

Excitation of Skinned Muscle Fibers by Imposed Ion Gradients

IV. Effects of Stretch and Perchlorate Ion

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ABSTRACT Depolarizing ion gradients stimulate ^{45}Ca release in skeletal muscle fibers skinned by microdissection. Several lines of indirect evidence suggest that sealed transverse (T) tubules rather than sarcoplasmic reticulum (SR) are the locus of such stimulatory depolarization. Two implications of this hypothesis were tested. (a) A requirement for signal transmission was evaluated from the stimulation of ^{45}Ca efflux in fibers that had been highly stretched, an intervention that can impair the electrical stimulation of intact fibers. Length was increased over ~ 95 – 115 s, after loading with ^{45}Ca and rinsing at normal length; prestimulus ^{45}Ca loss due to stretch itself was very small. In the first study, stimulation of ^{45}Ca release by KCl replacement of K propionate was inhibited completely in fibers stretched to twice slack length, compared with fibers at 1.05–1.1 times slack length. Identical protocols did not alter ^{45}Ca release stimulated by caffeine or Mg^{2+} reduction, implying that SR Ca release per se was fully functional and inhibition was selective for a preceding step in ionic stimulation. In a second study, stimulation by choline Cl replacement of K methanesulfonate, at constant $[\text{K}^+][\text{Cl}^-]$ product, was inhibited strongly; total ^{45}Ca release decreased 69%, and stimulation above control loss decreased 78%, in segments stretched to twice the length at which sarcomere spacing had been $2.2\ \mu\text{m}$, compared with paired controls from the same fibers kept at $2.3\ \mu\text{m}$. (b) Perchlorate potentiation of T tubule activation was evaluated in fibers stimulated at constant $[\text{K}^+][\text{Cl}^-]$ at normal length ($2.3\ \mu\text{m}$); this anion shifts the voltage dependence of intramembrane charge movement and contractile activation in intact fibers. Perchlorate (8 mM) potentiated both submaximal stimulation of Ca^{2+} -dependent ^{45}Ca release by partial choline Cl replacement of K methanesulfonate and the small Ca^{2+} -insensitive ^{45}Ca efflux component stimulated by nearly full replacement in the presence of 5 mM EGTA. These results provide independent support for the hypothesis that the T tubules are the locus of stimulation by depolarizing ion gradients, with junctional transmission of this signal causing SR ^{45}Ca release.

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INTRODUCTION

The specific mechanisms of signal transmission between transverse (T) tubules and sarcoplasmic reticulum (SR) and of SR Ca release during excitation-contraction coupling in skeletal muscle fibers are unresolved. This problem could be addressed in mechanically skinned fibers if the T tubule system, which remains after removal of the sarcolemma, can be stimulated. Depolarizing ion gradients, imposed at constant $[K^+][Cl^-]$ product, stimulate ^{45}Ca efflux from these fibers; stimulated release has a small sustained Ca^{2+} -insensitive component that appears to grade the much larger Ca^{2+} -dependent component and may reflect intermediate steps in activation (Stephenson, 1985*a, b*). A crucial question about the mechanism and physiological significance of this activation is whether the site of ion gradient action is the T tubule system or the SR (for reviews see Endo, 1977; Stephenson, 1981*a*; Martonosi, 1984). The implications for the specific mechanisms of excitation-contraction coupling and Ca release are important. If the primary site is the T tubules, the activation follows the physiological pathway, the properties of this stimulated ^{45}Ca release are directly applicable to intact fibers, and T-SR coupling mechanisms can be studied in this preparation.

The inaccessibility and small size of T tubules make this question difficult to resolve directly. The principal support for the SR as the direct target is the responsiveness of fibers split by a procedure thought to leave the T tubules patent on the outer surface, and therefore already depolarized in high $[K^+]$ internal solutions (Endo and Nakajima, 1973; Thorens and Endo, 1975); however, the evidence does not conclusively exclude many sealed T tubules. The possibility that T tubules can seal, maintain Na^+ and K^+ gradients and polarization, and be depolarized to stimulate the SR arose from observations on partially skinned fibers under oil (Costantin and Podolsky, 1967); for example, cardiac glycoside pretreatment inhibited responses to current pulses. Recent studies with completely skinned preparations in aqueous solution support this possibility. Force responses produced by depolarizing ion gradients at constant $[K^+][Cl^-]$ product are inhibited when the impermeant cardiac glycoside ouabain is applied before, but not after, mechanical skinning (Donaldson, 1985). The Na^+/K^+ ionophores monensin and gramicidin D, which would dissipate both Na^+ and K^+ gradients, stimulate force responses and ^{45}Ca release. These are inhibited selectively by the permeant glycoside digitoxin, while caffeine stimulation is unaffected (Volpe and Stephenson, 1986). Depolarizing gradients also stimulate Ca efflux from isolated triads before, but not after, the attachment of terminal cisternae to T tubules is disrupted (Ikemoto et al., 1984). The known SR properties do not account for these observations. Isolated vesicles from T tubules, but not from SR, have Na^+/K^+ pump activity and cardiac glycoside-binding sites (Lau et al., 1979). In intact fibers, electron probe microanalysis shows no elemental Na, K, or Cl gradients between SR and myoplasm (Somlyo et al., 1977). The distribution of permeant tracer ions in skinned fibers gives no evidence of either resting SR polarization or sustained negative polarization concomitant with the stimulation of Ca^{2+} -insensitive ^{45}Ca efflux by depolarizing gradients noted above (Stephenson, E. W. submitted for publication).

The present studies explored two independent lines of evidence for the T tubule locus of depolarization by ion gradients. First, the hypothesis implies that transmis-

sion of a T tubule signal is required; this requirement was evaluated with an intervention to impair such signal transmission. When fibers were highly stretched, which can reduce electrically stimulated Ca release in intact fibers (Frank and Winegrad, 1976; Blinks et al., 1978), ionic stimulation of ^{45}Ca efflux was inhibited selectively. Stimuli thought to act directly on the SR were unaffected, implying impairment of a preceding step. Second, the hypothesis predicts potentiation by an intervention that potentiates T tubule activation. This prediction was evaluated with the chaotropic anion perchlorate, which shifts the voltage dependence of intramembrane charge movements and associated contractile activation in intact or cut fibers (Luttgau et al., 1983). Perchlorate potentiated both Ca^{2+} -dependent and Ca^{2+} -insensitive components of the stimulated ^{45}Ca efflux, but not control efflux.

Preliminary reports of these studies have been made (Stephenson, 1978*b*, 1986; Stephenson and Lerner, 1986*a, b*; Stephenson, 1987).

METHODS

The basic methods of fiber preparation and flux analysis were described previously (Stephenson, 1978*a*, 1981*b*, 1985*a*). In brief, single semitendinosus fibers from the southern grass frog (*Rana berlandieri*) were isolated, and segments were cut, stored, and skinned by microdissection in cold paraffin oil just before use. Mounted segments were loaded for ~40 s in ^{45}Ca buffer (0.375 mM ^{45}Ca EGTA, 0.125 mM EGTA) prepared with high specific activity ^{45}Ca (New England Nuclear, Boston, MA) and rinsed in dilute EGTA solution before collection of measured rinses and washes. Isometric force (recorded on one of 2 chart recorders: Honeywell Elektronik 193; Honeywell Inc., Minneapolis, MN; or Linseis 2021; Linseis Inc., Princeton Junction, NJ) and ^{45}Ca efflux were measured simultaneously; the fiber was transferred through bathing solutions maintained at 19°C in the wells of a spring-mounted thermoregulated chamber. Efflux was followed for ~2 min before and ~1 min after stimulation, when residual ^{45}Ca was extracted in solution with 0.05% Triton X-100; the extraction solution contained either 5 mM EGTA (in the earlier experiments on stretched fibers) or 5 mM CaEGTA, which additionally provided the maximum force for the segment. Fiber ^{45}Ca was obtained by sequentially back-adding the wash contents to residual content, and the release into each wash and total release were expressed as fractions of fiber ^{45}Ca at the time of stimulation; fractional efflux and apparent first-order rate coefficients [k , (^{45}Ca efflux)_{*t*}/(fiber ^{45}Ca content)] were calculated from these. (Fractional efflux approximates k in single washout protocols; k provides direct comparisons with recycling, see Results). Force responses and Ca^{2+} -dependent stimulation of ^{45}Ca efflux were assessed with the addition of 5 mM EGTA a few seconds after the stimulus to minimize ^{45}Ca reaccumulation (interrupted responses). Ca^{2+} -insensitive stimulation was assessed with 5 mM EGTA added 10 or 15 s before the stimulus and thereafter (pretreated responses); control efflux was measured with the same protocol.

All bathing solutions (pH 7.00) contained 120 or 122.5 mM salt (K propionate, K methanesulfonate [Mes], or choline Cl), 10 mM imidazole, 5 mM Na_2ATP , and 1–5 mM MgSO_4 and EGTA as indicated. In the earlier studies on stretched fibers (Figs. 1–4), the ^{45}Ca buffer (in K propionate) contained 1 mM MgSO_4 (~20 μM free [Mg^{2+}]); either (a) KCl replaced K propionate (mole for mole) after MgSO_4 was increased to 3 mM (~110 μM free [Mg^{2+}]) (see Stephenson, 1978*a*), (b) 5 mM caffeine was added, or (c) MgSO_4 was reduced to 0.25 mM (~4 μM free [Mg^{2+}]) (see Stephenson, 1981*b*). In the later studies on stretched fibers and on perchlorate (ClO_4) potentiation (Figs. 5–10), all solutions contained 5 mM MgSO_4 (600–800 μM free [Mg^{2+}]; see Stephenson, 1981*a*; Volpe and Stephenson, 1986) and ionic stimulation was by choline Cl replacement of KMes at 300 mM² [K^+][Cl^-] (see Stephenson, 1985*a*); pro-

pionate was replaced by Mes after ^{45}Ca loading. When 8 mM Tris ClO_4 was added, 15 s before and during stimulation (or control), the maximum Cl was reduced to 112 mM; Tris Mes was used to substitute for Tris ClO_4 and to adjust total salt at intermediate Cl replacements. Reagent grade chemicals were used throughout; low-Ca Na_2ATP , EGTA, and caffeine were obtained from Sigma Chemical Co., St. Louis, MO.

In the earlier studies on stretched fibers (Figs. 1–4), segments were loaded with ^{45}Ca and rinsed at a normal length defined as ~ 105 – 110% slack length, and stimulated either at this length or after the length between ties was increased to twice the estimated slack length (presumably $>4.0\ \mu\text{m}$ sarcomere spacing, but see below and Results). Stretch was applied slowly in 0.1 mM EGTA solution over ~ 90 s, and the same time protocol was used on the unstretched segments; ^{45}Ca loss was monitored during these rinses. In the later studies on stretched fibers (Figs. 5–6) and on ClO_4 , sarcomere spacings (SL) were estimated with a helium-neon laser (model 145; Spectra-Physics, Inc., Mountain View, CA). Segments were loaded and rinsed at a normal length defined as SL $2.3\ \mu\text{m}$, and stimulated at either the same length or after slow stretch (over ~ 95 s) to twice the length at which SL had been $2.2\ \mu\text{m}$, nominally $4.4\ \mu\text{m}$. Laser measurements were not feasible during efflux measurement, but in similar segments this procedure gave $4.0\text{-}\mu\text{m}$ average SL (but see Results); straightening and compliance where the fiber ends lay on the holders and were tied probably accounts for the difference. The nominal length increase was larger because a preliminary (later) study using twice the length at which SL had been $2.0\ \mu\text{m}$ showed no inhibition (data not shown); slack length may have been overestimated in the earlier studies (without laser measurements). Again the same time protocol was used for the unstretched segments and ^{45}Ca loss was monitored during the stretch or control periods. A similar long measured rinse (~ 90 s) was used in the ClO_4 experiments.

Average data shown are the mean \pm SEM for the number of segments indicated in parentheses. The significance of differences between means or paired segments was evaluated with Student's *t* tests; $P < 0.05$ was considered significant.

RESULTS

Selective Inhibition of Cl Stimulation in Stretched Fibers

If depolarizing ion gradients act specifically on the T tubules, this stimulation should be inhibited selectively by interference with T tubule depolarization or the transmission of this signal to the SR. The intervention used to test for inhibition of stimulated ^{45}Ca efflux was a high degree of stretch; in intact stretched fibers, electrically stimulated ^{45}Ca efflux can decrease selectively, with no inhibition of caffeine-stimulated efflux (Frank and Winegrad, 1976), and the aequorin signal be reduced (Blinks et al., 1978).

In the first study of ^{45}Ca efflux from highly stretched and unstretched fibers, the ionic stimulus was Cl replacement of propionate anion; this gradient was the first shown to increase Ca^{2+} -dependent ^{45}Ca efflux as well as force, and the data here at normal length overlap with those in the original study (Stephenson, 1978a). This replacement does not appear to be an appreciable osmotic stimulus, as discussed previously (Stephenson, 1978a, 1985a). The segments were loaded with ^{45}Ca (at 1 Mg:5 ATP) and rinsed at normal length (105–110% estimated slack length); they were stimulated (at 3 Mg:5 ATP) either at the same length or after increasing the length between ties slowly (over ~ 90 s) to twice the initial slack length. The response was interrupted by the addition of 5 mM EGTA near the force peak (or equivalent

time), because maximal ^{45}Ca release is measured when reaccumulation is minimized by chelation (Stephenson, 1978a).

Representative force traces and the simultaneous ^{45}Ca efflux from the same segment are shown in Fig. 1, for unstretched (left) and stretched (right) fibers. Force development was completely inhibited, due not merely to decreased myofilament overlap but to a drastic reduction in stimulated Ca efflux. The mean cumulative ^{45}Ca release during the washout period in a series of such experiments, summarized in Fig. 4, was reduced from $26.8 \pm 3.1\%$ (12) in unstretched segments to $8.2 \pm 0.5\%$

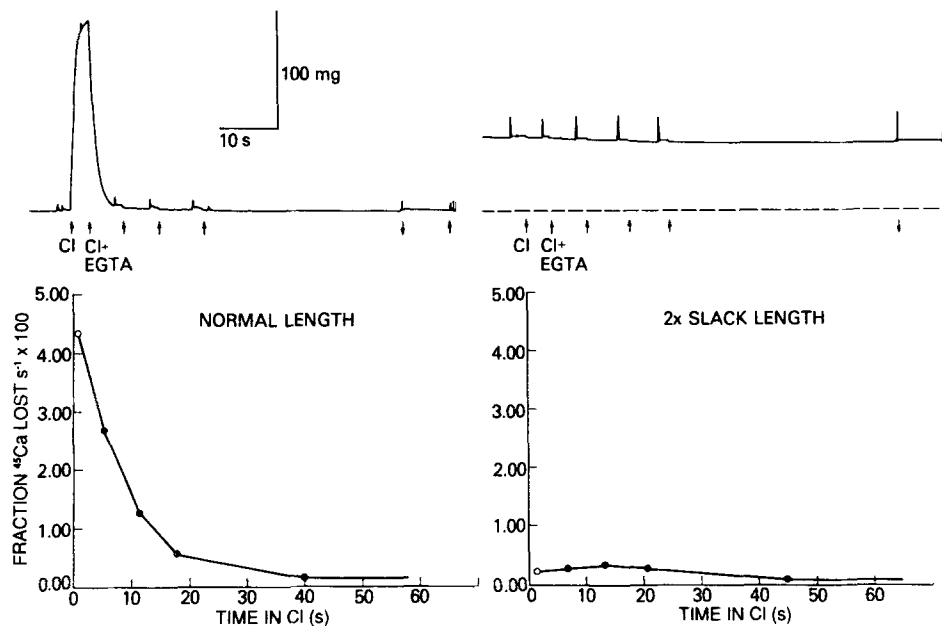


FIGURE 1. Inhibition of the ionic stimulation of ^{45}Ca efflux by KCl replacement of K propionate in stretched fiber segments. The responses were interrupted by EGTA addition shortly after the stimulus. Representative force traces (upper) and simultaneous efflux curves (lower) are shown for a fiber segment at normal length ($\sim 10\%$ > slack length), on the left, and a segment stretched to twice slack length before stimulation, on the right. Fiber segments were stretched slowly after ^{45}Ca loading and rinsing; unstretched segments had the same time protocol. See text for further details.

(10) in stretched segments. Tracer loss in the Cl-stimulated stretched fibers was indistinguishable from that measured in unstimulated (control) fibers at normal length under similar conditions (Stephenson, 1978a), which suggests complete inhibition. This result also implied that long sarcomere length had little or no effect on the passive Ca permeability of the SR.

To evaluate the possibility that SR Ca release per se was inhibited in highly stretched skinned fibers, the identical protocols were applied with each of two stimuli thought to act directly on the SR, caffeine or the sudden reduction of free $[\text{Mg}^{2+}]$. These experiments were carried out during the same period as other studies with these stimuli and the data here at normal length overlap with those published

previously (Stephenson, 1981*b*). Again, the segments were ^{45}Ca loaded and rinsed at normal length, and stimulated either at unchanged length or after slow stretch to twice the initial slack length.

Representative force traces and the simultaneous ^{45}Ca efflux from the same fiber segments are shown in Fig. 2 for unstretched (left) and stretched (right) fibers stimulated by 5 mM caffeine. The normally large rapid force spike was greatly reduced by the decreased myofilament overlap at twice slack length, but it was not completely eliminated, and ^{45}Ca efflux was not reduced. The residual stimulated force

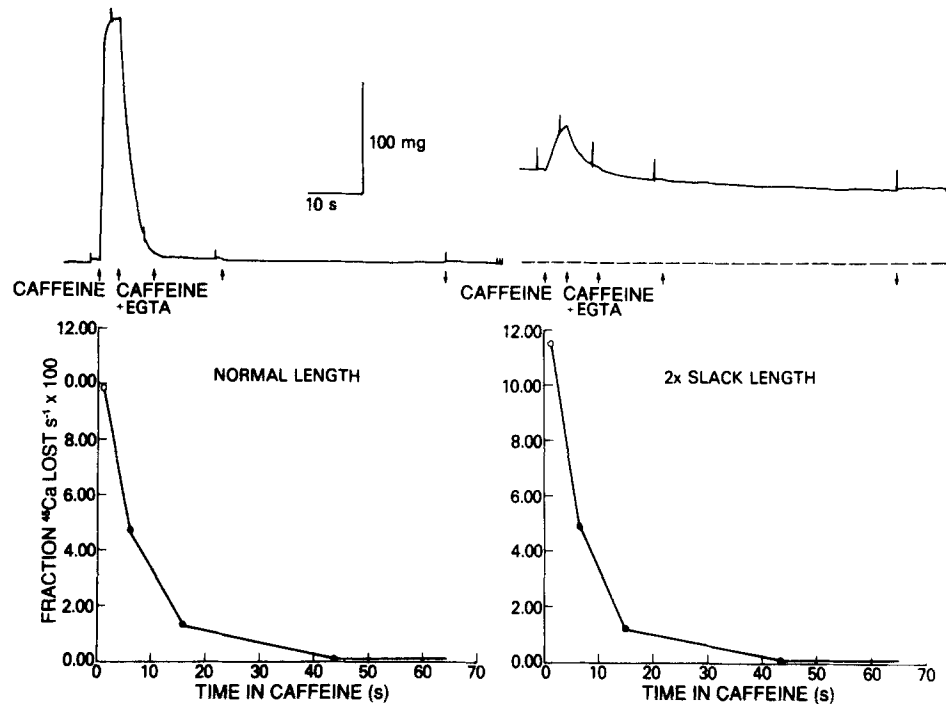


FIGURE 2. Uninhibited stimulation of ^{45}Ca efflux by 5 mM caffeine in stretched fiber segments (from the same study as Fig. 1). Representative force traces (upper) and simultaneous efflux curves (lower) are shown for a fiber segment at normal length (left) and twice slack length (right). Protocol was as in Fig. 1; see text for further details.

development implies that some sarcomeres had not been completely out of overlap (also see below). The mean cumulative ^{45}Ca release during the efflux period in a series of such experiments (summarized in Fig. 4), $68.0 \pm 1.0\%$ (5) in unstretched fibers and $72.8 \pm 1.3\%$ (4) in stretched fibers, was not changed significantly. Thus, caffeine stimulation of skinned fibers, like intact fibers, was not inhibited by the stretch intervention.

Similar results were obtained when the fibers were stimulated by reduction of Mg from 1 mM ($\sim 20 \mu\text{M}$ free $[\text{Mg}^{2+}]$) to 0.25 mM ($\sim 4 \mu\text{M}$ free $[\text{Mg}^{2+}]$) (Stephenson, 1981*b*). Fig. 3 shows representative force traces and simultaneous ^{45}Ca efflux from unstretched (left) and stretched (right) fiber segments. As in the case of caffeine,

force development was greatly reduced but not completely eliminated in the stretched fibers, and ^{45}Ca efflux was not reduced. The mean cumulative ^{45}Ca release in a series of such experiments (summarized in Fig. 4), which was $60.3 \pm 1.8\%$ (10) in unstretched segments and $58.4 \pm 4.7\%$ (6) in stretched segments, was not altered significantly.

The cumulative ^{45}Ca release data summarized in Fig. 4 show that the same length change that strongly inhibited stimulation by Cl replacement of propionate, did not inhibit stimulation of SR Ca release by either caffeine or $[\text{Mg}^{2+}]$ reduction. The

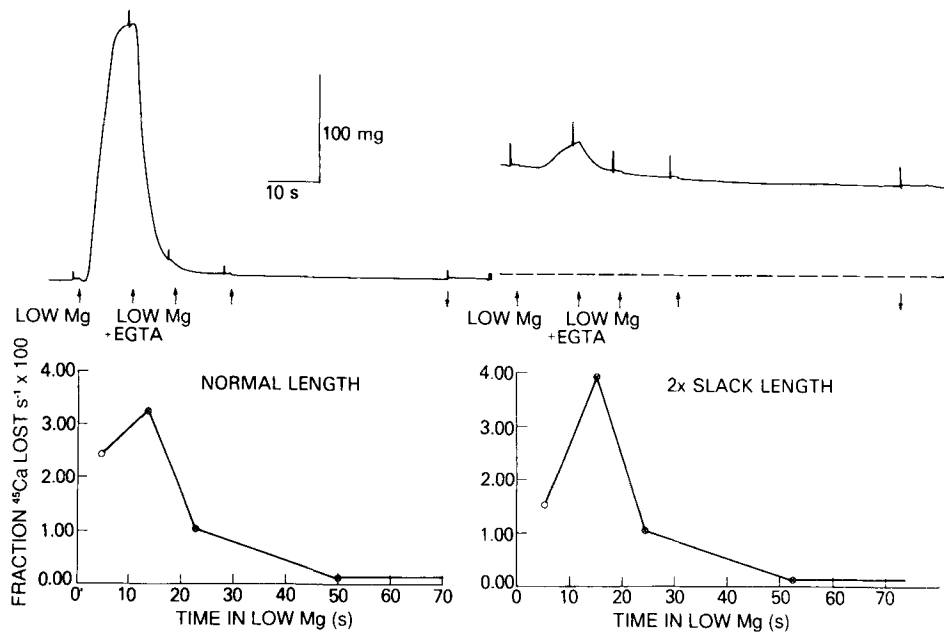


FIGURE 3. Uninhibited stimulation of ^{45}Ca efflux by Mg^{2+} reduction in stretched fiber segments (from the same study as Fig. 1). The stimulus was a sudden reduction in the concentration ratio of $\text{Mg}:\text{ATP}$ from 1:5 to 0.25:5. Protocol was as in Fig. 1; see text for further details.

possibility was considered that this differential inhibition was due to substantial prior loss of SR Ca during the stretch period, with a differential effect on Cl stimulation. However, this was not the case, as seen in Table I, which compares ^{45}Ca loss during the stretch period with that during the parallel time interval in unstretched fibers, pooled for the three stimuli (applied subsequently to the identical prestimulus wash conditions). Loss was slightly larger in the stretched fibers, but they contained only 2.4% less total ^{45}Ca at the time of stimulus application than the unstretched fibers. This small a difference in initial SR Ca content would not account for the strong inhibition of Cl stimulation.

Inhibition of Cl stimulation in stretched fibers, therefore, was highly selective and was not attributable to direct inhibition of the SR Ca release mechanism per se. This selectivity implies that the stretch intervention impaired a step (or steps) preceding

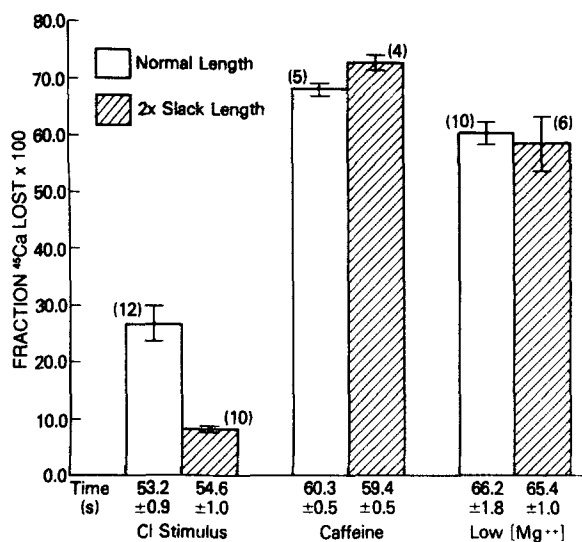


FIGURE 4. Differential inhibition of the ionic (Cl^-) stimulus. Data from experiments of the type illustrated in Figs. 1–3 are summarized as mean cumulative ^{45}Ca loss after stimulation (% initial fiber ^{45}Ca) for unstretched (open bars) and stretched (hatched bars) fiber segments. Cumulative efflux times are given beneath. In this and subsequent figures, results are shown as the mean \pm SEM for the number of determinations in parentheses. In the stretched fiber segments, ^{45}Ca loss with the Cl^- stimulus was reduced to unstimulated control level; there was no significant change in ^{45}Ca loss stimulated by caffeine or Mg^{2+} reduction.

SR Ca release and that Cl stimulation required such steps, which is consistent with the hypothesis that Cl acts to depolarize the T tubules.

Inhibition of Ionic Stimulation in Stretched Fibers under More Rigorous Conditions

Under the conditions of the first study, stimulation would be expected to be transient due to the dissipation of the Cl^- gradient with propionate as the initial impermeant anion, and the continued presence of the permeant K^+ cation. Therefore, the stretch interaction was examined in a second study with stimulation by more sustained depolarizing ion gradients; Cl^- replaced a less permeant initial anion, methanesulfonate (Mes), and initial K^+ was replaced by the much less permeant choline cation, at constant $[\text{K}^+][\text{Cl}^-]$ product (Stephenson, 1985a). In addition, the second study was made at 5 mM Mg :5 mM ATP, which gave a higher free $[\text{Mg}^{2+}]$ in the physiological range.

The protocols were similar to those in the first study, but SL was measured before ^{45}Ca loading (see Methods) to provide more accurate and reproducible estimates of

TABLE I
⁴⁵Ca Loss during Stretch or Control Period before Stimulation

	Stretched fibers (n = 15)	Control fibers (n = 32)	Effect of stretch
Percentage ^{45}Ca lost* \pm SEM	8.30 \pm 0.63	5.86 \pm 0.27	+2.44 [†]
Duration of period (seconds) \pm SEM	95.1 \pm 3.7	92.4 \pm 0.7	2.7
Percentage ^{45}Ca lost*/second \pm SEM	0.09 \pm 0.01	0.06 \pm 0.00	+0.03 [†]

*Percentage of total ^{45}Ca in the segment at the time of stimulus application.

[†]Difference significant at $P < 0.01$.

the normal and long lengths. Also, additional information on the effect of the stretch procedure was obtained from the maximal force developed in a final ^{45}Ca elution solution (with Triton X-100) that contained CaEGTA rather than EGTA (5 mM).

Segments from the same fibers were stimulated by the replacement of 112 mM KMes by choline Cl, either at normal length, SL $2.3\ \mu\text{m}$, or after they had been stretched to twice the length at which SL had been $2.2\ \mu\text{m}$. This stimulus usually produced a large rapid rise at normal length and undetectable force development in the paired stretched segment. However, the stretched segments developed appreciable sustained force in 5 mM CaEGTA in the final elution solution, an indication that many sarcomeres were not out of overlap when the overall length increase nominally corresponded to SL $4.4\ \mu\text{m}$ (but see Methods). The final maximal force developed (above the immediately preceding baseline) in stretched seg-

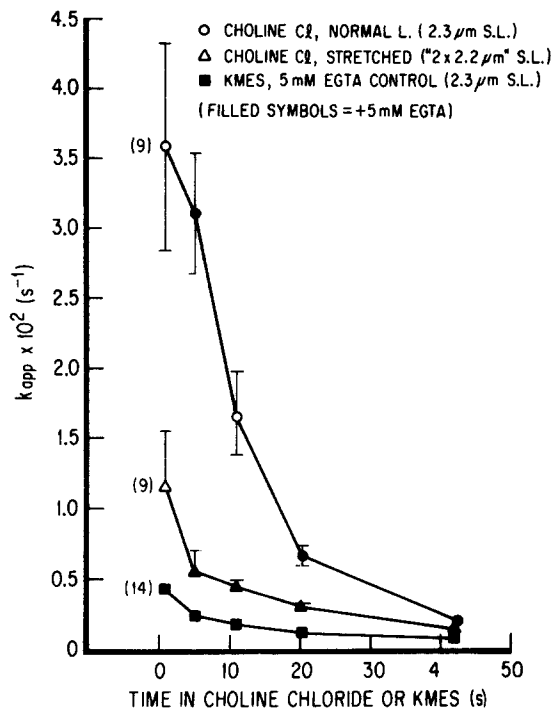


FIGURE 5. Depolarization-stimulated ^{45}Ca efflux in unstretched and stretched fiber segments under more rigorous conditions. The mean apparent first-order rate coefficients $[(^{45}\text{Ca}\ \text{efflux})_i/(\text{fiber } ^{45}\text{Ca}\ \text{content})_i]$ are plotted against time after stimulation. The stimulus was choline Cl (112 mM) replacement of KMes at constant $[\text{K}^+][\text{Cl}^-]$ and 5 mM Mg:5 mM ATP, applied at normal length (SL $2.3\ \mu\text{m}$) (circles) or after slow stretch to twice the length at which SL had been $2.2\ \mu\text{m}$ (triangles); initial SL was estimated with a helium-neon laser. Unstimulated (control) efflux in KMes is shown for comparison. Filled symbols indicate presence of 5 mM EGTA. See text for further details.

ments averaged $22.7 \pm 0.93\%$ (11) of that in paired unstretched segments from the same fiber in these and additional mechanically similar experiments.

The mean ^{45}Ca efflux in a series of such experiments is compared in Fig. 5, where apparent first-order rate coefficients $[(^{45}\text{Ca}\ \text{efflux})_i/(\text{mean } ^{45}\text{Ca}\ \text{content})_i]$ are plotted against time in choline Cl. Stimulation was inhibited strongly in the stretched fiber segments (triangles) compared with those at normal length (circles); however, residual stimulation remained significant during most of the efflux period compared with unstimulated efflux in KMes from fiber segments at normal length under similar conditions (squares). The cumulative ^{45}Ca loss during the efflux

period from responsive fibers under these conditions is summarized by the open bars in Fig. 6. (These data exclude occasional weakly responsive fibers in which cumulative release in the segments at normal length was <15% fiber ^{45}Ca .) Stimulation induced an additional ^{45}Ca release (above control release) of 24.4% fiber tracer at normal sarcomere length but only 6.7% in stretched fiber segments. The decrease in total loss due to the stretch intervention was 59% between mean values and 69% between paired segments from the same fibers; this corresponds to ~72 and 78% inhibition of the stimulation of ^{45}Ca release above control loss. The residual stimulation of release in stretched segments was 22% of stimulation in paired unstretched fiber segments, which is similar to the fraction of final developed force (in CaEGTA) noted above.

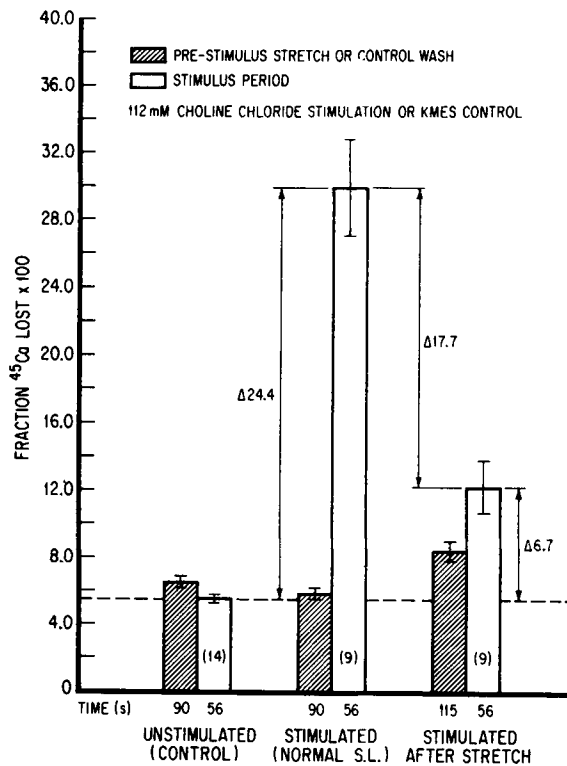


FIGURE 6. Cumulative ^{45}Ca release in unstretched, stretched, and control fibers, from the data shown in Fig. 5. Hatched bars represent cumulative loss during the long dilute EGTA wash preceding stimulation when slow stretch was applied (right bars) or the corresponding control period. Open bars represent cumulative loss during the stimulated efflux period or corresponding control period (left bars), with conditions shown beneath. Cumulative exposure times are indicated beneath each bar. The dashed line is the mean control loss (first open bar). The decrease in stimulated ^{45}Ca release in the stretched fiber segments was highly significant; residual stimulation above control loss in these segments was also significant (see text for related details).

The ^{45}Ca loss during the prestimulus stretch period or the corresponding prestimulus wash period at normal length is shown by the hatched bars in Fig. 6, with the mean wash duration indicated beneath. This ^{45}Ca loss was slightly larger in the stretched fiber segments, as in the earlier series (Table I), but again the difference of only 2.6% fiber tracer would not account for the inhibition. Moreover, in these experiments the difference was attributable to slightly longer wash durations in the stretched fiber segments, since ^{45}Ca efflux ($0.07\% \pm 0.01$ [9] and $0.06\% \pm 0.01$ [9] s^{-1} , respectively) was not increased significantly. This result suggested that a slowly applied length increase usually has little or no effect on SR Ca permeability. In some

additional segments, stretch (or especially reversal of stretch, see below) did seem to be associated with increased ^{45}Ca loss; results in either stretched or unstretched segments were rejected when ^{45}Ca loss during the long prestimulus wash exceeded 10% fiber tracer in order to avoid ancillary effects of abnormally low initial SR ^{45}Ca content or abnormally high permeability.

There was some indication that inhibition of stimulation in stretched fibers was reversible, but this was technically difficult to demonstrate. Seven stretched fiber segments were slowly returned to the initial normal length after stimulation and efflux (but were not extracted in Triton X-100); they were restimulated (without ^{45}Ca reloading) in a second cycle with the same protocol as the initial cycle, terminated by ^{45}Ca extraction. The force produced directly by 5 mM CaEGTA in the extraction solution in such reshortened segments was $76.7 \pm 7.5\%$ (5) of that in paired unstretched segments from the same fiber, i.e., restored substantially (see above) but incompletely. However, the restoration of efflux stimulation in such recycling experiments was compromised by substantial Ca loss before the second stimulus was applied, a complication not present in the intact fiber studies. Only two fiber segments met the criterion of $<10\%$ fiber ^{45}Ca loss during the reshortening period alone, and even these already had lost 29 and 24% tracer (during the preceding stretch and stimulus cycle). Nevertheless, ^{45}Ca release after reshortening (normalized to ^{45}Ca content at the time of stimulation) was 41 and 170% larger than in the preceding stimulation cycle at long length.

The inhibition of stimulation at constant $[\text{K}^+][\text{Cl}^-]$ and higher free $[\text{Mg}^{2+}]$ was in agreement with the earlier selective inhibition of stimulation by KCl replacement of K propionate, and related it more definitively to depolarizing ion gradients as such. Inhibition was less complete, but most or all of the residual stimulation in the stretched fibers could be related to the nonhomogeneous length increase indicated by the residual active force described above (see Discussion).

Potentiation of Submaximal Ionic Stimulation by the Perchlorate Anion

If the depolarizing ion gradients act on the T tubules, stimulation at constant $[\text{K}^+][\text{Cl}^-]$ should be potentiated by the chaotropic anion perchlorate (ClO_4^-). Recent studies have shown that relatively low concentrations of ClO_4^- shift the voltage dependence of mechanical activation and of the intramembrane charge movements that have been associated with the T tubule signal to the SR in parallel (Gomolla et al., 1983; Luttgau et al., 1983).

Submaximal choline Cl substitutions were made in analogy to the submaximal voltage-clamp steps imposed on intact or cut fibers, where 8 mM ClO_4^- produced the largest changes in the steep activation curve at small depolarizations. The force responses of skinned frog fibers to systematically varied choline Cl substitution under fairly similar conditions suggested that 60 mM choline Cl is close to threshold (Moblely, 1979). Therefore, the effect of 8 mM ClO_4^- on stimulation by 60 and 70 mM choline Cl was examined, using the "interrupted" response protocol.

In the absence of ClO_4^- , the force responses to these substitutions were small and variable; the mean ratios of peak force response to maximal force in the same segment (in the terminal extraction solution with 5 mM CaEGTA) were 0.03 ± 0.03 (5) in 60 mM choline Cl and 0.10 ± 0.06 (6) in 70 mM choline Cl. However, stimulation

of ^{45}Ca release above control was significant, although the increase produced by 60 mM choline Cl was barely detectable. The open bars in Fig. 7 summarize the cumulative ^{45}Ca loss in 60 and 70 mM choline Cl, and in 120 mM choline Cl, the maximal substitution; the dashed line indicates the mean control loss under similar conditions (see Fig. 9). The hatched bars show the cumulative ^{45}Ca loss with submaximal choline Cl in the presence of 8 mM ClO_4^- . In 60 mM choline Cl, the effect of ClO_4^- was small, variable, and not significant. In 70 mM choline Cl, ClO_4^- increased ^{45}Ca loss significantly, potentiating the small stimulated release above control loss more than twofold. Expressed relative to the maximal stimulated ^{45}Ca release above control with full choline Cl replacement (120 mM), submaximal stimulation by 70 mM choline Cl was increased from ~13 to ~32%.

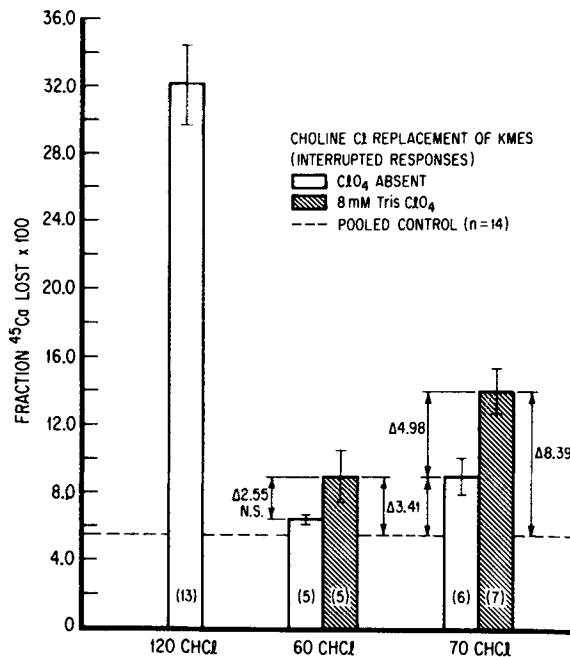


FIGURE 7. Potentiation by ClO_4^- of stimulation by submaximal depolarizing ion gradients of Ca^{2+} -dependent ^{45}Ca release (interrupted responses). Open bars, mean ^{45}Ca release (in the absence of ClO_4^-) stimulated by 60 or 70 mM choline Cl replacement, or 120 mM choline Cl (shown for comparison). Unstimulated (control) release is shown by the dashed line (see Fig. 9). Stimulation above control was significant for both partial replacements. Hatched bars, mean ^{45}Ca release in the presence of 8 mM ClO_4^- , which produced a significant increment with 70 mM choline Cl replacement.

The time course of ^{45}Ca efflux in fibers stimulated by 70 mM choline Cl in the absence (square symbols) or presence (triangles) of 8 mM ClO_4^- is shown in Fig. 8, and is compared with efflux during maximal stimulation (120 mM choline Cl, circles). The submaximal stimulation was characterized by low initial efflux to the bath before the addition of EGTA, as would be expected with effective binding of the relatively small amount of released Ca in the myoplasm, by troponin (reflected in force development) and other Ca sinks. The subsequent efflux must represent mainly the outward diffusion, as $^{45}\text{CaEGTA}$, of ^{45}Ca released during the first few seconds, because in the presence of 5 mM EGTA even a large stimulus produced only very small increases in efflux under the present conditions (see below). The initial submaximal stimulation and time course of subsequent chelation and outward diffusion were variable, so that the mean increase in efflux with ClO_4^- during the

first high EGTA wash was not significant, although it was the largest; the absolute increase in flux was significant only during the next EGTA wash. The consistently higher mean values, however, resulted in the significant increase in cumulative ^{45}Ca loss (Fig. 7).

The observation that ClO_4^- did not significantly affect the marginal stimulation by 60 mM choline Cl (Fig. 7) suggested that it did not potentiate unstimulated Ca efflux during this type of protocol. Similarly, ^{45}Ca loss in the 15 s dilute EGTA wash (0.01 mM) preceding submaximal stimulation was not altered by the added ClO_4^- . No effect on unstimulated efflux was detectable in the control measurements in 5 mM EGTA (see Fig. 9). Therefore, ClO_4^- did not appear to act directly on SR Ca efflux.

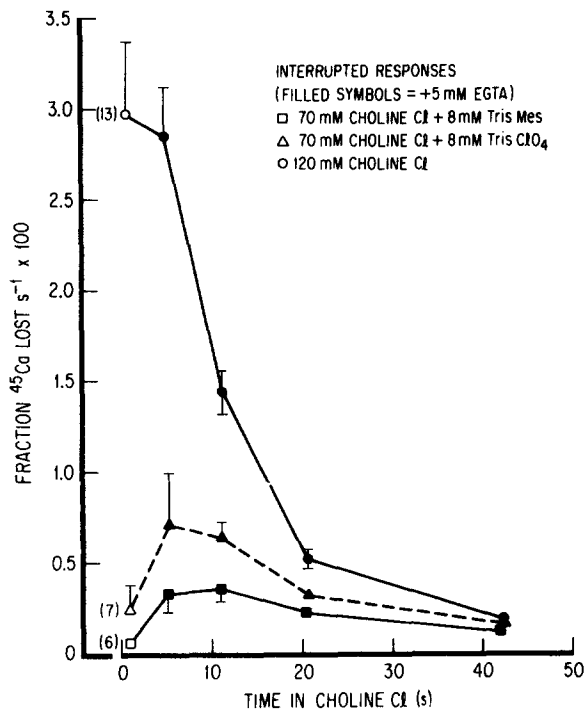


FIGURE 8. Mean time course of Ca^{2+} -dependent ^{45}Ca efflux stimulated by submaximal (70 mM) choline Cl replacement in the absence (squares) or presence (triangles) of 8 mM ClO_4^- (the data are summarized in Fig. 7). Efflux stimulated by maximal (120 mM) choline Cl replacement (circles) is shown for comparison. Filled symbols indicate addition of 5 mM EGTA (interrupted responses).

These results indicated that ClO_4^- potentiated the submaximal stimulation of Ca^{2+} -dependent ^{45}Ca efflux by depolarizing ion gradients, which is consistent with its T tubule-mediated effects on intact fibers, and supported the hypothesis that the gradients act on the T tubules.

ClO_4^- Potentiation of Ca^{2+} -insensitive Ionic Stimulation

Depolarizing ion gradients at constant $[\text{K}^+][\text{Cl}^-]$ product stimulate ^{45}Ca efflux with a small sustained Ca^{2+} -insensitive component that appears to grade the much larger Ca^{2+} -dependent component, and may reflect intermediate coupling steps (Stephenson, 1985a). Potentiation of the Ca^{2+} -insensitive component by ClO_4^- would be con-

sistent with such a role and would further support a T tubule locus of depolarization.

This possibility was examined under the same conditions as the submaximal Ca^{2+} -dependent ^{45}Ca release above (interrupted responses), with 5 mM Mg:5 mM ATP throughout, but with 5 mM EGTA added 10 s before the stimulus and maintained throughout efflux. The stimulus was the replacement of KMes by 112 mM choline Cl (a nearly maximal gradient); the small responses obtained (see below) precluded

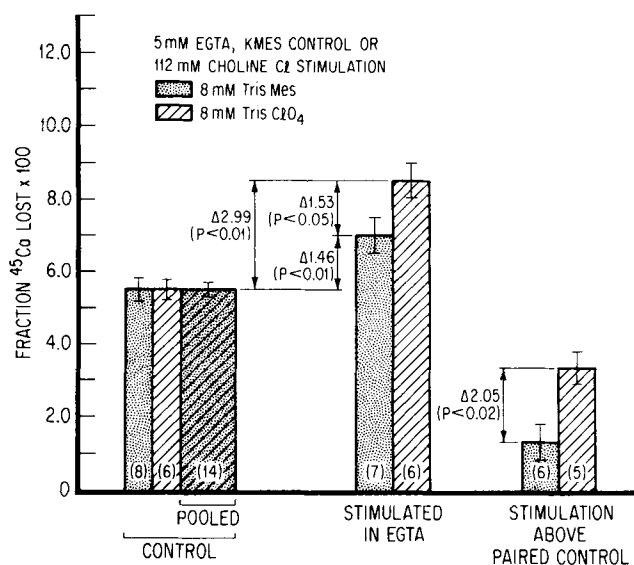


FIGURE 9. Potentiation by ClO_4^- of stimulation of Ca^{2+} -insensitive ^{45}Ca release. The stimulus was nearly maximal choline Cl replacement (112 mM) in the presence of 5 mM EGTA (applied 10 s before the stimulus and maintained throughout). Left bars, unstimulated (control) cumulative loss in KMes with identical protocol; ^{45}Ca loss was the same in the absence (stippled bar) or presence (hatched bar) of 8 mM ClO_4^- , so the data were pooled. Middle bars, cumulative ^{45}Ca release under the stimulatory conditions given above. Stimulation above control loss and the increase produced by ClO_4^- , both were significant. Right bars, stimulation of ^{45}Ca release above control loss in paired segments from the same fiber. Both stimulation and potentiation were significant.

the use of submaximal gradients. In control (unstimulated) fiber segments, identical protocols were used without the replacement of KMes.

The results of this study are summarized as cumulative ^{45}Ca release in Fig. 9. In the absence of stimulation (control fiber segments), shown by the first set of bars, fractional ^{45}Ca loss was the same in the absence (stippled bar) or presence (hatched bar) of 8 mM ClO_4^- , even when paired segments from the same fibers were compared (not shown); these data were pooled for comparison with mean losses from stimulated fiber segments. In the absence of ClO_4^- , choline Cl replacement in the presence of 5 mM EGTA increased ^{45}Ca loss (second stippled bar) significantly above control, but stimulation was very small; the increase above control loss under

the present conditions was considerably less than in the earlier study (Stephenson, 1985a), which was possibly related to the higher $[Mg^{2+}]$ (see Discussion). This small stimulation was potentiated significantly by ClO_4^- (second hatched bar); comparison of both the mean data and paired differences from control segments from the same fiber (last two bars) indicated that stimulation above control was potentiated about twofold.

The mean time course of the small Ca^{2+} -insensitive ^{45}Ca efflux from these fibers is shown in Fig. 10. Mean efflux was consistently higher in stimulated fibers (circles, squares) than in unstimulated fibers (triangles, solid curve), but the initial wash was

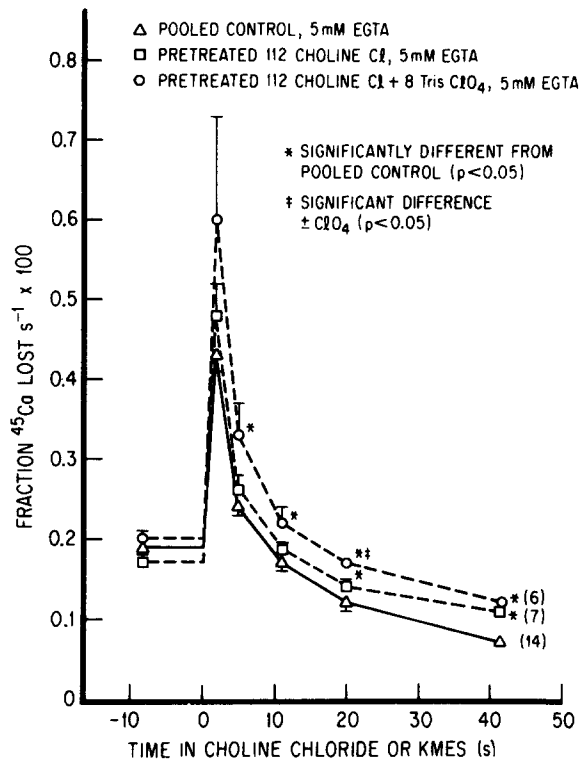


FIGURE 10. The mean time course of Ca^{2+} -insensitive ^{45}Ca efflux under stimulatory conditions in the presence or absence of 8 mM ClO_4^- ; control efflux is shown for comparison. These are the data summarized in Fig. 9 (stimulus, 112 mM choline Cl, 5 mM EGTA, 5 mM MgATP). Asterisks denote significant differences from control, and the dagger indicates significant difference between the presence and absence of ClO_4^- . The mean differences in these small fluxes, not always significant, are sustained and result in the significant differences in cumulative ^{45}Ca release shown in Fig. 9.

variable and the later small sustained difference was not always statistically significant in the absence of ClO_4^- . Similarly, the increase in mean stimulated efflux produced by ClO_4^- was consistent but usually not significant. The sustained nature of these small differences resulted in the significant increments in cumulative ^{45}Ca loss shown in Fig. 9.

Although the absolute increments were small under present conditions, these results clearly showed that ClO_4^- potentiated the stimulated Ca^{2+} -insensitive ^{45}Ca loss without increasing unstimulated loss. The selective potentiation was consistent with ClO_4^- action on T tubule processes and activation in electrically depolarized intact or cut fibers.

DISCUSSION

Several lines of indirect evidence have supported the hypothesis that sealed T tubules are the immediate target site for activation by depolarizing gradients. The present studies provide new and independent results, which are consistent with two predictions of a T tubule locus compared with direct SR activation. Thus a substantial body of circumstantial evidence now supports T tubule mediation of this stimulus.

Inhibition of Ionic Stimulation in Highly Stretched Fibers

The implied requirement for junctional transmission of a T tubule signal was examined in highly stretched fibers. This intervention to impair T tubule-mediated stimulation was suggested by observations in intact fibers. Frank and Winegrad (1976) found that ^{45}Ca efflux from tetanically stimulated muscle is reduced by 70% after stretch to 160% in vivo length, and that this inhibition is selective because caffeine-stimulated or unstimulated efflux is unaltered. Blinks et al. (1978) also found that the aequorin signal during twitches and tetani decreases progressively at longer sarcomere lengths, as much as 70% at 3.6 μm striation spacing, although the $[\text{Ca}^{2+}]$ change would be smaller. Inhibition was reversible in both cases and was assumed to result from the disturbance of T-SR transmission or the normal activation of Ca release, but the exact mechanism was not pursued. It could relate to impairment of T tubule depolarization or of junctional transmission per se, with minor configurational changes or a physical separation more analogous to the disruption of isolated triads (Ikemoto et al., 1984).

In the present studies the stretch intervention was also applied empirically, and its ability to inhibit steps preceding SR Ca release is inferred from the selectivity of inhibition. Caffeine and $[\text{Mg}^{2+}]$ reduction are thought to act directly on the SR (Stephenson, 1981*b* and references); consistent with this interpretation, intramembrane charge movement in cut or intact fibers is unaltered by caffeine (Kovacs and Szucs, 1983; Huang, 1986), caffeine stimulates at least as much Ca release from purified SR terminal cisternae as from intact triads (Palade, 1987), and caffeine increases the frequency and duration of single channel events without changing the unit conductance of the isolated SR Ca^{2+} -activated Ca release channel (Meissner et al., 1987). Stimulation of ^{45}Ca release by these agents is unaffected by protocols identical to those that inhibit Cl^- stimulation completely (Fig. 4). The selectivity is not due to a differential effect of increased Ca loss during the stretch procedure, because this increase is normally very small (Table I and Fig. 6). Striation spacing and directly activated force were not measured in the first study, but the force developed by stretched fibers stimulated by caffeine or Mg^{2+} reduction (Figs. 2 and 3) indicates that not all sarcomeres were out of overlap and suggests that SL was not increased uniformly, as in intact fibers (see Blinks et al., 1978). The more complete inhibition of Cl^- stimulation in the first study than of choline Cl stimulation (Figs. 1 and 4 compared with Figs. 5 and 6) may be related to greater stretch and/or more transient and weaker ionic gradients with initial propionate anion and constant $[\text{K}^+]$ (see Stephenson, 1985*a*); any osmotic component of this stimulus would be expected to reduce, not increase, the overall effect of the selective inhibition.

Under the more rigorous conditions of the second study, inhibition of stimulation of ^{45}Ca efflux by ionic gradients at constant $[\text{K}^+][\text{Cl}^-]$ and higher $[\text{Mg}^{2+}]$ is large but incomplete, similar to that in stretched intact fibers (see above). The residual stimulated ^{45}Ca release is very similar to the residual maximal force produced directly by high $[\text{Ca}^{2+}]$ (after efflux measurement), which indicated that many sarcomeres had not been out of overlap. The residual stimulation could derive entirely from these inadequately stretched sarcomeres, but a relationship between sarcomere length and impairment cannot be inferred from the present studies; nonuniformity could be different from that in intact fibers, and the distribution of stresses, as well as other factors leading to impairment, is not known. A fixed relationship is unlikely because optically monitored Ca release is surprisingly large in voltage-clamped cut fibers that have been highly stretched to minimize movement artifacts (e.g., Melzer et al., 1986), compared with the inhibition of release in both intact and skinned fibers. For present purposes, these questions are ancillary. The primary result is that ionic stimulation under rigorous conditions, like electrically stimulated Ca efflux in intact fibers, is strongly inhibited. In conjunction with the selectivity of inhibition, this result implies that a step or steps preceding the SR Ca release channel is required and is consistent with the hypothesis that a T tubule signal is transmitted.

Potentiation of Ionic Stimulation by T Tubule Potentiation

The effect of ClO_4^- on depolarizing stimulation was examined because this anion selectively potentiates contractile activation while shifting the voltage dependence of the putative T tubule voltage sensor, with either extracellular or intracellular application (Gomolla et al., 1983; Luttgau et al., 1983; Csernoch et al., 1987). ClO_4^- specifically shifts the threshold and voltage dependence of the delayed charge movement component most closely associated with Ca release (Huang, 1986), and potentiation can be accounted for by its action on charge movement without significant direct effects on subsequent SR Ca release (Csernoch et al., 1987). Chaotropic anions are well known to potentiate the twitch, and at higher concentrations to alter the electrical properties of the sarcolemma (see Dani et al., 1983). ClO_4^- acts on excitation-contraction coupling much more specifically, with large effects at <10 mM, but the precise mechanism of alterations in intramembrane charge movement and coupling is not known.

The twofold potentiation of submaximal stimulation of ^{45}Ca release by partial choline Cl substitution (Figs. 7 and 8) is qualitatively analogous to the potentiation of stimulation by submaximal voltage steps in intact or cut fibers (Gomolla et al., 1983; Luttgau et al., 1983; Csernoch et al., 1987). By itself, 8 mM ClO_4^- has no significant effect on ^{45}Ca efflux. This result is consistent with the hypothesis that the imposed ion gradients are acting similarly to impose a voltage step on the T tubules.

Stimulation in the presence of EGTA, the small Ca^{2+} -insensitive stimulation, clearly is potentiated as well (Figs. 9 and 10), which is consistent with the idea that it reflects an intermediate step in excitation-contraction coupling that controls Ca^{2+} -dependent ^{45}Ca release (Stephenson, 1985*a, b*). This component is significant but extremely small in the present studies; stimulated ^{45}Ca loss above control is only $\sim 30\%$ of that measured previously (Stephenson, 1985*a, b*). The present conditions

differ in two respects, a small reduction in maximum choline Cl replacement (from 120 to 112 mM, to allow for Tris ClO_4^- or control Tris Mes additions) and an increased Mg:ATP ratio throughout of 5:5, compared with previous initial ratios of 1:5 that were increased to 3:5 before stimulation. The changes in free $[\text{Mg}^{2+}]$ are more likely bases for the reduced Ca^{2+} -insensitive ^{45}Ca release; this point is particularly interesting because Ca^{2+} -dependent stimulation is not reduced (Figs. 5–8), and requires direct investigation. In any case, the very small stimulation with nearly maximal choline Cl substitution precluded a direct comparison of potentiation of Ca^{2+} -dependent and Ca^{2+} -insensitive components with the submaximal ionic stimulus. The potentiation factor for Ca^{2+} -insensitive stimulation at nearly maximal substitution is not appreciably different from that for the submaximal Ca^{2+} -dependent stimulation. The significance of this apparent similarity remains to be determined.

The extremely small Ca^{2+} -insensitive stimulation also suggests that Ca^{2+} dependence at physiological free $[\text{Mg}^{2+}]$ is even larger than the eightfold factor at lower free $[\text{Mg}^{2+}]$ (Stephenson, 1985a). An important implication of T tubule-mediated potentiation of both components of this stimulated ^{45}Ca release is that the physiological pathway between T tubule depolarization and effective SR Ca release is similarly Ca^{2+} dependent.

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