted response was compared with the dashed curve, which represents the approximate time-course that tracer loss would follow if it were the sum of the control loss and the outward diffusion of the extra ^{45}Ca as $^{45}\text{CaEGTA}$. The extra ^{45}Ca was assumed to be in the MFS at the time EGTA was applied, to be chelated instantaneously,

and to diffuse out of an infinite cylinder (Hill, 1928) of radius $61 \mu\text{m}$ (see Methods) with an effective diffusion coefficient $\sim 1.5 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$. The dashed curve starts with the amount of tracer remaining in the stimulated fiber at the end of the first wash and the time EGTA was applied; the small time displacement is the transfer time. The agreement is fairly good and would be improved by taking account of the time for transfer and for the inward diffusion of EGTA. This result is consistent with the assumption that essentially all of the extra ^{45}Ca released by stimulation was already in the MFS when EGTA was applied (3 s after the Cl stimulus) in the interrupted response.

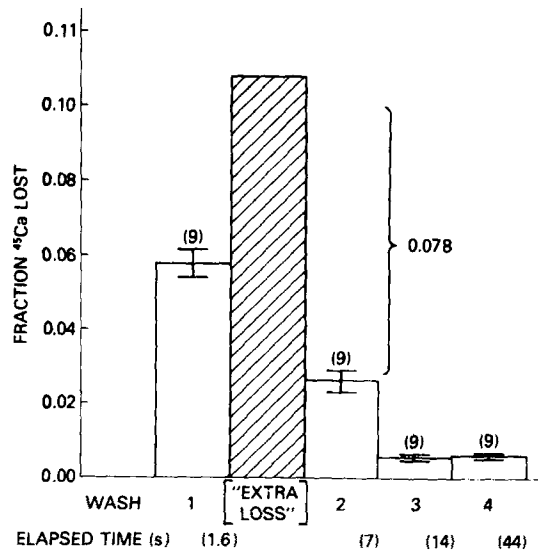


FIGURE 9. Analysis of ^{45}Ca loss and reaccumulation during the completed Cl response. Open bars show the fraction of ^{45}Ca lost into each wash (\pm SEM). The average time between Cl application and the end of each wash is given beneath. The hatched bar shows the extra ^{45}Ca released by initial stimulation, assumed to be in the MFS at the start of the second wash (Fig. 8). The curved bracket indicates the amount of the extra ^{45}Ca still in the fiber at the end of the second wash; this Ca is largely reaccumulated (see text for details).

Analysis of ⁴⁵Ca Movement during the Completed Response

The results of the preceding analysis of the interrupted response can be used to estimate the minimum release and reaccumulation of Ca by the SR in the absence of EGTA. The fraction of fiber tracer lost into each Cl wash during the completed responses is shown in Fig. 9; the time between application of the stimulus and the end of each wash is given beneath. The extra released Ca, shown by the interrupted response to be in the MFS at the start of the second wash, about 3 s after the Cl stimulus was applied, is indicated after the first wash. During the first 3 s, at least 16% of the fiber tracer was released from the SR by stimulation; 6% diffused into the bath during the first wash and 10% was still in the MFS at the start of the second wash, when force was close to its peak

value. At the end of the second wash, at about 7 s, nearly 8% was still in the fiber, although force had declined to low values (Fig. 4). Tracer loss in the subsequent washes was small. About 7% of the fiber tracer, or 70% of the tracer which had been in the MFS at the start of the second wash, was transported back into the SR and never diffused out of the fiber. This large fractional reaccumulation confirms previous observations on the ability of the SR to compete with diffusion for MFS Ca (Ford and Podolsky, 1972*a, b*; Stephenson and Podolsky, 1977*a, b*).

Most of the reaccumulation took place during the second wash, while the force was falling to about a third its peak value. The mean ratio of the final force in the second wash to peak force was 0.36 ± 0.12 ($n = 9$), and the mean peak force in these fibers was 0.97 ± 0.10 kg/cm² ($n = 9$), which is 70% of the maximum isometric force developed by skinned fibers (Hellam and Podolsky, 1969). From previous measurements of steady force at pCa 6.2 and 6.5 in Ca-EGTA buffer solutions with 3 mM Mg (Stephenson and Podolsky, 1977*a* and unpublished experiments), these force levels correspond to about 0.7 μ M and 0.3 μ M free Ca in the MFS. Skinned fibers are capable of high rates of ⁴⁵Ca uptake at these Ca⁺⁺ levels (Ford and Podolsky, 1972*a*); the tracer reaccumulation during the completed response is well within this capability, and in fact appears to be surprisingly slow (see Discussion).

DISCUSSION

Unstimulated Efflux

All components of the unstimulated efflux are substantially slower than the stimulated net flux, but the faster component probably is not attributable to the intact SR. This component is very small at low [EGTA] and increases with [EGTA] to a maximum of ~10% fiber tracer (Figs. 1 and 2). The dependence on [EGTA] is not consistent with the impermeability of isolated SR vesicles to EGTA at neutral pH (Weber et al., 1966; Duggan and Martonosi, 1970), and the maximum size is too small to correspond to the Ca content of the SR elements. The faster component may have multiple origins. Tracer loss from the apparatus during the 1st min is equivalent to 1-2% fiber tracer in the control experiments (see Methods). A small amount might come from mitochondria, although the affinity of mitochondria for Ca is low under the loading conditions used (Weber et al., 1966), and mitochondrial volume is <20% SR volume in frog muscle (Moblely and Eisenberg, 1975). A small fraction of the fiber is likely to be damaged; for example, the monofilament silk ties (18 μ m diameter) compress about 2% fiber volume. The faster component may be the sum of such small sources and other Ca binding sites accessible to the MFS.

The early ⁴⁵Ca loss in EGTA solutions was estimated from residual ⁴⁵Ca by a difference method by Ford and Podolsky (1972*a*). Their results in 0.1 mM EGTA were consistent with the present self-normalized measurements. However, in 1 or 3 mM EGTA they found 25-50% losses within 30 s; the basis for this large discrepancy from the present series of measurements is not known.

The rate of the slower component(s) is more likely to reflect unstimulated loss from intact SR, and be the appropriate reference for comparison with the stimulated ⁴⁵Ca flux. In 0.01 mM EGTA, the MFS [Ca⁺⁺] appears to be high

enough to permit substantial reaccumulation, but from 0.1–5 mM EGTA, the later efflux shows little or no dependence on (EGTA), and the average fraction lost per second under all conditions is about 0.0003. If the initial Ca content is 1.6 mM·liter⁻¹ (see Methods), this fraction corresponds to 4.8×10^{-4} mM·liter⁻¹·s⁻¹. The Ca flux can also be expressed per unit membrane area, using the surface: fiber volume ratios measured for the terminal cisternae and the total SR by Mobley and Eisenberg (1975). The resting efflux is 0.089 pmole·cm⁻²·s⁻¹ if restricted to the terminal cisternae, and 0.024 pmole·cm⁻²·s⁻¹ if distributed along the entire SR membrane. These values are low compared to the resting Na influx across the sarcolemma of intact fibers (Hodgkin and Horowitz, 1959), but may not differ much from the resting Ca influx (Curtis, 1966), if allowance is made for the surface of the transverse tubules.

The unstimulated efflux is not strongly influenced by free Mg. A 65-fold change in [Mg⁺⁺], between 20 μM and 1.3 mM, has no detectable effect in 0.1 mM EGTA; there may be a twofold effect in high [EGTA] on the later efflux. The existence of this effect in the absence of EGTA is doubtful, because the combination of 20 μM Mg⁺⁺ and high [EGTA] is likely to reduce the divalent cation associated with the SR membrane to abnormally low levels, a condition which tends to increase membrane permeability. In any case, the change in efflux is small relative to the change in [Mg⁺⁺]. These results have a limited significance with respect to the strong inhibitory effect that a fivefold change in [Mg⁺⁺] can have on stimulated Ca release from the SR (Stephenson and Podolsky, 1977*a, b*; see Endo, 1977). It is not known that stimulated and unstimulated efflux use the same type of pathway. If the pathway is the same, then an undetectable or very small direct effect of Mg⁺⁺ on efflux would have to account for practically complete inhibition of stimulated release. If the pathway is different, inhibition of efflux is not excluded by these observations, but neither is it suggested, because previous work has shown that Mg⁺⁺ could act on net Ca release by enhancement of reaccumulation by the SR (Stephenson and Podolsky, 1977*a, b*).

Stimulated Efflux

The stimulation of ⁴⁵Ca efflux proper, under the present conditions, could not be resolved directly by use of EGTA, but the stimulated net flux gives a lower limit for this quantity. Immediately after Cl application, the rate of fractional tracer loss from fiber to bath is 0.04 s⁻¹, and the simultaneous force development indicates additional Ca movement from SR to MFS. Analysis of the response interrupted by EGTA application at about 3 s after the stimulus gives a mean rate of fractional tracer loss from the SR of 0.16/3 or 0.05 s⁻¹ (Figs. 8 and 9). The stimulated net flux is at least 100–200 times the unstimulated (resting) efflux from the SR.

It is clear that the large net flux cannot be due to cessation of Ca transport into the SR (also see Endo, 1977). If the Ca transport system were totally inoperative, the stimulated efflux would be equal to the stimulated net flux. However, if the active Ca transport system were fully operative during the response, then the stimulated efflux would have to be another order of magnitude larger than the net flux (as discussed in a later section). These

considerations place the transient stimulation of Ca efflux from the SR at >2 , and possibly 3, orders of magnitude.

The stimulated net flux corresponds to about $0.085 \text{ mM} \cdot \text{liter}^{-1} \cdot \text{s}^{-1}$. Expressed per unit surface (Mobley and Eisenberg, 1975), the net flux is $15.8 \text{ pM cm}^{-2} \cdot \text{s}^{-1}$ if restricted to the terminal cisternae, and $4.2 \text{ pM cm}^{-2} \cdot \text{s}^{-1}$ if distributed along the entire SR. This is, of course, an average rate during the rising phase of the response (the initial 3 s) for the total net Ca release, $\sim 47 \text{ pM} \cdot \text{cm}^{-2}$; it is reasonable to suppose that during the rising phase of the twitch in the intact fiber, the total net release from the SR is similar (but 50–100 times faster). Therefore, it is interesting to note that the total net Ca release during the rising phase is more than 500 times the resting efflux (per second), whereas in the intact muscle fiber the Na entry per impulse is only about 5 times the resting influx (per second) (Hodgkin and Horowitz, 1959) and the extra Ca entry per twitch is about 3 times the resting influx (per second) (Curtis, 1966). Cast in these terms, the difference between sarcotubular and SR membrane areas is removed from the comparison. The time scales of signal and response are different: Na entry occurs within a few milliseconds, associated with the very large increase in conductance, while Ca release during the rising phase may be prolonged over perhaps 50 ms. Ca efflux from the SR, per unit membrane area, appears to be stimulated for a much longer time and possibly by a larger factor than the flux across the sarcotubular membranes. The net effect is a 100-fold increase in the total stimulation of ionic movement ($[\text{active flux} \times \text{duration}] / \text{resting flux}$). A number of differences in membrane properties or intervening steps in the coupling process could be responsible for this amplification; one possible mechanism is secondary stimulation by released Ca, as discussed below.

The Ca Dependence of Cl Stimulation

The inhibitory effect of EGTA strongly suggests that the stimulation of Ca release depends on the presence of unchelated Ca. Chelation of Ca, rather than some unknown trace metal, seems particularly likely as the cause of inhibition because Ca^{++} applied in the bath is known to be an adequate stimulus under suitable conditions (Ford and Podolsky, 1970, 1972*b*; Endo et al., 1970). Previous work has shown that EGTA inhibits ^{45}Ca release after a weak Cl stimulus at $20 \mu\text{M Mg}^{++}$ (Stephenson and Podolsky, 1977*b*); inasmuch as this $[\text{Mg}^{++}]$ permits bath Ca^{++} to stimulate net Ca release, the effect of EGTA could be attributed to inhibition of secondary stimulation by a small amount of released Ca. The present experiments utilized Cl stimulation in the presence of 0.11 mM Mg^{++} , which inhibits the response to bath Ca nearly completely (Stephenson and Podolsky, 1977*a*). A direct conclusion to be drawn from the EGTA inhibition under these conditions is that the responsiveness of skinned fibers to Ca applied in the bath is not an adequate measure of the role of Ca in the release process.

Inhibition by EGTA of Cl-induced release was not evident in split muscle fibers from *Xenopus laevis* stimulated at 0°C (Thorens and Endo, 1975). Release was estimated from the residual Ca in the SR, assayed by the size of caffeine contractures in the presence of EGTA. The conditions of these experiments (temperature, buffer) clearly reduced the effectiveness of Ca chelation by

EGTA, because large caffeine contractures were recorded in solutions with 2 mM or even 10 mM free EGTA, and it seems possible that Ca required for the Cl response was not sufficiently chelated. A second possibility is that Cl acts at different sites in the split fiber and skinned fiber preparations. In the split fiber, Cl is thought to act directly on the SR membranes, because the open transverse tubules are already depolarized by the relaxing solution (Nakajima and Endo, 1973). In the skinned fiber, the transverse tubules (T-tubules) are thought to seal over and repolarize (Costantin and Podolsky, 1967), and Cl may act directly on these membranes. This site of action seems more likely in view of new evidence that the monovalent ion gradients between SR and MFS appear to be small or nonexistent in intact fibers (Somlyo et al., 1977), but more information on this important point is needed.

In the present experiments, stimulation of ⁴⁵Ca efflux by Cl was undetectable in the presence of 5 mM EGTA, although a small increase due to the washout protocol per se could be distinguished readily (Fig. 7). This brackets the amount of Ca that might have been released between zero and <1% fiber tracer ($\cong 0.01 \text{ mM} \cdot \text{liter}^{-1}$), a very small quantity with respect to activation of the myofilaments. The basis of the Ca dependence of the release process requires further characterization. Three possible mechanisms might be involved: (a) Ca might act as a chemical transmitter between T-tubules and SR (see Ford and Podolsky, 1972*b* and references); although the normal twitch does not seem to require external Ca (Armstrong et al., 1972), Ca-binding sites at the T-SR junction (Politoff et al., 1974) could be altered by depolarization; (b) Ca associated with the SR membrane might activate a Ca channel or carrier, in analogy with the strong influence of Ca influx and internal Ca on K permeability and active K conductance in snail neurons and many other cells (see Heyer and Lux, 1976; Thomas and Gorman, 1977); (c) Ca released from the SR might act within the fiber as a secondary stimulus to amplify the Ca efflux, in a similar way to stimulation by bath Ca at low Mg (Ford and Podolsky, 1970). These possibilities overlap and are not mutually exclusive; indeed, control of a Ca channel or carrier by membrane Ca could form the basis for stimulation by extrinsic Ca.

Reaccumulation of released Ca

Analysis of the completed Cl response, utilizing the data from the interrupted and inhibited responses, showed that the SR reaccumulated about 7% of the fiber tracer, or 70% of the tracer in the MFS near the peak of the force response (Fig. 9). Reaccumulation took place mostly during the second wash; an upper limit for the reaccumulation rate is obtained by assuming that the entire 7% is reaccumulated during the 4-s period of the second wash, while the force is falling to low values. The initial Ca content of the fibers at the specific activity of the loading solution is estimated to be $1.6 \text{ mM} \cdot \text{liter}^{-1}$ (see Methods), so the reaccumulated tracer corresponds to about $0.11 \text{ mM} \cdot \text{liter}^{-1}$ and the average reaccumulation rate to $0.028 \text{ mM} \cdot \text{liter}^{-1} \cdot \text{s}^{-1}$.

It is striking that this rate is much smaller than the influx predicted when MFS $[\text{Ca}^{++}]$ is in the range $0.7\text{--}0.3 \mu\text{M}$, the $[\text{Ca}^{++}]$ indicated by the force during the second wash (see Results). The rate constant for ⁴⁵Ca uptake by relaxed

skinned fibers is at least $1.4 \times 10^3 \text{ s}^{-1}$, estimated in $^{45}\text{CaEGTA}$ buffer solutions containing $20 \mu\text{M Mg}^{++}$ (Ford and Podolsky, 1972*a*), and would be even higher in 0.11 mM Mg^{++} (Stephenson and Podolsky, 1977*a, b* and references). This rate constant predicts an average influx that is about 25 times the observed reaccumulation rate. The rate of force relaxation during the second wash deviates even more from prediction; if 4 s is required to reduce MFS [Ca^{++}] from $0.7 \mu\text{M}$ to $0.3 \mu\text{M}$ and remove the appropriate amount of Ca from the myofilaments, the apparent rate constant for Ca uptake (see Eq. 5, Ford and Podolsky, 1972*a*) is 46 times smaller than the rate constant above (even if no Ca were lost by diffusion). The discrepancy between the reaccumulation rate estimated from the tracer data and the predicted influx is so much larger than the uncertainties in the calculations (such as the initial Ca content and the precise MFS [Ca^{++}]) that it must be concluded that the effect is real.

The relatively low rate of reaccumulation could be due to an abnormally low influx, which raises the possibility that the active transport system for Ca uptake by the SR is inhibited by depolarization. This possibility is attractive, because the operation of normal active influx during stimulation could require substantial energy expenditure for a counter-productive process. Normal pump operation would require also a rapid turnover of SR Ca, with both unidirectional fluxes much larger than the rates of net release and reaccumulation. Calculations with the predicted high influx and efflux suggest that the amount of Ca moving out of the SR during the first 3 s would exceed the total initial Ca content by at least $0.6\text{--}1.2 \text{ mM} \cdot \text{liter}^{-1}$. The ability of the SR to recycle released Ca at the required rate is questionable, in view of the slow time-course of Ca redistribution after a tetanus, as indicated by radioautography (Winegrad, 1968).

A second possible explanation for the low reaccumulation rate is that the stimulated efflux remains high during the reaccumulation phase of the response. There is no evidence of a sustained high efflux from the SR when EGTA is applied near the force peak (Fig. 8), but this observation does not definitively rule out an elevated efflux in the absence of EGTA, a quantity which is difficult to measure. A sustained high efflux can account for the results only with the large unidirectional fluxes and rapid Ca turnover discussed above, and therefore a primary contribution of this mechanism to the low rate is less likely.

Inhibition of active Ca transport by depolarization is an interesting possibility that appears to be more consistent with observation and has considerable adaptive value. It should be possible to test this hypothesis in the skinned fiber.

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