

# Excitation of Skinned Muscle Fibers by Imposed Ion Gradients

## *I. Stimulation of $^{45}\text{Ca}$ Efflux at Constant $[\text{K}][\text{Cl}]$ Product*

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**ABSTRACT**  $^{45}\text{Ca}$  efflux from skinned muscle fibers is stimulated transiently, by a highly  $\text{Ca}^{2+}$ -dependent mechanism, by KCl replacement of K propionate. In the present studies, Cl replaced the much less permeant anion methanesulfonate (Mes) either (a) at constant  $[\text{K}]$ , in which increased  $[\text{K}][\text{Cl}]$  permits net KCl and water flux across internal membranes, or (b) at constant  $[\text{K}][\text{Cl}]$  (choline substitution), in which the imposed gradients and diffusion potentials should dissipate slowly.  $^{45}\text{Ca}$  efflux and isometric force were measured simultaneously on segments of frog semitendinosus fibers skinned by microdissection. EGTA was applied to chelate released  $^{45}\text{Ca}$  either (a) shortly after high  $[\text{Cl}]$  (interrupted response), to minimize reaccumulation, (b) before high  $[\text{Cl}]$  (pretreated response), to evaluate  $\text{Ca}^{2+}$  dependence, or (c) under control conditions in KMes. KCl replacement of KMes stimulated release of 65% fiber  $^{45}\text{Ca}$  within 1 min in interrupted responses; EGTA pretreatment was only moderately inhibitory with substantial residual stimulation. In contrast, choline Cl replacement of KMes induced release of 26–35% fiber  $^{45}\text{Ca}$  in interrupted responses; EGTA pretreatment was strongly inhibitory, but release significantly exceeded control with a small, sustained increase in  $\text{Ca}^{2+}$ -insensitive efflux. These differences in  $^{45}\text{Ca}$  release and EGTA inhibition suggest that Cl replacement of Mes at constant  $[\text{K}]$  stimulates efflux by osmotic effects as well as imposed diffusion potentials; at least half the stimulated  $^{45}\text{Ca}$  loss (above control) in interrupted KCl responses is attributable to an osmotic component with low  $\text{Ca}^{2+}$  sensitivity. In the highly  $\text{Ca}^{2+}$ -sensitive stimulation at constant  $[\text{K}][\text{Cl}]$ ,  $^{45}\text{Ca}$  release (above control) in interrupted responses correlated well with that in the pretreated responses of segments from the same fiber, with a slope of 8.4. This relationship suggests that imposed diffusion potentials stimulate a small  $\text{Ca}^{2+}$ -insensitive component that gradates a much larger  $\text{Ca}^{2+}$ -dependent efflux. The  $\text{Ca}^{2+}$ -insensitive component apparently reflects intermediate steps in the excitation-contraction coupling that require positive feedback to result in sufficient Ca release for contraction.

## INTRODUCTION

Since the original observations by Natori (1954) and Costantin and Podolsky (1967), much evidence has accrued that skinned skeletal muscle fibers and some fragmented sarcoplasmic reticulum (SR) preparations can be stimulated to release Ca by ionic substitutions in the medium that produce an electrically negative diffusion potential (see Stephenson, 1981*a*, for a review). The supposition has been that some component of the internal membrane systems is depolarized or becomes negative with respect to the rest of the myoplasm, but the mechanism and site(s) of stimulation and its relationship to the physiological pathway of excitation-contraction coupling have been uncertain. Imposed ion gradients have been suggested to act on or mimic physiological changes at the transverse (T)-tubules, the SR, or the T-tubule-SR junction (e.g., Costantin and Podolsky, 1967; Endo and Nakajima, 1973; Stephenson and Podolsky, 1977; Stephenson, 1978; Campbell and Shamoo, 1980; Caswell and Brandt, 1981; Miyamoto and Racker, 1982). Recent evidence supports the interpretation that the T-tubules are sealed and polarized (Volpe and Stephenson, 1986) and are the locus of permeant anion stimulation (Donaldson, 1983; also see Ikemoto et al., 1984). In this case, the mechanisms that increase Ca efflux would encompass the normal pathway: T-tubule depolarization, T-SR coupling, and activation of SR Ca channels.

The present studies were undertaken to analyze  $^{45}\text{Ca}$  efflux in skinned fibers stimulated by anion replacement under conditions that would favor a large, sustained potential change. Previous studies have shown that KCl replacement of K propionate stimulates  $^{45}\text{Ca}$  efflux transiently by a highly  $\text{Ca}^{2+}$ -dependent mechanism; stimulation is undetectable when  $\text{Ca}^{2+}$  outside the membrane-bounded compartments is maintained at low levels by EGTA (Stephenson and Podolsky, 1977; Stephenson, 1978). The anion pair Cl/propionate may generate a weak and transient stimulus, because of a relatively small permeability difference, while the continued presence of high  $[\text{K}^+]$  as counterion could contribute to rapid dissipation of the anion gradient. It seemed possible that a  $\text{Ca}^{2+}$ -insensitive component could be identified and studied with the stronger stimulus generated by Cl replacement of a less permeant anion such as methanesulfonate (Mes). Furthermore, the anionic diffusion potential would be prolonged and osmotic water entry would be minimized with simultaneous replacement of  $\text{K}^+$  by a relatively impermeant cation such as choline, since the chemical potential difference for KCl across the internal membranes, determined by the initial and stimulating solutions, is minimized when the  $[\text{K}^+][\text{Cl}^-]$  product in these solutions is kept constant. From studies of net Ca release after KCl replacement of KMes in *Xenopus* fibers, using different methods, Thorens and Endo (1975) concluded that "depolarization"-induced release has little  $\text{Ca}^{2+}$  dependence or similarity to " $\text{Ca}^{2+}$ -induced" release. However, similar studies on frog fibers (Mobley, 1979) have suggested that osmotic effects contribute substantially to stimulation by Cl/Mes replacement at constant  $[\text{K}]$ , where the greatly increased  $[\text{K}^+][\text{Cl}^-]$  product can drive salt and water entry into the SR during the relatively slow re-equilibration of  $\text{Mes}^-$ . Therefore, analysis of stimulation by the Cl/Mes diffusion potential per se, and its  $\text{Ca}^{2+}$  dependence, required  $^{45}\text{Ca}$  efflux measurements with a less permeant cation, at constant  $[\text{K}][\text{Cl}]$  product.

In the present studies, stimulation by Cl replacement of the ambient anion Mes at constant [K] was compared to that with choline substitution at constant [K][Cl], in order to evaluate osmotic and voltage effects. At constant [K][Cl], the stimulated efflux was smaller and much more  $\text{Ca}^{2+}$  dependent, which is consistent with the presence of a substantial osmotic component with low  $\text{Ca}^{2+}$  sensitivity at constant [K]. However, it was possible to identify a small  $\text{Ca}^{2+}$ -insensitive component of the "voltage-stimulated" efflux that could provide important information on the mechanisms coupling the stimulus to the large  $\text{Ca}^{2+}$ -dependent efflux. Some properties of this  $\text{Ca}^{2+}$ -insensitive component are described in the following article (Stephenson, 1985).

A preliminary report of this work has been presented (Stephenson, 1983).

#### METHODS

Microdissection and mounting of skinned fiber segments, isometric force measurement, general procedures, and tracer methods were as described previously (Stephenson, 1978, 1981b), except that force was recorded on a chart recorder with automatic suppression (2021, Linseis Inc., Princeton Junction, NJ) and  $^{45}\text{Ca}$  samples were counted on a liquid scintillation spectrometer (SL32, Intertechnique, Plaisir, France). In brief, semitendinosus muscles of the Southern grass frog (*Rana berlandieri*) were isolated and suspended in cold, low-Cl Ringer solution containing (mM): 217 sucrose, 2.5 KCl, 1.8  $\text{CaCl}_2$ , 3.1  $\text{NaH}_2\text{PO}_4$  plus  $\text{Na}_2\text{HPO}_4$ . A bundle of fibers was transferred to cold paraffin oil, a single fiber was isolated from tendon to tendon, and segments were cut to be skinned by microdissection just before use. The skinned segment was tied with monofilament thread to small stainless steel rods, one fixed and the other attached to a leaf-spring photodiode force transducer, for continuous measurement of isometric force and transfer of the segment between bathing solutions maintained at 19°C in the wells of a spring-mounted, thermoregulated chamber. Segments were 2–3 mm long and 75–150  $\mu\text{m}$  wide (measured at a magnification of 40); assuming a cylindrical cross-section, the mean diameter of segments giving interrupted responses at constant [K][Cl] (see Results) was  $111 \pm 6 \mu\text{m}$  (10).

Bathing solutions (pH 7.00) contained 120 or 122.5 mM salt, 10 mM imidazole, 5 mM  $\text{Na}_2\text{ATP}$ , 1 or 3 mM  $\text{MgSO}_4$ , and EGTA as indicated. For constant [K][Cl] studies, Tris base was used instead of KOH for preparation of the EGTA stock solution and other base additions for pH adjustment in order to prevent increased [K]. In the study with 10 mM EGTA, KCl was reduced to 110 mM. The  $^{45}\text{Ca}$ EGTA buffer and dilute EGTA rinse solutions contained 120 mM K propionate and 1 mM  $\text{MgSO}_4$  (20  $\mu\text{M}$   $\text{Mg}^{2+}$ ) to maintain the loading conditions of previous experiments, while the stimulating (and control) solutions and the KMes solutions preceding them contained 3 mM  $\text{MgSO}_4$  (110  $\mu\text{M}$   $\text{Mg}^{2+}$ ; see Stephenson, 1978). In experiments with stimulation at constant [K], 120 mM KCl replaced 120 mM KMes. In experiments with stimulation at constant [K][Cl], 120 mM choline Cl plus 2.5 mM KMes replaced 120 mM KMes plus 2.5 mM choline Cl ([K][Cl] product, 300  $\text{mM}^2$ ). The solution for extraction of residual  $^{45}\text{Ca}$  also contained 0.05% Triton X-100 plus 5 mM EGTA or CaEGTA. Reagent grade chemicals were used throughout; EGTA and low-Ca  $\text{Na}_2\text{ATP}$  were obtained from Sigma Chemical Co., St. Louis, MO.

Fiber segments were loaded for 40 s as described previously (Stephenson, 1978) in  $^{45}\text{Ca}$ EGTA buffer solution containing 0.375 mM CaEGTA with 0.5 mM total EGTA (a ratio nominally giving pCa 6.2 at pH 7.00; Sillen and Martell, 1964), prepared with high-specific-activity  $^{45}\text{CaCl}_2$  (New England Nuclear, Boston, MA); the final activity was 19–46  $\mu\text{Ci/ml}$ . After rinsing two to three times with 0.1 mM EGTA, K propionate solution ( $\sim 10$  s each), once with 0.1 mM EGTA, KMes solution ( $\sim 90$  s), and finally with 0.01 mM

EGTA, KMes solution (10 s), the segment was transferred through a series of washout solutions for 60 or 70 s and then extracted in solution with 0.05% Triton X-100 plus 5 mM EGTA or 5 mM CaEGTA, which removes the remaining  $^{45}\text{Ca}$  (Stephenson, 1978, and present study). The amount of  $^{45}\text{Ca}$  lost into each washout solution and into the KMes rinse solutions was expressed as a fraction of the total  $^{45}\text{Ca}$  in the segment at the time of stimulation (or corresponding time in control segments), which is the sum of the  $^{45}\text{Ca}$  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 residual Triton extract, and the fraction lost into each wash was expressed as a flux by dividing by the dwell time (i.e., transfer times were excluded). The flux divided by the mean  $^{45}\text{Ca}$  in the fiber during the interval gives an apparent first-order rate coefficient for efflux.

Results are expressed as means  $\pm$  SEM, with the number of determinations in parentheses. The significance of differences 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

### *Stimulation of $^{45}\text{Ca}$ Loss at Constant [K]*

In skinned *Xenopus* fibers (Thorens and Endo, 1975) and frog fibers (Mobley, 1979), KCl replacement of KMes induces force responses indicative of a very large Ca release from the SR. The present studies on frog fibers skinned by the Natori method confirmed this finding directly with  $^{45}\text{Ca}$  efflux measurements, and showed that this response had a moderate  $\text{Ca}^{2+}$  dependence.

The total  $^{45}\text{Ca}$  released during a 1-min washout after stimulation at constant [K] is shown in Fig. 1. The  $^{45}\text{Ca}$  loading and rinsing procedures are described in the Methods. In interrupted responses, the same washout solution plus 5 mM EGTA was applied within a few seconds after Cl replacement of Mes, when the large isometric force rise (not shown) was close to its maximum. The total  $^{45}\text{Ca}$  release under these conditions averaged 65% of the initial content (at the time of stimulation), which is more than twice the fractional release observed previously when KCl replaced K propionate under the same conditions (Stephenson, 1978).

When Cl replaced Mes in the presence of 5 mM EGTA, after pretreatment in 5 mM EGTA, KMes solution,  $^{45}\text{Ca}$  release was reduced more than twofold (Fig. 1); however, residual stimulation was substantial when release was compared with the small control loss in KMes solution with 5 mM EGTA (followed by the same efflux protocol) (Fig. 1). The residual  $^{45}\text{Ca}$  efflux was reduced somewhat further by pretreatment and stimulation in the presence of 10 mM EGTA; the mean fraction lost in 10 mM EGTA did not differ significantly from that in 5 mM EGTA, but the difference between paired segments from the same fiber ( $n = 4$ ) was significant ( $P < 0.05$ ). In 10 mM EGTA,  $^{45}\text{Ca}$  loss was more than three times that in control solution (5 mM EGTA, KMes), which is a residual stimulation in marked contrast to the undetectable effect of KCl replacement of K propionate in the presence of 5 mM EGTA under otherwise identical conditions (Stephenson, 1978). However,  $\text{Ca}^{2+}$  dependence was much greater than inferred by Thorens and Endo (1975) for stimulation by KCl replacement of KMes at 2–5°C.

The mean time course of  $^{45}\text{Ca}$  efflux during the interrupted and pretreated responses under these conditions is shown in Fig. 2. The initial flux from fiber to bath in the absence of EGTA,  $8.8\% \text{ s}^{-1}$ , was much higher than observed previously after KCl replacement of K propionate (Stephenson, 1978), as reflected in the large total release. With EGTA pretreatment, efflux was reduced during the first 30 s of washout and had a similar time course in 5 and 10 mM EGTA. Parallel control efflux measurements (5 mM EGTA, KMes) are included in Fig. 5.

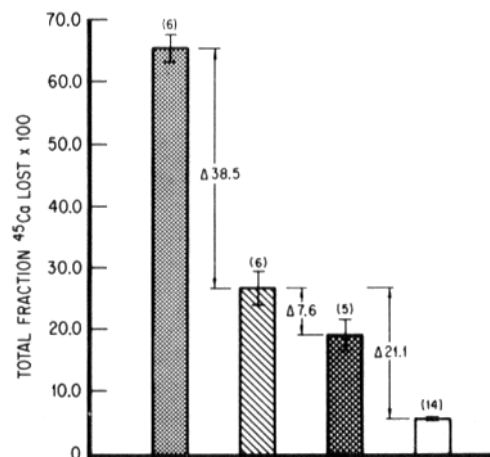


FIGURE 1. Stimulation of  $^{45}\text{Ca}$  release by KCl (constant [K]). Each bar shows the cumulative  $^{45}\text{Ca}$  loss (mean  $\pm$  SEM) during a 1-min washout after stimulation by Cl replacement of Mes at constant [K], using different washout protocols as described in the text. The first bar (stippled) shows interrupted responses with 5 mM EGTA applied soon after stimulation; the second and third bars show pretreated responses with 5 (hatched bar) or 10 (cross-hatched bar) mM EGTA applied before stimulation; the fourth bar (open) shows control responses in 5 mM EGTA, KMes. Mean differences between release with different protocols indicated with vertical arrows are significant ( $P < 0.01$ ), except that the difference between pretreatment with 5 or 10 mM EGTA was significant only with comparison between paired segments from the same fiber ( $P < 0.05$ ).

#### *Stimulation of $^{45}\text{Ca}$ Loss at Constant [K][Cl]*

Contractures induced in skinned frog muscle fibers by maximal Cl replacement of Mes are reduced at constant [K][Cl]; the maximum tension is 70% and the time-tension integral is only 31% of the values at constant [K] (Mobley, 1979). The difference was attributed to osmotic effects of KCl entry on SR Ca release, which was supported by additional osmotic studies. The stimulation of  $^{45}\text{Ca}$  release in the present experiments directly confirmed this difference and showed a much different  $\text{Ca}^{2+}$  dependence of the response.

The total  $^{45}\text{Ca}$  released during a 1-min washout after stimulation by choline Cl is shown in Fig. 3. The fractional release was much smaller and more variable than at constant [K]. For all fibers tested in this series, the mean loss during

interrupted responses, with 5 mM EGTA applied near the force peak, was 26.1% fiber tracer. Excluding minimally responsive fibers that released <15%, the mean loss was 34.6%; this value is about half the release in response to KCl, which is consistent with the expectation that constant [K][Cl] conditions minimize osmotic effects on the SR. While it was not feasible to keep the [Na][Cl] product constant, the osmotic effect of 10 mM Na appears to be negligible (see Discussion). In addition, K removal with choline substitution would be expected to enhance and

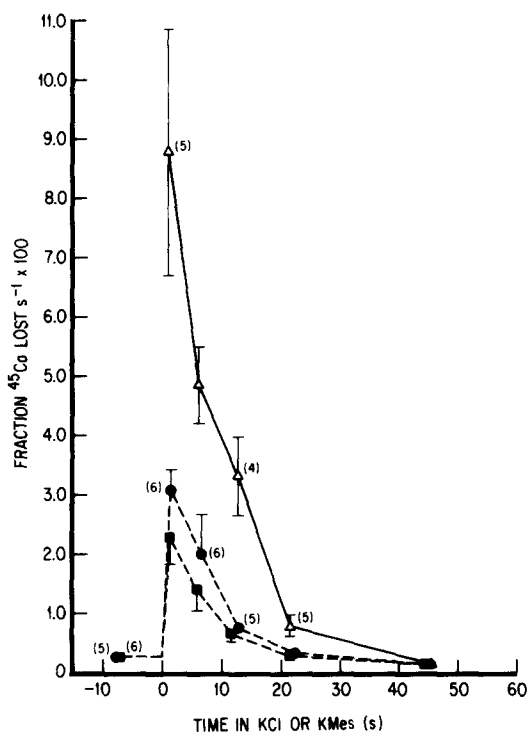


FIGURE 2. The time course of  $^{45}\text{Ca}$  efflux to the bath after stimulation by Cl replacement of Mes at constant [K]. Mean efflux ( $\pm$  SEM) during each wash interval is plotted against time after Cl replacement for interrupted responses (EGTA application soon after Cl), shown by the solid curve and open triangles, and for pretreated responses (EGTA application preceding Cl), shown by the dashed curves and solid circles (5 mM EGTA) or solid squares (10 mM EGTA). The efflux interval during EGTA pretreatment is assigned a negative value.

prolong the ionic diffusion potential at both T-tubular and SR membranes and to maximize voltage-dependent stimulation.

In some segments, EGTA application was delayed until the last 10–15 s of washout. The mean net  $^{45}\text{Ca}$  release, 30.9% fiber tracer, did not differ significantly from that in responses interrupted by early EGTA application to minimize reaccumulation of released tracer (Fig. 3), and paired segments from the same fiber ( $n = 5$ ) did not differ. The time course of  $^{45}\text{Ca}$  efflux also resembled that

in interrupted responses (see Fig. 6). These fibers maintained high force levels until EGTA application. Both the force pattern and tracer release differed from the transient "completed" responses that follow KCl replacement of K propionate (Stephenson, 1978), in which the fibers relax spontaneously and net  $^{45}\text{Ca}$  release is substantially smaller than in interrupted responses because of reaccumulation by the SR. It is possible that SR reaccumulation was less effective because the stimulatory diffusion potential was more sustained (Stephenson, 1978), but further studies, including an ATP-regenerating system to prevent ATP depletion during the prolonged contraction, are needed to evaluate this possibility.

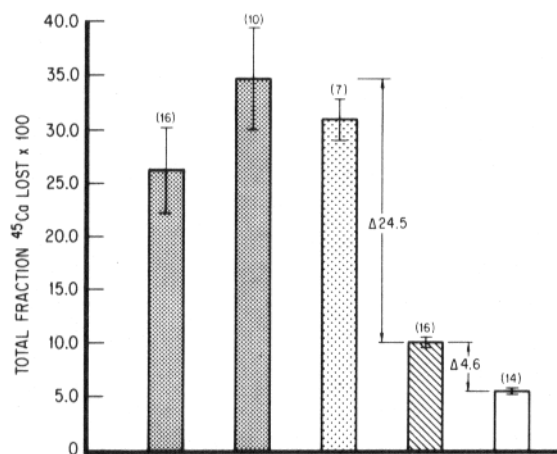


FIGURE 3. Stimulation of  $^{45}\text{Ca}$  release by choline Cl at constant [K][Cl]. Each bar represents the cumulative  $^{45}\text{Ca}$  loss during a 1-min washout after choline Cl replacement of KMes (mean  $\pm$  SEM) with various protocols described in the text. The first bar (stippled) shows mean release during the interrupted response (early EGTA application after choline Cl) from all segments tested in this series; in the second bar, unresponsive segments (releasing  $<15\%$  initial fiber  $^{45}\text{Ca}$ ) are excluded. The third bar (lightly stippled) shows the mean release with delayed EGTA application after choline Cl; the fourth bar (hatched) shows the mean release during pretreated responses with EGTA application preceding choline Cl; the fifth bar (open) shows mean control release in KMes, EGTA. Mean differences between different protocols shown with vertical arrows are highly significant ( $P < 0.01$ ).

In fibers pretreated with 5 mM EGTA,  $^{45}\text{Ca}$  release at constant [K][Cl] product was reduced to 10% (Fig. 3), which indicates much stronger inhibition than at constant [K]. However, the mean release was significantly larger than the small control loss seen in 5 mM EGTA, KMes with the same efflux protocol (Fig. 3; also see below). This residual stimulation differed from  $^{45}\text{Ca}$  release induced by KCl replacement of K propionate under otherwise similar conditions, where stimulation in 5 mM EGTA is undetectable (Stephenson, 1978), and was an important new result of the present study.

A more closely controlled comparison of stimulation initiated in the absence

or presence of EGTA was possible with measurements of  $^{45}\text{Ca}$  efflux during the interrupted, pretreated, and control protocols on segments from the same fiber. In Fig. 4, the stimulated  $^{45}\text{Ca}$  release, above the control loss, during the interrupted response is plotted against the stimulated release, above control loss, during the pretreated response. The stimulated release under the two conditions displayed a good linear correlation ( $r = 0.922$ ,  $P < 0.01$ ), with a slope of 8.4. This relationship suggested that choline Cl replacement of KMes initiated a small, rather variable,  $\text{Ca}^{2+}$ -insensitive efflux component that controlled in a

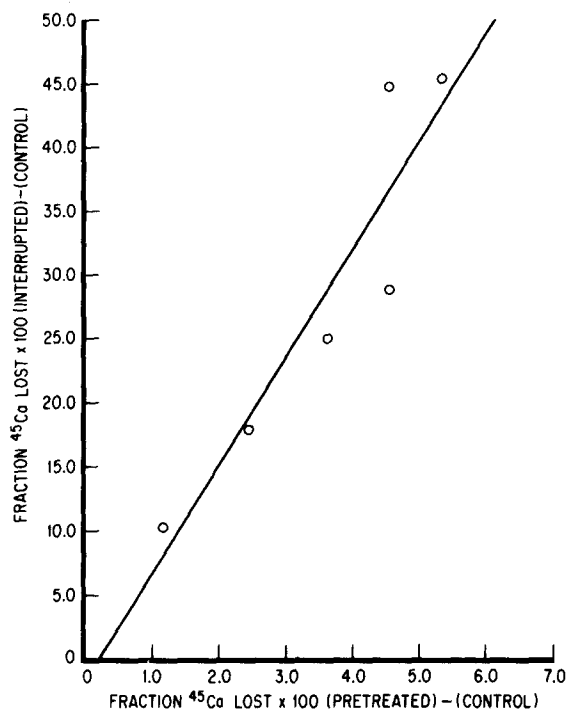


FIGURE 4. Correlation between stimulation of  $^{45}\text{Ca}$  release during interrupted and pretreated responses at constant  $[\text{K}][\text{Cl}]$ .  $^{45}\text{Ca}$  loss during interrupted, pretreated, and control protocols was compared on segments from the same fiber, to give a better-controlled comparison of stimulation (above control loss) initiated in the absence or presence of 5 mM EGTA. The extra (stimulated)  $^{45}\text{Ca}$  loss during 1 min after choline Cl in the interrupted response is plotted against the stimulated loss in the pretreated response. The line drawn from the linear regression ( $r = 0.922$ ) has a slope of 8.4.

graded manner a much larger  $\text{Ca}^{2+}$ -dependent component in the initial absence of EGTA.

#### *Comparison of Release at Constant $[\text{K}]$ and Constant $[\text{K}][\text{Cl}]$*

Table I summarizes the extra  $^{45}\text{Ca}$  release, above control loss, stimulated by Cl replacement of the much less permeant Mes anion under both conditions. At constant  $[\text{K}]$ , stimulation in interrupted responses was much larger than when Cl replaced propionate under the same conditions (Stephenson, 1978). However,



when choline simultaneously replaced K at constant [K][Cl], stimulation was less than half that at constant [K]. The difference between the two conditions is attributable to KCl and water entry into the SR when the large anion permeability increase was made in the presence of a high concentration of the permeant K cation.

KCl stimulation thus appeared to consist of a diffusion potential component, which can be estimated from release at constant [K][Cl] product, and an equally large osmotic component. EGTA reduced release in both cases, but the much larger inhibition at constant [K][Cl] product implied greater  $\text{Ca}^{2+}$  dependence of the component stimulated by the diffusion potential. The difference between the stimulated release when [K][Cl] increased or was constrained reflects the osmotic component induced by KCl entry; the small ratio of this difference in the absence and the presence of EGTA indicated that the osmotic component had low  $\text{Ca}^{2+}$  dependence. The substantial contribution of the osmotic component to Ca release induced by KCl replacement of KMes would thus obscure at least one important property of the Ca efflux stimulated by imposed diffusion potentials.

TABLE I  
*Extra  $^{45}\text{Ca}$  Loss (%) Stimulated by Cl Substitution for Mes*

	(A) Interrupted (- control)	(B) Pretreated (- control)*	(A/B) $\text{Ca}^{2+}$ dependence ratio
Constant $[\text{K}^+]$	59.6	21.1	2.8
Constant $[\text{K}^+][\text{Cl}^-]$	29.1	4.6	6.3-8.4 <sup>‡</sup>
Difference = osmotic component	30.5	16.5	1.8

\* 5 mM EGTA.

<sup>‡</sup> From the slope of linear correlation in paired segments from the same fiber.

#### *$^{45}\text{Ca}$ Efflux at Constant [K][Cl]*

The mean time course of  $^{45}\text{Ca}$  efflux to the bath during the interrupted, pretreated, and control responses at constant [K][Cl] product is shown in Fig. 5A. The large initial stimulation in the interrupted response declined rapidly, as expected if efflux after EGTA application (after the first wash) is due largely to outward diffusion as  $^{45}\text{CaEGTA}$  of the  $^{45}\text{Ca}$  released from the SR itself during the first wash, in the absence of EGTA. The initial efflux from SR to myoplasm during the first wash in the interrupted response was estimated by using  $^{45}\text{Ca}$  release in the pretreated response to evaluate the small contribution of stimulation during the subsequent EGTA washes. This calculation gives an estimated initial loss of 25.4% fiber  $^{45}\text{Ca}$  from the SR, and a minimum rate of  $6.85\% \text{ s}^{-1}$  for the initial stimulated efflux in the absence of EGTA.

The calculated rate is a lower limit. First, the time interval must include not only the initial wash time (with efflux to the bath), but also a similar duration transfer time (when diffusion conditions are unfavorable and poorly defined) into the EGTA trapping wash. Second, no correction is applied for  $^{45}\text{Ca}$ , EGTA, and  $^{45}\text{CaEGTA}$  diffusion delays, which are qualitatively predictable and evident

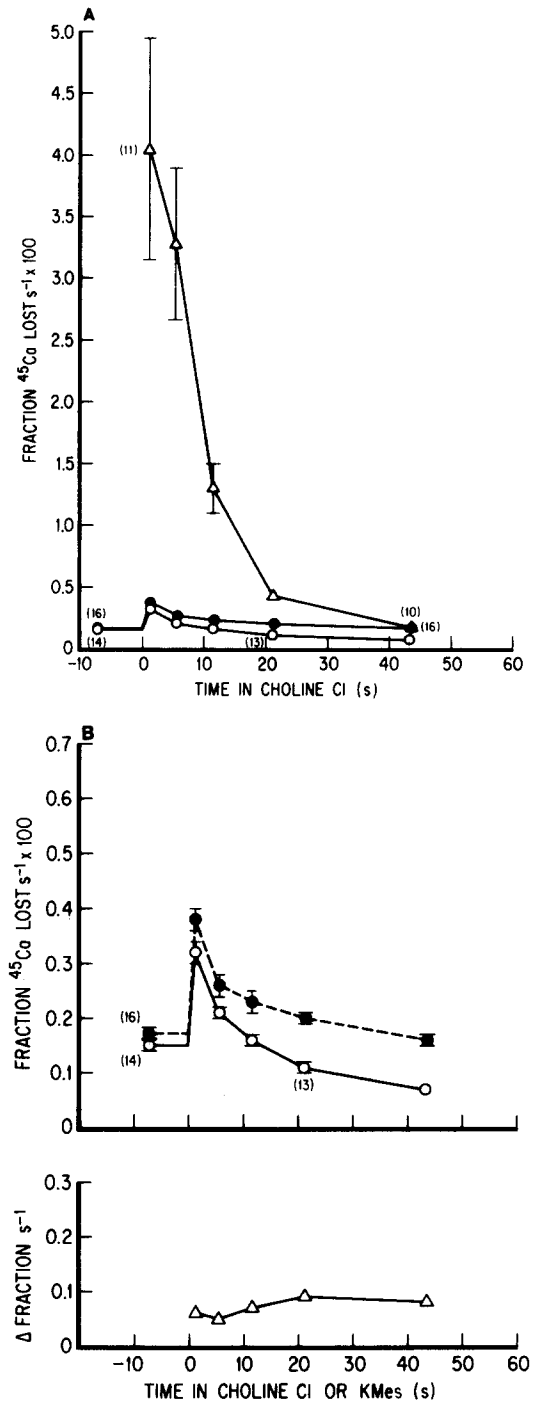


FIGURE 5.

in these relatively large preparations. In seven responsive segments with estimates of fiber diameter, efflux to the bath during the first wash decreased with increasing fiber radius squared (linear correlation coefficient, 0.792;  $P < 0.05$ ). In two additional fibers, efflux was compared in segments that were microdissected to give either normal or small diameter. In segments from fiber N170, all  $^{45}\text{Ca}$ -loaded for 39.5 s, the initial efflux to the bath from a small-diameter segment was 4.9 times the mean rate of two large-diameter segments, and the total fractional  $^{45}\text{Ca}$  release was 2.9 times as large. When  $^{45}\text{Ca}$ -loading times were adjusted by 10% (fiber N171), the mean initial efflux from two small-diameter segments loaded for only 36 s was 2.1 times that in a large-diameter segment loaded for 43 s, and the total  $^{45}\text{Ca}$  release was 1.7 times as large. Diffusion limitations and other factors likely to lead to a minimum estimation of SR efflux are considered in the Discussion.

In the interrupted response, the mean efflux during the initial wash and the first EGTA wash had large standard errors (Fig. 5), in part because of a second type of time course in some fibers. Examples of the two efflux patterns, seen with both early (interrupted response) and delayed EGTA addition, are shown in Fig. 6. The upper part shows data from segments with efflux decreasing monotonically, as in the mean data (Fig. 5). The lower part shows data from segments with a transiently increasing rate coefficient, giving a biphasic efflux pattern. The basis for this complex time course is unclear, but it might result from spatio-temporal dispersion of the ionic stimulus coupled to its  $\text{Ca}^{2+}$  dependence. Each pattern is represented by segments with the interrupted or delayed EGTA protocol; the segments were not pairs from the same fiber but were selected for similarity of the initial efflux (in the absence of EGTA) before the protocols diverged. With both patterns,  $^{45}\text{Ca}$  efflux in the absence of EGTA (delayed addition) did not decline more rapidly than efflux in the presence of EGTA (interrupted response with EGTA addition after the first wash). These results illustrate why the cumulative total release (Fig. 3) was not significantly smaller than when early EGTA addition minimized reaccumulation of the large initial efflux to the myoplasm, as is the case with KCl replacement of K propionate (Stephenson, 1978).

The efflux curves in Fig. 5 also illustrate the basis for the  $\text{Ca}^{2+}$ -insensitive

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FIGURE 5. (*opposite*) The time course of  $^{45}\text{Ca}$  efflux during stimulation at constant  $[\text{K}][\text{Cl}]$ . *A* shows the mean efflux ( $\pm$  SEM) during interrupted (open triangles) and pretreated (closed circles) protocols with choline Cl replacement of KMes at time zero, and control protocol with continued KMes (open circles). Interrupted responses from unresponsive segments (see Fig. 3) are not included. *B* shows the small  $\text{Ca}^{2+}$ -insensitive  $^{45}\text{Ca}$  efflux ( $\pm$  SEM) on an expanded scale to display the difference between pretreated (closed circles) and control (open circles) responses. The apparent sharp peak following time zero is attributable to artifacts of the washout procedure (see Stephenson, 1981*b*). Except for this first brief wash, the efflux during the pretreated response was significantly larger than control; the difference (open triangles) is plotted below.

component of the cumulative stimulated  $^{45}\text{Ca}$  release (Fig. 3). Fig. 5B shows the time course of the smaller  $^{45}\text{Ca}$  efflux in the presence of 5 mM EGTA on an expanded scale. Efflux during the pretreated response was only modestly increased above control efflux in KMes, but the difference was sustained throughout the washout period (and was more prominent after the usual artifactual peak of the first brief wash [see Stephenson, 1978]). The lower part of Fig. 5B shows the difference curve between pretreated and control responses; the small, sustained stimulation was consistent with the expectation that choline Cl replace-

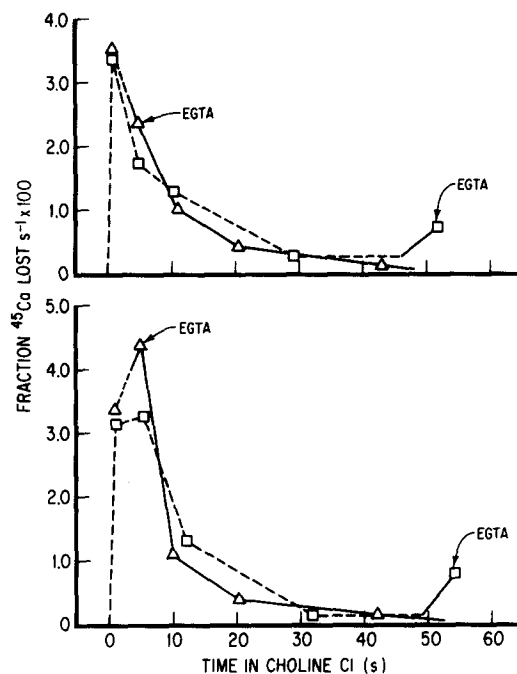


FIGURE 6. Examples of monotonic (upper) or biphasic (lower) time course of  $^{45}\text{Ca}$  efflux during stimulation by choline Cl with either early (triangles) or delayed (squares) EGTA application. Segments exhibiting each pattern were selected for similar initial efflux in the absence of EGTA, before the protocols diverged. Arrows indicate the first wash that contained 5 mM EGTA, actually applied earlier since efflux points are plotted at the mid-interval.

ment of KMes would impose sustained ionic gradients and resultant diffusion potentials, and showed that these could increase  $^{45}\text{Ca}$  efflux at very low initial free  $[\text{Ca}^{2+}]$  in the myoplasm.

#### DISCUSSION

These studies show that  $^{45}\text{Ca}$  efflux in fibers skinned by the Natori method is stimulated much more vigorously when Cl replaces Mes rather than the more permeant propionate as an initial anion. After Mes replacement at constant  $[\text{K}]$ ,

in which the greatly increased [K][Cl] product permits KCl and water entry, approximately half of the large increase in  $^{45}\text{Ca}$  release can be attributed to osmotic effects on the SR. At constant [K][Cl] product, in which Cl movement and osmotic effects are minimized, the imposed ion gradients stimulate efflux with a small, sustained,  $\text{Ca}^{2+}$ -insensitive component that appears to grade a much larger  $\text{Ca}^{2+}$ -dependent component. The results suggest that a relatively sustained negative potential difference is generated across T-tubule and possibly SR membranes, which initiates a small increase in SR Ca efflux; positive feedback is required to deliver substantial amounts of Ca to the myofilament space.

#### *Stimulation by Cl/Mes Replacement*

KCl replacement of K propionate, in skinned frog fibers at 19°C, produces transient,  $\text{Mg}^{2+}$ -sensitive, and highly  $\text{Ca}^{2+}$ -dependent stimulation, with rather modest net  $^{45}\text{Ca}$  release (Stephenson and Podolsky, 1977; Stephenson, 1978). The amount and ion dependences differ from release estimated from force responses after KCl replacement of KMes, in frog fibers at room temperature (Mobley, 1979) or *Xenopus* fibers at 0–2°C (Thorens and Endo, 1975). The present results directly relate this difference to the initial “impermeant” anion; neither propionate nor Mes is absolutely impermeant (see below), but a larger increase from an initially lower anion permeability at target membranes should produce a larger and more prolonged net diffusion potential.

Both internal membrane systems are candidates for effects of the imposed gradients, in view of indirect evidence that the T-tubules can seal over and repolarize in skinned fibers (see Stephenson, 1981*b*; Donaldson, 1983; Volpe and Stephenson, 1986) and in isolated membrane preparations (Lau et al., 1979; Ikemoto et al., 1984). In the case of the SR membrane, K propionate and KCl permeate rabbit SR vesicles at similar rates (limited by  $P_{\text{K}}$ ), which are fivefold faster than for KMes (Kometani and Kasai, 1978); with such relative permeation, KCl replacement of K propionate would have a much smaller osmotic effect on frog SR in situ than replacement of KMes and less electrical effect from the relative anion permeabilities. In the case of the T-tubular membrane, the relative permeabilities are uncertain, but the overall propionate/Cl conductance ratio in intact frog fibers is  $\sim 0.12$  at pH 7.0, while the Mes/Cl ratio is  $< 0.05$  (Woodbury and Miles, 1973). Thus, the observation that stimulation of  $^{45}\text{Ca}$  release (above control loss) by Cl/Mes replacement at constant [K] (Figs. 1 and 2) is more than three times as large as previous results with propionate is consistent by itself with evidence that Mes is substantially less permeant across both types of membrane.

#### *Osmotic Effects at Constant [K]*

Cl replacement at constant [K] (a 48-fold increase in [K][Cl] product) increases the chemical potential of a permeant salt and can permit osmotic water entry into the SR (Kometani and Kasai, 1978). The effect of net water entry requires evaluation because sufficiently hypo-osmotic solutions cause Ca release in skinned fibers (Endo and Thorens, 1975; Mobley, 1979). With propionate as the initial anion, relatively rapid anion exchange would minimize net water entry, and the

strong inhibition by osmotically negligible amounts of Mg and EGTA, noted above, argues against an appreciable osmotic stimulus. With Mes as the initial anion, however, stimulation by the larger predicted osmotic effect (Kometani and Kasai, 1978) is supported by inhibition by osmotic buffering in some SR membrane preparations (McKinley and Meissner, 1978; Campbell and Shamoo, 1980) as well as in skinned fibers.

The evidence of a large osmotic contribution to KMes/KCl contractures in frog fibers (Mobley, 1979) is confirmed as an SR effect by the present analogous twofold difference in  $^{45}\text{Ca}$  release (above control loss) between constant [K] and constant [K][Cl] at 120 mM anion replacement (Table I). At constant [K], the present results differ from the Mes/Cl response in *Xenopus* fibers at 0–2°C in that the total  $^{45}\text{Ca}$  release is smaller and the apparent  $\text{Ca}^{2+}$  dependence is much larger (Figs. 1 and 2) than the 100% net Ca release reported by Thorens and Endo (1975) with 82 mM Cl in 10 mM EGTA. However, the osmotic component alone, inferred from the difference between  $^{45}\text{Ca}$  release at constant [K] and constant [K][Cl], is less  $\text{Ca}^{2+}$  dependent than the overall response to KCl (Table I). The *Xenopus* responses to KCl are inhibited completely by the addition of 40 mM sucrose, while the frog fiber responses (Mobley, 1979) are inhibited only partially by even 200 mM sucrose. Osmotic effects may not be the sole basis of differences between constant [K] and constant [K][Cl]; for example, slower diffusion of choline Cl could decrease the stimulation rate and effectiveness (see below). However, the Mes/Cl response at constant [K] (a rather widely used stimulus) appears to include a substantial osmotic component that introduces uncertainty into the interpretation of properties such as ion dependence. In addition, Cl entry accompanied by K implies that imposed potential changes would be more transient and might contribute a smaller component than estimated from the release at constant [K][Cl] (see Hodgkin and Horowicz, 1959).

#### *Stimulation by Cl/Mes Replacement at Constant [K][Cl]*

The anion permeability difference between Mes and Cl could be exploited to maximize the diffusion potentials and minimize osmotic effects by replacing K with the poorly permeant choline cation. The passive permeability of isolated SR vesicles to choline is relatively low (Kometani and Kasai, 1978; McKinley and Meissner, 1978; Yamamoto and Kasai, 1982). Since their permeability to Na is much larger, the presence of Na (in  $\text{Na}_2\text{ATP}$ ) in the choline Cl solutions did not permit a completely constant “diffusible” ion product with respect to the SR. A constant [Na]Cl product or Na removal would have been incompatible with reasonable ionic strength and T-tubule excitability. However, with the half-time of NaCl permeation in SR vesicles of 13 s (Kometani and Kasai, 1978) and an external  $[\text{Na}^+]$  of 10 mM, the increase in SR salt and water content would be very small on the time scale of the rapid  $^{45}\text{Ca}$  release. A negligible osmotic effect of 10 mM Na in skinned fibers is indicated by observations that Ca release is not stimulated by either 30 mM Cl with 9 mM Na at constant [K][Cl] (Mobley, 1979) or by removal of 20 mM KMes (Mobley, 1979; also see Endo and Thorens, 1975), although water entry is very rapid in SR vesicles (Kometani and Kasai, 1978).

With respect to the T-tubules, no osmotic effect of the choline Cl replacement would be expected. Intact fibers are relatively impermeable to Na as well as choline, and it is well known that when the ion product of the primary "diffusible" ions K and Cl is kept constant, imposed transmembrane gradients and the diffusion potentials generated by them dissipate slowly, forming an "ionic clamp" (see Hodgkin and Horowitz, 1959). While the diffusion potential generated across the T-tubules in the present studies cannot be equated with those measured across the surface membrane (see Stephenson, 1981*a*), substantiating evidence for sustained gradients is provided by the sustained stimulation of efflux in the presence of 5 mM EGTA. The difference between stimulated efflux and control efflux does not decrease during the measurement period (Fig. 5*B*), although absolute rates and rate coefficients decline with time as in previous studies (Stephenson, 1978, 1981*a*).

#### *Ca<sup>2+</sup>-dependent Stimulation at Constant [K][Cl]*

In the absence of EGTA, choline Cl usually stimulates ample Ca release to saturate the myofilaments (Figs. 3 and 5*A*). In interrupted responses, <sup>45</sup>Ca efflux to the bath during the initial EGTA-free wash averaged 4.05% s<sup>-1</sup>, with a wide variation that apparently relates to variation in the pattern of stimulus propagation (Fig. 6) and in the fiber radius (see Results and discussion below). The initial efflux from the SR itself, including <sup>45</sup>Ca released initially (and generating force) that appears as <sup>45</sup>CaEGTA in subsequent washes, is estimated as at least 6.85% s<sup>-1</sup> (see Results). This lower limit of the tracer flux corresponds to 110–160 μmol Ca/liter fiber·s<sup>-1</sup> (Stephenson, 1978), which is equivalent to a rate of [Ca] increase in the myofilament space water (~0.58 liter/liter fiber; see Baylor et al., 1983) of ~190–270 μmol·s<sup>-1</sup> in a closed system. Uniformly distributed, this increase would saturate troponin in <1 s, but force developed more slowly and submaximally because of spatio-temporal dispersion of the stimulus and Ca flux to the bath.

This minimum average efflux may be ~100 times slower than release rates in electrically stimulated intact fibers, as estimated recently from a modeling of arsenazo III calcium transients (Baylor et al., 1983). It is important to note that the discrepancy reflects different rate-limiting steps rather than mechanisms; a difference of this magnitude is predictable simply from the slow stimulus propagation and <sup>45</sup>Ca flux assay in these relatively large preparations. In skinned fibers, potential gradients are imposed by diffusional exchange to membranes distributed throughout the fiber volume. The diffusion time estimated from the fiber diameter, assuming a cylindrical cross-section, and appropriate diffusion coefficients (Hill, 1928) is long. From the average fiber diameter in the interrupted responses, 111 μm (see Methods), and apparent diffusion coefficients in the myoplasm (about half the free diffusion coefficients [Kushmerick and Podolsky, 1969]) of 8.4 × 10<sup>-6</sup> cm<sup>2</sup>·s<sup>-1</sup> for KCl and ~6.1 × 10<sup>-6</sup> cm<sup>2</sup>·s<sup>-1</sup> for choline Cl and KMes (from the KCl value and the relative molecular weights), the estimated time required for 90% equilibration would be 1.2 s for KCl alone and ~1.7 s for choline Cl and KMes. In contrast, T-tubular depolarization in electrically stimulated intact or cut fibers is at least two orders of magnitude faster. The tubular

depolarization time estimated from potentiometric dye signals is  $\sim 3$  ms, and increases 10-fold when tetrodotoxin blocks the active Na conductance (Heiny and Vergara, 1982).

$^{45}\text{Ca}$  efflux from the SR also is underestimated, because of (a) outward diffusion delay, and (b) inclusion of transfer times in this flux calculation. A correlation between a larger initial efflux into EGTA-free solution and a smaller fiber radius was detectable despite other sources of variability (see Results), which presumably reflects the effects of stimulus diffusion and dispersion, as well as outward  $^{45}\text{Ca}$  diffusion per se.

In addition, the slow rate of stimulus propagation in the skinned fiber is likely to have an important secondary effect, decreased effectiveness of the imposed diffusion potentials. Tetrodotoxin treatment decreases the rate of Ca release estimated from arsenazo III signals in cut fibers, and appears to decrease the amount released at smaller depolarizations (Palade and Vergara, 1982); it can markedly reduce mechanical activation by action potential waveforms (Bastian and Nakajima, 1974). Stimulation of Ca release from skinned fibers by applied  $\text{Ca}^{2+}$  is rate dependent (see Fabiato, 1983), and the  $\text{Ca}^{2+}$  dependence of the present response makes it plausible that spatio-temporal dispersion of the ionic stimulus would dissipate the local effects of Ca efflux.

This assignment of the relatively slow measured release rates in the present studies to ancillary rate-limiting steps is strongly supported by recent studies of Ca release from SR-T-tubular triad suspensions, stimulated by choline Cl replacement of K gluconate (Ikemoto et al., 1984). In those rapid-flow studies, where the diffusion time is minimized and Ca release is assayed rapidly by optical methods, the rate constant for the initial release component, which requires associated T-tubules, is comparable to that in intact fibers.

#### *Ca<sup>2+</sup>-insensitive Stimulation by Sustained Ion Gradients*

Two important new results of these studies were the inhibition by EGTA of stimulation at constant  $[\text{K}][\text{Cl}]$  product, which indicates strong  $\text{Ca}^{2+}$  dependence of the total efflux, and the small but sustained stimulation in 5 mM EGTA, which reveals a  $\text{Ca}^{2+}$ -insensitive component. The  $\text{Ca}^{2+}$ -insensitive component may not be entirely  $\text{Ca}^{2+}$  independent, if rapid EGTA buffering is incomplete in sequestered regions. A stimulated  $^{45}\text{Ca}$  efflux component in EGTA was not detected after KCl replacement of K propionate (Stephenson, 1978), perhaps because that stimulus was more transient.

The  $\text{Ca}^{2+}$ -insensitive efflux is much too small for contractile activation and presumably reflects an intermediate step in activation of Ca release. Its source almost certainly includes the SR; the T-tubules alone are unlikely to provide sufficient Ca for this component, as seen by the following calculation. Stimulated release above control loss during the washout period in 5 mM EGTA averaged 5% fiber  $^{45}\text{Ca}$  (Fig. 3), which corresponds to at least  $80 \mu\text{mol Ca/liter fiber}$  under these loading conditions (Stephenson, 1978). The fractional volume of the T-tubules of frog fibers is only 0.3% (Mobley and Eisenberg, 1975) and its entire luminal free  $\text{Ca}^{2+}$  content (at 1.8 mM Ca in Ringer) would be only  $5 \mu\text{mol/liter fiber}$ . If the volume fraction of sealed T-tubules in skinned fibers were doubled,



the remaining eightfold discrepancy from the minimum Ca lost would still exceed various uncertainties in the estimates. An additional source could be Ca associated with the tubular membrane and T-SR junction; however, release of a large "bound" component would be required to account for the entire stimulated  $^{45}\text{Ca}$  release in EGTA and would be even larger during the potentiation described in the following article (Stephenson, 1985). A further possibility is that the initial T-tubule-related  $^{45}\text{Ca}$  content is replenished directly by stimulated efflux from the terminal cisternae, resulting in an apparently expanded source during the washout period.

The probability of SR efflux does not exclude the possibility that tubule-related Ca contributes, perhaps crucially, to the  $\text{Ca}^{2+}$ -insensitive stimulated efflux. If the imposed diffusion potentials act directly on sealed, polarized T-tubules, which then stimulate the SR (Ikemoto et al., 1984; also see Stephenson, 1981*b*), a T-tubule or junctional component of the  $\text{Ca}^{2+}$ -insensitive  $^{45}\text{Ca}$  efflux could provide important information on the role of "trigger  $\text{Ca}^{2+}$ " (Bianchi, 1968) in T-SR coupling. Alternatively, stimulated tubules could communicate with the SR by a  $\text{Ca}^{2+}$ -independent mechanism and the entire  $\text{Ca}^{2+}$ -insensitive efflux might derive from the SR. The present studies cannot distinguish between these possibilities. First, complete EGTA buffering of the restricted T-SR junctional space may be difficult to achieve, so the EGTA-resistant component is not necessarily entirely  $\text{Ca}^{2+}$  independent. Second, the diffusion potentials could conceivably act on both T-tubules and SR directly. However, while some direct SR potential change would be consistent with the relative Cl/Mes permeabilities implied by the osmotic component of  $^{45}\text{Ca}$  release at constant [K], as well as with the SR vesicle studies discussed above, no direct stimulation of release at constant ion product was observed in recent studies on associated and dissociated triads (Ikemoto et al., 1984) or on skinned rabbit fibers with T-tubules inhibited by cardiac glycoside (Donaldson, 1983). The effects of ATP removal on the present system, described in the following article, are consistent with the interpretation that at most a release of a few percent in EGTA might be attributable to direct SR stimulation (Stephenson, 1985). Direct electrical coupling also is improbable, according to recent evidence that putative SR potential signals in intact fibers accompany rather than precede Ca release (see Baylor et al., 1984, and references therein). Electron probe studies of elemental distributions are consistent with the possibility that Ca release generates an SR transmembrane potential (Somlyo et al., 1981; Kitazawa et al., 1984). The hypothesis has been advanced that the control mechanism of the SR efflux channel is voltage, which results physiologically from the electrogenicity of a small Ca release initiated by a  $\text{Ca}^{2+}$  signal (Miyamoto and Racker, 1982). However, the present results suggest that the  $\text{Ca}^{2+}$ -insensitive stimulation is much too small for a secondary voltage change alone to produce functional Ca release. Even if the  $\text{Ca}^{2+}$ -insensitive  $^{45}\text{Ca}$  release reflected direct voltage dependence of the efflux channel,  $\text{Ca}^{2+}$  would be required for its expression and regulation.

Although the results do not distinguish the details of the mechanism, they are consistent with the interpretation that the  $\text{Ca}^{2+}$ -insensitive component reflects a graded coupling step between T-tubular depolarization and the large efflux

increase required for contractile activation. Stimulated  $^{45}\text{Ca}$  release associated with the variable responses initiated in the absence of EGTA is highly correlated with the  $\text{Ca}^{2+}$ -insensitive stimulated  $^{45}\text{Ca}$  release component measured in the same fiber (Fig. 4). The grading of the  $\text{Ca}^{2+}$ -dependent component implies positive feedback from  $\text{Ca}^{2+}$  released by the "primary" mechanism, which leads to an amplification that need not be all or none, as noted previously (Endo, 1977; Stephenson, 1981*a*). The  $\text{Ca}^{2+}$ -dependent flux is thus likely to be mediated by the same channel described previously for stimulation of  $^{45}\text{Ca}$  efflux by KCl replacement of K propionate (Stephenson, 1978) and by caffeine or  $\text{Mg}^{2+}$  reduction (Stephenson, 1981*a*), and to be related to stimulation by applied  $\text{Ca}^{2+}$  (see Endo, 1977; Stephenson, 1981*a*; Fabiato, 1983). The  $\text{Ca}^{2+}$ -stimulated channel can also exhibit negative feedback at high  $[\text{Ca}^{2+}]$  (see Fabiato, 1983; Meissner, 1984, and references therein), which would provide direct control of the amplification at lower  $\text{Ca}^{2+}$  and might influence the time course of Ca release in intact fibers (Baylor et al., 1983). Negative feedback should be minimized under the present conditions, where endogenous  $\text{Ca}^{2+}$  is being released into an open system. By minimizing the positive feedback with EGTA, it has been possible to isolate a  $\text{Ca}^{2+}$ -insensitive  $^{45}\text{Ca}$  efflux stimulated by imposed diffusion potentials. Characterization of this component may provide important insight into the sequence of steps that activate Ca release.

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