Influence of Magnesium on Chloride-Induced Calcium Release in Skinned Muscle Fibers

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ABSTRACT Chloride-induced Ca release in skinned muscle fibers was studied by measuring isometric force transients and ^{45}Ca loss from fiber to washout solutions. Skinned fibers prepared from muscles soaked in normal Ringer solution made large force transients in 120 mM Cl solution with 5 mM ATP and 1 mM Mg, but 3 mM Mg was inhibitory. Mg inhibition was antagonized by low temperature and by Cd, agents which slow active Ca uptake by the sarcoplasmic reticulum (SR). In low Mg^{++}, Cl stimulated rapid ^{45}Ca release from the SR in sufficient amounts to account for the force response. The increased ^{45}Ca release was inhibited by EGTA, suggesting that release requires free Ca under these conditions. The ^{45}Ca initially released was partially reaccumulated later. Reaccumulation was increased in higher Mg^{++}. These results provide additional evidence that the Ca uptake rate is an important determinant of net release, and suggest that Mg^{++} acts primarily on this mechanism. Skinned fibers prepared from muscles soaked in low Cl solutions could give force responses to Cl solutions with 3 mM and 6 mM Mg. This observation suggests that the Cl stimulus varies with the [Cl] gradient across the internal membranes, and supports the hypothesis that applied Cl causes membrane depolarization.

INTRODUCTION

Intact muscle fibers contract when the sarcolemma is depolarized by solutions with high potassium or low chloride concentrations (Hodgkin and Horowicz, 1959; Foulks et al., 1965). Skinned muscle fibers can contract transiently when exposed to solutions with high chloride concentrations (Costantin and Podolsky, 1967; Ford and Podolsky, 1970; Nakajima and Endo, 1973). High chloride in the myofilament space (MFS) of skinned fibers has been presumed to increase Ca efflux from the sarcoplasmic reticulum (SR) by depolarization of internal membranes (Costantin and Podolsky, 1967). Direct evidence of depolarization is lacking, but recent aequorin measurements confirm that the MFS Ca ion concentration rises under these conditions (Endo and Blinks, 1973).

Chloride responses in skinned fibers may correspond to a step in the physiological activation pathway. In addition, they provide a useful system for analysis of the net Ca movement that controls the contractile response. This net flux includes both the stimulated efflux and Ca influx back into the SR. The SR reaccumulates Ca from the MFS in intact fibers (Winegrad, 1968) and in skinned

fibers (Ford and Podolsky, 1972*b*). In vesicles of SR membranes, the active transport system for Ca influx is stimulated by Mg^{++} in a complex way (see Yamada and Tonomura, 1972). The effect of Mg^{++} on Cl responses is thus of special interest. The Mg^{++}-dependent influx rate can have a large influence on net Ca movement in skinned fibers, and might account for Mg^{++} inhibition of the net Ca release induced by Ca^{++} (Ford and Podolsky, 1972*b;* Stephenson and Podolsky, 1977). The interpretation of Cl responses is more direct than with a Ca stimulus, because applied Cl diffuses uniformly into the fiber, while the diffusion of applied Ca can be influenced by the Ca transport system of the SR. Furthermore, the large source of Ca in the bathing solution is avoided.

In the present study of Cl responses, Ca movement was followed by measurement of isometric force transients and ^{45}Ca release. The results provide further evidence regarding the role of Ca reaccumulation and Mg^{++} in net release, and also further characterize the Cl stimulus. Preliminary reports of the results have appeared (Stephenson and Podolsky, 1974; Stephenson, 1975).

METHODS

Fiber Preparation

Single intact fibers from frog semitendinosus muscle were isolated and skinned in paraffin oil as described in the preceding paper (Stephenson and Podolsky, 1977) except that the whole muscles were suspended in cold Ringer solution of varied composition. No other solutions were applied before skinning and mounting the fiber segments. Normal Ringer solution contained (mM) NaCl 115.5, KCl 2.5, $CaCl_2$ 1.8, NaH_2PO_4 + Na_2HPO_4 3.1, and *d*-tubocurarine 9 mg/liter, with a total Cl concentration 121.6. In low-Cl Ringer solutions NaCl was reduced to 5.8 mM or omitted, leaving 11.9 or 6.1 mM total Cl; osmolarity was maintained by sucrose (207 or 217 mM) or, in a few cases, by equimolar Na propionate. Muscles were kept in the low-Cl solutions for at least 1 h before removal of a fiber bundle, to insure equilibration.

Fiber Mounting and Experimental Chamber

For nonradioactive experiments, the skinned fiber segment was mounted between clamps, one attached to a leaf-spring transducer for measurement of isometric force, as described in the preceding paper (Stephenson and Podolsky, 1977). For radioactive experiments, preliminary tests showed that the amount of tracer held and released by the clamps (the tips of jeweller's forceps) was unsatisfactorily large with respect to the tracer in the small piece of tissue. A new mounting method was developed in which the fiber segment was tied with monofilament silk (about 20 μm diam) to fine rods made from 14 mil stainless steel wire (Fig. 1). One rod was attached to a transducer of the same design for measurement of isometric force. The mounted fiber was exposed to experimental solutions in the wells of the spring-mounted thermoregulated chamber described in the preceding paper.

Bathing Solutions

All bathing solutions for skinned fibers contained 120 mM K propionate or KCl, 10 mM imidazole, and 5 mM Na_2ATP. The Mg concentration was set at 1, 3, or 6 mM with $MgCl_2$; free Mg^{++} was about 20 μM, 110 μM, or 1.3 mM (Stephenson and Podolsky, 1977). The Mg of the final propionate rinse before exposure to Cl was matched to that of the Cl solution; in some experiments, the extra Mg was made up with $MgSO_4$, keeping

the total Cl constant at 2 mM. No differences were observed. Additional constituents are described in the preceding paper and in the text. Triton-X100 and high-purity, low-Ca Na_2ATP were obtained from Sigma Chemical Co., St. Louis, Mo. High specific activity $^{45}CaCl_2$ was obtained from New England Nuclear, Boston, Mass. The specific activity of this stock was reduced 10-fold with $CaCl_2$ to reduce any error due to the supplier's assay of total Ca. The labeled buffered Ca solution prepared from this contained CaEGTA 0.375 mM, total EGTA 0.5 mM, pCa 6.2, and other constituents as described above, and had a final activity of about 14–26 μCi/ml.

Tracer Experiments

For direct measurements of Ca release, skinned fiber segments were loaded with ^{45}Ca in $^{45}CaEGTA$ buffer solution, rinsed three times in dilute EGTA solutions (0.1 and 0.01

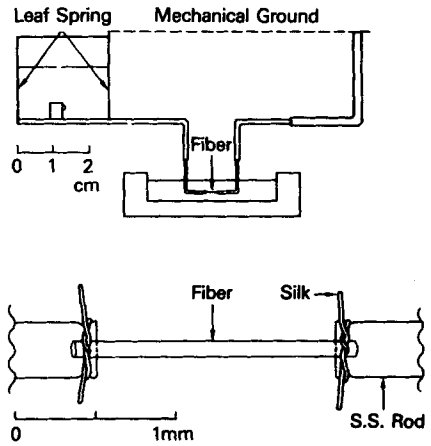

FIGURE 1. Modified mounting of skinned fibers for ^{45}Ca experiments. The upper diagram shows the fiber attached to fine stainless steel rods, which connect with a leaf-spring force transducer. The lower diagram is an enlarged view of the fiber tied to the rods with monofilament silk.

mM), and then exposed sequentially to the following measured washout solutions: (*a*) stimulating or control solution; (*b*) 1 mM EGTA solution to trap MFS ^{45}Ca (Ford and Podolsky, 1972*a*); and (*c*) 0.05% Triton-X100 + 5 mM EGTA solution to extract remaining ^{45}Ca from the fiber segment. The sum of the tracer collected in each of these washout solutions gives the total ^{45}Ca in the segment before washout, and the tracer lost into each washout solution can be expressed as a fraction of the total. This method requires that the tracer in the washout solutions originate in the tissue itself (contamination is small), and final tracer extraction from the tissue be complete. Extraction was monitored in 14 segments by counting the extracted fiber segment after NaOH digestion; the mean activity remaining after Triton-EGTA extraction (6–7 min) was 0.3 ± 0.4% (SEM) of the total activity of the segment. The contribution of contaminant tracer carried over from the loading solution as adherent fluid or adsorbed on the apparatus was evaluated in several ways. With the modified mounting method and rinsing procedures, the disintegrations per minute per milliliter of the final EGTA rinse were less than 10^{-6} that of the loading solution and were 2.4 ± 0.05% of the total disintegrations per minute in the fiber segments (n = 13), so that the small fraction of a milliliter adhering to muscle and apparatus could contribute negligible activity to the subsequent experimental washout

solutions. Tracer adsorbed on the apparatus was a small source of contamination. In five blank runs, without a fiber segment, the average total activity washed from the mounting rods and monofilament silk ties was 3.3% of the average total activity obtained from 13 mounted muscle segments. About half the adsorbed tracer appeared in the first two washout solutions, and the remainder in the Triton-5 mM EGTA extraction solution. Mounted muscle segments which were carried through the washout procedure without any stimulus for Ca release formed a complete control for the stimulated muscles (see Fig. 6). The tracer collected from control muscle segments includes the apparatus contamination and adherent fluid of the segment as well as the control efflux of sequestered tissue Ca. The adherent fluid has a negligible effect (see above); the tracer desorbed from the apparatus contributes appreciably to the small tracer washout from control tissue, but is a small fraction of the larger tracer loss from stimulated tissue.

Aliquots of the washout solutions (1 ml of an original volume of 1.2 ml), and diluted aliquots of loading solution, were assayed by liquid scintillation counting (Tri-Carb Automatic Liquid Scintillation Spectrometer System, Model 3375 or 3330, Packard Instrument Co., Downers Grove, Ill.) using 10 ml Aquasol scintillation solution (New England Nuclear).

RESULTS

Inhibition of Cl-Induced Force Spikes by Mg

Skinned fibers from muscles which had been dissected in normal Ringer solution responded to application of 120 mM Cl at 20°C with large force transients, provided the Mg level of the bathing medium was low. A typical response in 1 mM Mg solution is shown in Fig. 2*a*; after the fiber had been loaded by exposure to CaEGTA buffer solution (see Methods) and rinsed in dilute EGTA solutions, all containing 1 mM Mg, the peak force of the transient induced by Cl was approximately the maximum force for that fiber segment. Maximum force was determined by subsequent exposure to high exogenous Ca (seen at the right of the record) in order to normalize the transient responses reflecting Ca release. The Cl response was inhibited strongly by moderate Mg levels; Fig. 2*b* shows that a segment from the same muscle fiber developed little or no force when exposed to Cl in the presence of 3 mM Mg, while the rate and amount of force development when exposed to exogenous Ca seemed unimpaired. Similar results on a number of fibers stimulated in 3 or 6 mM Mg are included in Fig. 10. In these fibers, the ratio of [Cl] in the solution stimulating the skinned fibers to [Cl] in the normal Ringer solution which had bathed the intact fiber was about 1. The lumen of at least part of the internal membrane system was equilibrated with high extracellular [Cl], and the transmembrane [Cl] gradient during the stimulus presumably would be relatively small.

Mechanism of Mg Inhibition

Mg could reduce net Ca release by inhibition of the release mechanism itself or by stimulation of active Ca reaccumulation by the SR. In order to differentiate between these possibilities, the effect of conditions that should inhibit active Ca transport was studied. If increased Mg blocks the release mechanism, its effect should be similar in the presence of high or low rates of Ca reaccumulation. If increased Mg acts by promoting active Ca reaccumulation, its effect should be antagonized by inhibition of this transport.

Since active Ca transport by vesicles of SR membranes is known to have a large activation energy (see Inesi, 1972), the temperature dependence of Mg inhibition was examined. Fig. 3 illustrates the effect of low temperature on the Cl responses of a fiber with a [Cl] ratio of 1, as defined above. In traces (*a*) and (*b*), at 2–5°C, segments containing only their endogenous Ca gave large responses to Cl solutions containing 3 or 6 mM Mg. In traces (*c*) and (*d*), at 18–20°C, segments

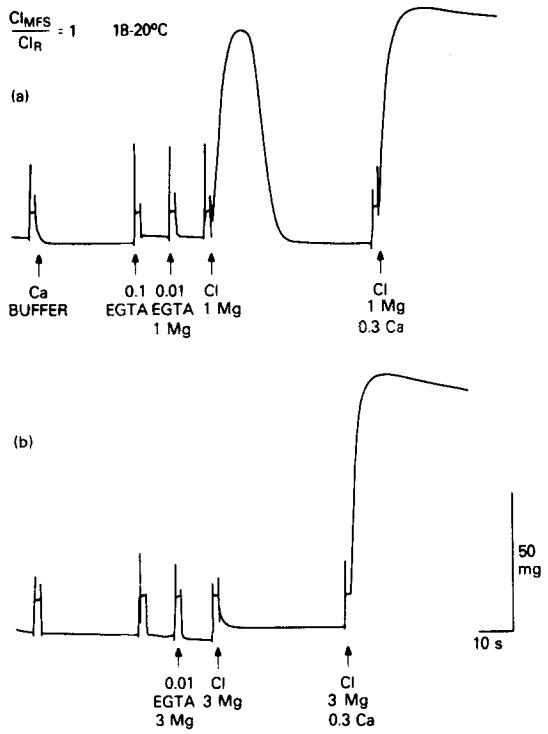

FIGURE 2. Cl-induced force responses at about 20°C in a fiber from a muscle bathed in normal Ringer solution. Ca-loaded segments from the same fiber were stimulated by replacing 120 mM propionate with Cl, in solution containing (*a*) 1 mM Mg, (*b*) 3 mM Mg. (Ca buffer and 0.1 mM EGTA solutions routinely contained 1 mM Mg.) Subsequent exposure to 0.3 mM Ca gives the maximum force of the segment.

from the same fiber, loaded with Ca buffer, produced no detectable responses to the same solutions. (A similar effect of low temperature was observed in fibers with large [Cl] ratios which were unresponsive in higher Mg [see below].) These results show first that Mg does not prevent the force response by direct action on the myofilaments, and second, that Mg does not block the release mechanism itself. The unmasked response at 5°C suggests that at 18–20°C Mg acts mainly, perhaps entirely, by stimulating active Ca reaccumulation.

At 18–20°C, the effect of Cd on Mg inhibition was examined. The Ca transport system of isolated SR membranes is strongly inhibited by Cd (Carvalho, 1968; MacLennan, 1970), and the effects of Cd described in the preceding paper are

consistent with this action. The antagonistic effect of Cd is illustrated in Fig. 4*a*. While fibers with a [Cl] ratio of 1 give negligible responses in 3 mM Mg (Fig. 2), immediate force development occurred in Cl with 3 mM Mg + 0.5 mM Cd, although the rise rate was slower than that of a typical Cl response. When a control segment from the same fiber was treated identically except for application of the Cl stimulus, as shown in Fig. 4*b*, force developed only after a substantial delay. (The possible basis of eventual force development in Cd solutions is discussed in the preceding paper [Stephenson and Podolsky, 1977].)

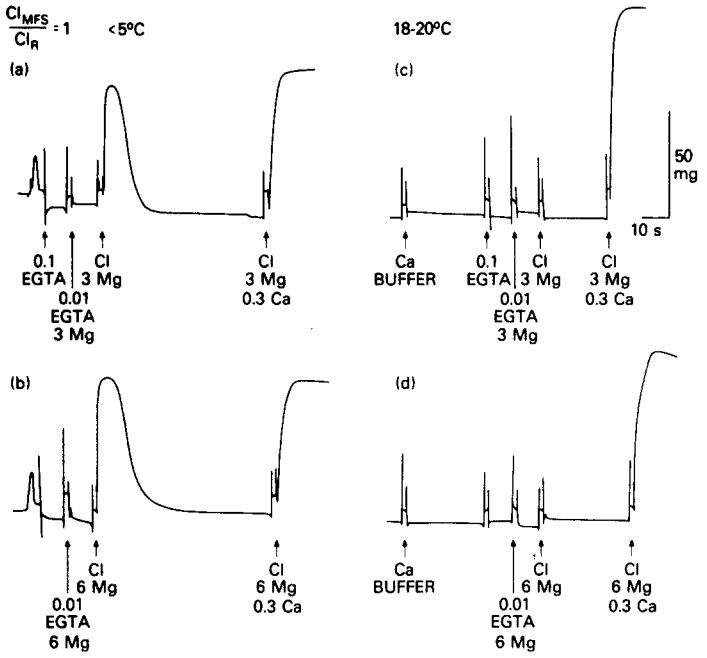

FIGURE 3. The effect of temperature on Mg inhibition in a fiber from a muscle bathed in normal Ringer solution. Segments from the same fiber were stimulated by Cl in solution with 3 mM Mg (*a*) and (*c*), or 6 mM Mg (*b*) and (*d*). Segments (*a*) and (*b*) contained only endogenous Ca and were stimulated at 2–5°C. Segments (*c*) and (*d*) were Ca loaded and stimulated at 18–20°C.

These results are consistent with the idea that Cd ion, like low temperature, unmasks a Cl effect at higher Mg levels, indicating that Mg does not block the release process itself.

Release and Reaccumulation of ^{45}Ca at Low Mg

In order to evaluate Ca release and reaccumulation more directly, ^{45}Ca movement during the response to Cl was measured. For these experiments, the apparatus and method used to mount the skinned fiber segments were modified (see Methods) to minimize tracer contamination from the high specific activity Ca buffer solution required to load the SR with sufficient ^{45}Ca for the assays. Force traces from one of these experiments are shown in Fig. 5 to illustrate the procedures used. The segments were loaded in ^{45}Ca buffer solution, rinsed

thoroughly in dilute EGTA solutions, and then exposed to stimulating or control solutions. In Fig. 5*a* the segment was exposed to Cl during a complete force spike, then to 1 mM EGTA Cl solution for about 10 s to trap ^{45}Ca in the MFS, and finally to a solution containing Triton-X100 and 5 mM EGTA, which extracts the tracer remaining in the segment, that is the SR ^{45}Ca. Each of these solutions was assayed for the tracer that had been washed out of the segment

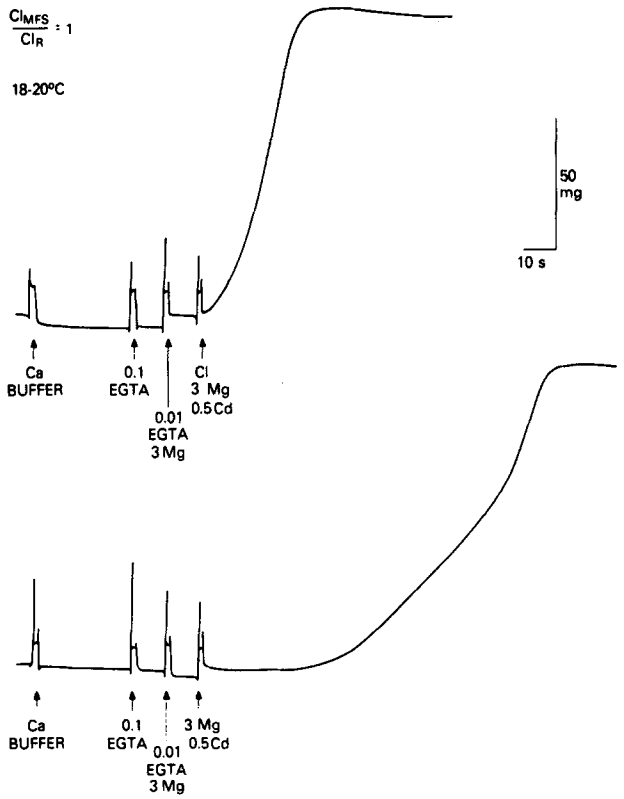

FIGURE 4. The effect of Cd on Mg inhibition at 18–20°C in a fiber from a muscle bathed in normal Ringer solution. Ca-loaded segments from the same fiber were (*a*) stimulated by Cl solution with 3 mM Mg + 0.5 mM Cd; (*b*) exposed to propionate solution with 3 mM Mg + 0.5 mM Cd.

during exposure. The sum of the tracer lost to all the washout solutions gives the total ^{45}Ca in the SR at the outset, and the amount lost into each washout solution can be expressed as a fraction of the total. In Fig. 5*b* the force spike was interrupted. The segment was exposed to Cl solution only during the rising phase of the force spike, to initiate release, then to 1 mM EGTA [Cl] solution to trap the ^{45}Ca which had been released to the MFS, and finally to Triton solution. If the Ca initially released to the MFS is subsequently reaccumulated during a completed spike, more tracer should be collected in this interrupted spike than in a completed spike. In control segments, this same procedure was used with all propionate solutions, that is, with no stimulus. In Fig. 5*c*, the segment was

briefly pre-exposed to EGTA in propionate solution before exposure to Cl solution with 1 mM EGTA, and then extracted in Triton. The continuous presence of EGTA prevents force development, but the full amount of released ^{45}Ca should be collected.

This group of experiments was carried out at 18–20°C in 1 mM Mg. The

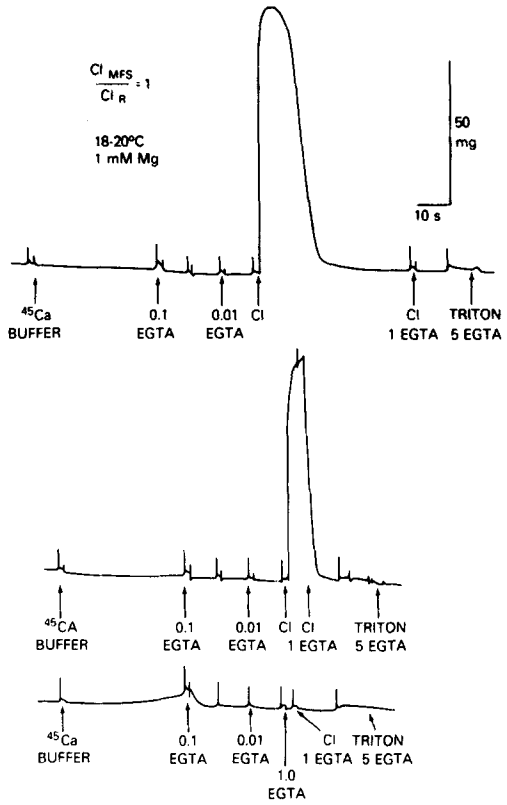

FIGURE 5. Force traces illustrating the protocol of ^{45}Ca release experiments (18–20°C). Segments were loaded in buffered ^{45}Ca solution, rinsed, and exposed sequentially to stimulating or control solution (Cl or propionate), trapping solution (1 mM EGTA), and extracting solution (0.05% Triton X100 + 5 mM EGTA). (*a*) Completed Cl response. (*b*) Cl stimulus followed by EGTA. (*c*) Cl stimulus in the presence of EGTA. Tracer sampling and analysis are described in the text.

pattern of tracer loss under these conditions is shown in Fig. 6. Each group of bars corresponds to one of the procedures outlined above. The first bar in each group is the fraction of ^{45}Ca lost into the stimulating or control solution, the second bar, the fraction lost into the EGTA trapping solution, and the stippled bar is the sum of the first two, that is the total fraction of the original ^{45}Ca in the SR which was lost during the maneuver. The first group shows the small control loss of tracer from muscle and apparatus in the absence of a Cl stimulus. The second group shows the tracer loss during a completed Cl force transient; when the spike was allowed to go to completion, net loss of tracer was about 17%. Most

of the tracer appeared in the Cl solution itself, and little was collected by EGTA after the fiber had relaxed. When the Cl spike was interrupted by transfer to the EGTA trapping solution shown in the third group, about 27% of the tracer was collected. About two-thirds of this was in the EGTA trapping solution applied at the peak of the spike. The substantial increment in total tracer collected shows that Ca reaccumulation affects the net response even in low Mg. Although more released Ca was collected by applying EGTA at the peak of the spike, maximal release was *not* measured when EGTA was present throughout the exposure to

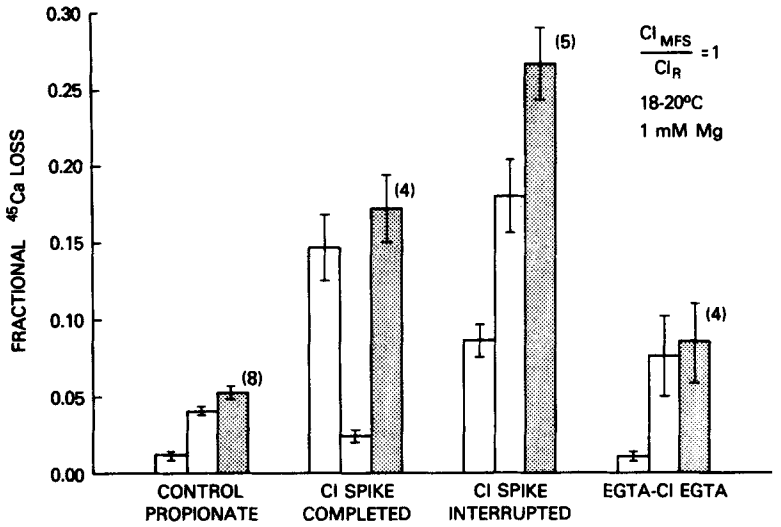

FIGURE 6. ^{45}Ca loss (mean ± SEM) in 1 mM Mg at 18–20°C in segments from fibers with a small Cl gradient. The first bar in each set shows the fraction of accumulated ^{45}Ca lost into the stimulating or control solution (first wash); the second bar shows the fraction lost into the trapping solution (1 mM EGTA); the third bar shows the total fraction lost. First set of bars: control loss in propionate solutions. Second set of bars: completed Cl response. Third set of bars: Cl response interrupted by EGTA-Cl. Fourth set of bars: Cl stimulus in the presence of EGTA. The number of segments is given at the right of each set.

Cl. Under these conditions (fourth group), tracer loss was drastically reduced, and in fact not significantly different from control loss. This result shows that some other factor intervened to prevent Ca release when the EGTA concentration was high and free Ca remained low. Under the conditions of these experiments (low Mg, small [Cl] ratio), Ca release induced by Cl appeared to require the presence of free Ca during the response.

Effect of Mg on ^{45}Ca Reaccumulation

In the experiments above, ^{45}Ca released early in the Cl response was partly reaccumulated and partly lost by diffusion to the bath during the later portion of the transient. If Mg stimulates the reaccumulation process, the diffusional loss of tracer during this period should be reduced by increased Mg. This prediction was tested by measuring the tracer loss into two sequential Cl washes during a

completed Cl response. Force records are shown in Fig. 7 to illustrate the protocol used. Release was initiated by exposure to Cl solution with 1 mM Mg; when force development (reflecting net release) was close to maximal the segment was transferred to a second Cl wash containing either 1 mM or 3 mM Mg.[1]

The results of these experiments are summarized in Fig. 8. Tracer loss into the first Cl wash, containing 1 mM Mg in both cases, was the same in both groups of segments. During the second Cl wash, 50% more tracer was lost to the bath in 1 mM Mg than in 3 mM Mg. In both cases, very little tracer was lost after the

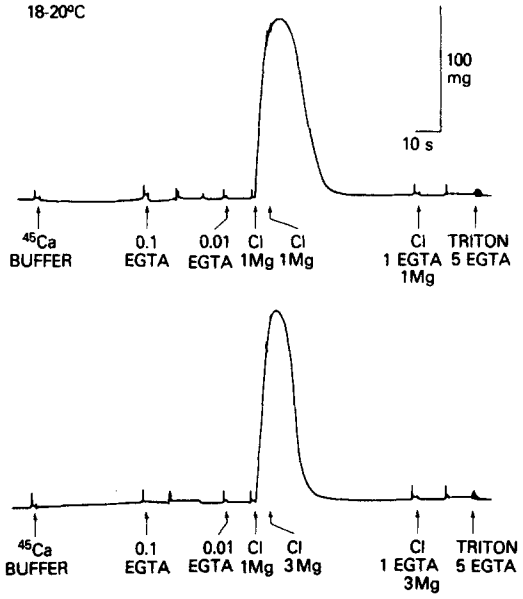

FIGURE 7. Force traces illustrating the protocol of ^{45}Ca release-reaccumulation experiments (18–20°C). Segments were loaded and stimulated with Cl as in Fig. 5*a*, except that the completed Cl response was divided into two sequential washes. The first washout solution contained 1 mM Mg; when force was close to maximal, the segment was transferred to a second washout solution containing 1 mM Mg (upper trace) or 3 mM Mg (lower trace). After relaxation the segment was exposed to trapping solution and extraction solution.

transient to the 1 mM EGTA trapping solution, and the tracer remaining in the segments, extracted by Triton solution, simply reflected the difference in reaccumulation during the second Cl wash. This difference gives a minimum estimate of the Mg stimulation of reaccumulation during this time period because a finite time is required for the increased Mg to diffuse and act at target sites

[1] This set of experiments was carried out on fibers prepared from muscles bathed in low [Cl] solutions; however, the Mg dependence of the Cl response in these fibers (from summer frogs) was the same as in fibers from fall and winter frogs prepared from muscles soaked in normal Ringer solution, presumably because of variability in the effect of a large Cl gradient (see below). When stimulated by Cl in the presence of 3 mM Mg, force and ^{45}Ca loss did not increase; in four segments, the total fractional tracer loss during 60 s in Cl solution followed by 10 s in 1 mM EGTA was only 0.038 ± 0.007, similar to the control loss in propionate shown in Fig. 6.

throughout the fiber early in the second Cl wash when the rates of diffusion and reaccumulation should be highest.

Dependence of Cl Responses on the Cl Gradient

If high Cl in the MFS acts by depolarization of the internal membrane system, then the strength of the Cl stimulus should depend on the electrochemical driving force for transmembrane Cl movement. The [Cl] gradient should be increased when the [Cl] in the lumen of the internal membrane system is reduced. Skinned fibers prepared from muscles soaked in low Cl solutions did respond to Cl at the higher Mg levels at 18–20°C. Fig. 9 shows records of Cl-

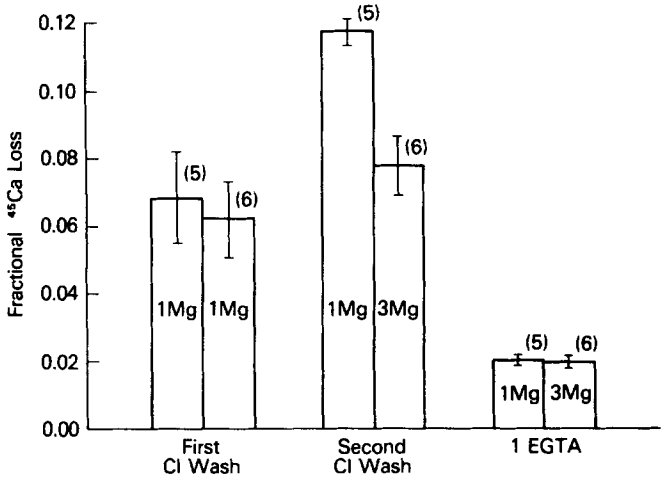

FIGURE 8. ^{45}Ca loss (mean ± SEM) in release-reaccumulation experiments. The first set of bars shows the fraction of accumulated ^{45}Ca lost into the first Cl wash (1 mM Mg in both groups). The second set of bars shows the fraction of accumulated ^{45}Ca lost into the second Cl wash, containing 1 mM Mg (first bar) or 3 mM Mg (second bar). The third set of bars shows the fraction of ^{45}Ca lost into the final EGTA trapping solution. The number of segments is given above each bar.

induced force spikes in three segments of the same fiber from a muscle which had been bathed in Ringer solution containing 6.1 mM Cl (NaCl replaced by sucrose, see Methods). The ratio of Cl in the solution stimulating the skinned fiber to Cl in the original Ringer solution is about 20. After loading the SR with Ca and rinsing, all in 1 mM Mg solutions, the Mg level was either left at 1 mM (Trace *a*), or raised to 3 mM (Trace *b*) or 6 mM (Trace *c*). The ionized Mg in each of these solutions is about 20 μM, 110 μM, and 1.3 mM, respectively. Replacement of propionate by Cl caused rapid force development in each case; with increased Mg, the transients were reduced in duration and height, but a substantial response remained in the highest Mg in this fiber.

Similar experiments were performed on fibers from muscles bathed in Ringer solution containing 12 mM Cl. The results of a series of such experiments on fibers from fall and winter frogs are summarized in Fig. 10. The height of the Cl-induced transient divided by the maximum force of the segment, the normalized peak force, is plotted against the ratio of [Cl] in the solution stimulating the

skinned fiber (about 120 mM) to [Cl] in the original Ringer solution. Responses at different Mg levels were measured in segments from the same fibers, which had a given [Cl] ratio. Responses at different [Cl] ratios could not be measured in segments from the same fibers, with this method of varying the [Cl] ratio. In a few cases fibers from the same muscle were compared at different [Cl] ratios, by removing a fiber bundle after the muscle had been soaked in different Ringer

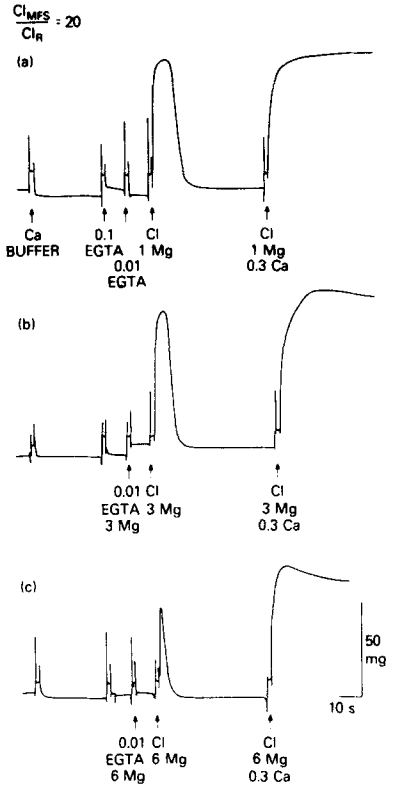

FIGURE 9. Cl-induced force responses at 20°C in a fiber from a muscle bathed in 6.1 mM Cl solution. Ca-loaded segments from the same fiber were stimulated by Cl in solutions containing (*a*) 1 mM Mg; (*b*) 3 mM Mg; (*c*) 6 mM Mg. Subsequent exposure to 0.3 mM Ca gives the maximum force of the segment.

solutions. The responses in these fibers followed the pattern of the mean responses. When Mg was only 1 mM, maximal responses were seen at all Cl ratios. In 3 mM Mg, responses were negligible at a [Cl] ratio of 1, but substantial responses were seen at higher ratios. In 6 mM Mg, responses were small or absent at the intermediate [Cl] ratio of 10; at the highest [Cl] ratio, 20, substantial responses were sometimes seen, as in Fig. 9*c*. However some fibers gave no response in 6 mM Mg and smaller responses in 3 mM Mg at this [Cl] ratio. The large standard error bars reflect a decrease in responsiveness to Cl toward the end of the experimental series.[2]

[2] These experiments were carried out between October and early February, with the highest [Cl] ratio tested only late in the series. In the last fibers tested, force spikes decreased in 3 mM Mg and

When responses at the different Mg levels are compared at a fixed [Cl] ratio, it is clear that increased Mg inhibited the Cl response at 18–20°C, even at the higher [Cl] ratios. When responses at different [Cl] ratios are compared at fixed Mg, it appears that net Ca release at higher Mg (3 or 6 mM) depended on the [Cl] in the internal membrane system at the time of skinning. The difference in response at 3 mM Mg between a Cl ratio of 1 and a ratio of 10 or 20 was highly significant. The curves suggest a progressive increase in response with increas-

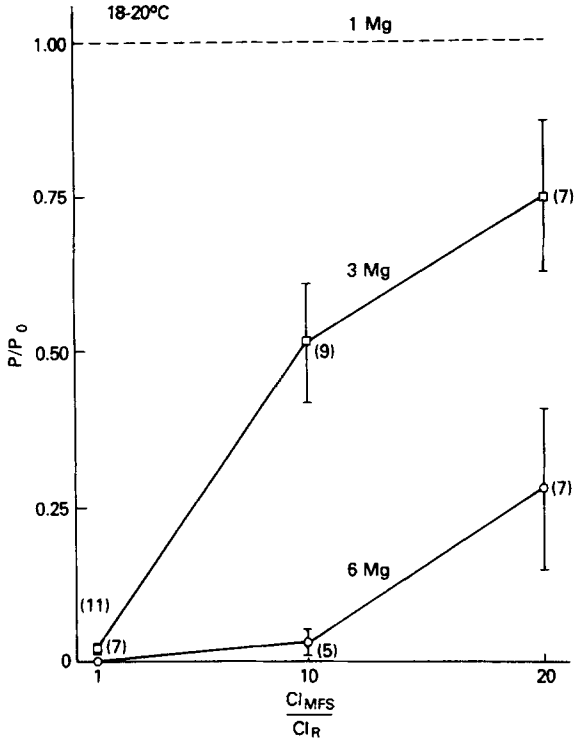

FIGURE 10. The dependence of the Cl response at 20°C on the Cl of the original Ringer solution and on the Mg of the Cl-stimulating solution. The normalized peak force of the Cl spike (mean ± SEM) is plotted against the ratio of stimulating Cl (120 mM) to Ringer Cl, for a series of experiments on winter frogs. The stimulating solution contained either 1 mM Mg (dashed line), 3 mM Mg (□), or 6 mM Mg (○). The number of segments is given next to each point.

ing [Cl] ratio, but further experiments on responsive fibers are required to verify this correlation.

The effect of the composition of the original Ringer solution on Ca-induced Ca release also was tested. Segments were Ca loaded, rinsed, and exposed to a 0.1 mM Ca stimulus in the presence of 1, 3, or 6 mM Mg. Responses were

were absent in 6 mM Mg even at the highest [Cl] ratio. Responsiveness remained low in spring and summer. It seems possible that the Cl response is subject to seasonal variation, perhaps due to changes in relative Cl permeability. In intact frog muscle, the size of contractures induced by low external Cl is variable (Foulks et al., 1965), and low K permeability during the winter has been reported (McCrea, 1975).

compared in two sets of fibers taken from the same muscles (see above); other segments from the same fibers showed marked differences in the Cl response in 3 mM Mg. The Ca-induced responses and their inhibition by Mg (Stephenson and Podolsky, 1977) were unaffected by the previous treatment of the intact fibers.

DISCUSSION

Chloride-Induced Ca Release

These observations verify that Cl causes sufficient net Ca release from the SR to account for the force transient in skinned fibers. The fractional ^{45}Ca release caused by Cl was at least 0.27 (Fig. 6); this fraction corresponds to at least 0.27 mM Ca/kg fiber, because the initial Ca content was at least 1 mM/kg fiber under the Ca-loading conditions used (Ford and Podolsky, 1972*b*). Since the regulatory proteins of the myofilaments are saturated by 0.1 mM Ca (see Ebashi et al., 1969), the net Ca release is more than sufficient to produce maximum force.

Role of Ca Influx and the Influence of Mg

It is clear that the net Ca release is strongly influenced by Ca influx back into the SR, as well as by the stimulated efflux. The effect of inhibitors of active Ca transport on force responses to a weak Cl stimulus ([Cl] ratio about 1) is evidence for the importance of the influx component during the initial response. Low temperature and Cd each caused responses to Cl solution with 3 mM Mg (110 μM Mg^{++}) in fibers from muscles bathed in normal Ringer solution, which were otherwise unresponsive under these conditions (Figs. 2–4). These agents both are known to inhibit the Ca transport system of isolated SR membrane. A temperature decrease from 18–20°C to 5°C should decrease influx by a factor of 4–10 based on the activation energy for Ca uptake by SR vesicles, 15–25 kcal mol^{-1} (see Inesi, 1972). Low Cd concentrations strongly reduce Ca uptake and ATPase activity in isolated SR vesicles (Carvalho, 1968) and block the activity of the purified enzyme (MacLennan, 1970). The Cd effect was not due to activation of the myofilaments since force did not develop immediately without a Cl stimulus (also see Stephenson and Podolsky, 1977).

The ^{45}Ca experiments show that influx is an important factor in net release even at low free Mg levels (20 μM). Overall, at least one-third of the early net ^{45}Ca release estimated with EGTA trapping near the force peak was reaccumulated during the later phase of the complete Cl response (Fig. 6). Since about one-third of the released tracer had diffused into the bath during the initial force rise, the reaccumulated tracer represents 50% of the tracer actually in the MFS at the start of the later phase. During Ca-induced responses in low Mg, Ford and Podolsky (1972*b*) found that the SR ^{45}Ca at the end of a completed spike was about the same as the control level in unstimulated segments, suggesting a large amount of reaccumulation.

The ^{45}Ca experiments also provide additional evidence that the reaccumulation rate increases with $[Mg^{++}]$, as would be expected from the Mg dependence of the rate-limiting step in the partial reactions of the Ca transport system of isolated SR (see Yamada and Tonomura, 1972). In the completed response in

low Mg, 0.09 of the original ^{45}Ca appeared to be reaccumulated after the force had peaked (Fig. 6, the difference between tracer loss in the interrupted and completed responses). When this later phase was examined separately (Fig. 8), increasing Mg to 3 mM (110 μM Mg^{++}) was found to alter the balance between ^{45}Ca reaccumulation and diffusion into the bath; the difference in tracer loss into 1 mM and 3 mM Mg solutions was 0.04 of the original fiber ^{45}Ca. The results together imply that reaccumulation in Cl solution with 3 mM Mg was at least 1.4 times greater than with 1 mM Mg—(0.09 + 0.04)/0.09—and therefore with 3 mM Mg, some 70% of the tracer in the MFS at the start of the later phase was reaccumulated. This increase agrees well with the effect of 3 mM Mg on relaxation from Ca-induced force transients described in the preceding paper; in those experiments, the rate of Ca reaccumulation appeared to increase by a factor of 1.6 (Stephenson and Podolsky, 1977). In both cases, the observed effect of increasing Mg to 3 mM is a lower limit, because substantial Ca movement, by outward diffusion and reaccumulation, can occur before the applied Mg equilibrates and acts throughout the fiber. In the intact fiber, free Mg is likely to be at least as high as in these solutions (110 μM), as discussed in the preceding paper, and Ca influx must be an important determinant of net release.

The substantial role of Ca influx, and its Mg dependence, provides a mechanism for the inhibitory effect of increased Mg on net Ca release, if the reaccumulation mechanism operates in a similar way during the entire time course of the response. This mechanism for inhibition should be particularly effective in the case of a weak Cl stimulus, where much of the response in 1 mM Mg is likely to be due to Ca-induced release (see below). Even in 1 mM Mg, a Ca efflux of 1.4×10^{-4} M s^{-1} (sufficient to saturate the myofilaments in 1 s) would be balanced by influx at 1×10^{-7} M $[Ca^{++}]$, because the rate constant for Ca influx is large, 1.4×10^{3} s^{-1} (Ford and Podolsky, 1972*a*). However, if Ca efflux after a weak Cl stimulus were, for example, 3×10^{-4} M s^{-1}, $[Ca^{++}]$ could rise to $>2 \times 10^{-7}$ M, exceed the thresholds for force development and Ca-induced release (Ford and Podolsky, 1972*a*), and cause an additional increase in efflux. In e mM Mg, with a 50% increase in the rate constant for influx, a Ca efflux of 3×10^{-4} M s^{-1} could be balanced by influx when $[Ca^{++}]$ rose to only 1.4×10^{-7} M, which is below the threshold for Ca-induced release. The influence of Mg on influx thus can reduce net release both by a direct effect on the amount of Ca that reaches the myofilaments at a given efflux, and by an indirect effect on the stimulation of efflux by $[Ca^{++}]$ at the SR membrane.

Properties of the Stimulated Ca Efflux

The tracer experiments show that a weak Cl stimulus, in the presence of low Mg, causes a large increase in the rate of Ca efflux from the SR. During 4–5 s of force rise in Cl nearly maximal force was developed (Fig. 5), reflecting the movement of about 0.1 mM Ca/kg from the SR to the myofilaments (Ebashi et al., 1969). At the same time, an additional 0.09 of the original ^{45}Ca appeared in the bath, corresponding to at least 0.09 mM Ca/kg fiber. On this basis, a minimum of 0.19 mM Ca/kg had left the SR, compared to about 0.01 mM/kg in the control fibers exposed to propionate solution for the same time (Fig. 6). Additional ^{45}Ca released to the MFS was collected by application of EGTA near the force peak,

giving a total loss of at least 0.27 mM/kg. When similarly loaded fibers are exposed to propionate solutions with 1 or 5 mM EGTA for long periods, 360 s are required for the same Ca loss (unpublished experiments). These comparisons indicate that stimulation increased the rate of net Ca loss 20–80-fold. Since this estimate includes the effects of diffusional delays and reaccumulation, the true efflux rate must have increased even more.

It is likely that Mg does not act primarily by direct inhibition of efflux, if the efflux mechanism is independent of the active transport system for influx. Inhibition by Mg appears to require a high rate of Ca accumulation; increased Mg does not block the Cl response when the effect of Mg on influx is counteracted by conditions which reduce influx, that is low temperature or Cd. While a similar antagonism would appear if both these conditions acted by increasing efflux, this possibility seems very unlikely. In the case of low temperature, a negative temperature dependence would be implied. However, one would expect the Cl stimulus to have a positive temperature dependence; the Cl permeability of intact frog muscle has a relatively large Q_{10} (Harris, 1965). In addition, the lower Ca content of the SR in the low-temperature fibers (which were not Ca loaded) would tend to decrease efflux (Nakajima and Endo, 1973). In the case of Cd, this ion does not appear to increase efflux in the manner of Ca-induced Ca release (Fig. 4; also see Stephenson and Podolsky, 1977).

The stimulated Ca efflux was blocked by EGTA under the experimental conditions used (Fig. 6). This important new result of the tracer experiments suggests that Cl-induced release is Ca dependent in fibers from muscles soaked in normal Ringer solution which gave large responses only in low Mg. Thorens and Endo (1975) have recently described Cl-induced Ca release in *Xenopus* fibers in the presence of EGTA at low temperature; Ca release was assayed by an indirect method. The preparation, conditions, and assay all differ from those in the present experiments, and it is difficult to relate these results at present. Two types of mechanism could account for the Ca dependence observed here. First, the basic response to Cl, and presumably to depolarization, may be amplified substantially by secondary Ca-induced Ca release. The operation of this mechanism seems almost certain in the present experiment, because the low Mg used facilitates Ca-induced Ca release and the response was suppressed as readily as the direct Ca-induced response by Mg (Fig. 2; Stephenson and Podolsky, 1977). Some implications of this interpretation are discussed below. Second, either the coupling of depolarization to Ca efflux or the efflux mechanism itself may include a Ca-dependent step. The existence of these primary mechanisms cannot be ascertained from the present experiment; the Ca requirement under other experimental conditions is under current investigation.

The inverse relation between the response to applied Cl and the [Cl] bathing the intact fiber before skinning (Fig. 10) suggests that stimulation of efflux depends on the [Cl] gradient across the internal membranes. The simplest effect of the original extracellular [Cl] is to influence the [Cl] in the lumen of the internal membrane system. The critical parameter appears to be the [Cl] gradient rather than high MFS [Cl] per se, because the applied [Cl] was constant. Nakajima and Endo (1973) found that the Cl response in split muscle fibers increased with increasing applied [Cl], and an analogous effect has been re-

ported for isolated SR vesicles (Kasai and Miyomoto, 1973). The present results support the assumption that high Cl applied to the MFS acts by depolarization of some portion of the internal membrane system (Costantin and Podolsky, 1967; Ford and Podolsky, 1970; Nakajima and Endo, 1973). By such a mechanism, the Cl stimulus in skinned fibers could simulate a step in the physiological activation pathway, although highly unphysiological Cl concentrations are applied. The present results also show that a Cl effect occurs in fibers with physiological [Cl] in the internal membrane systems. In all the earlier work cited above, the fibers studied had been exposed to solutions with low or zero Cl before skinning, so that the lumen of the transverse tubules (T tubules), and perhaps the SR, contained abnormally low [Cl].

The effect of the original extracellular [Cl] is retained in the skinned fiber through the initial exposure to propionate solutions. Retention of intraluminal Cl in very low [Cl] media implies a restriction of Cl diffusion out of the T tubules and across the internal membranes. The studies of Costantin and Podolsky (1967) on the electrical excitability of skinned fibers also implied that the internal membrane system becomes a closed compartment after skinning, presumably by sealing over of the mouths of the T tubules. The transmembrane potential difference indicated by those studies provides a basis for slow transmembrane Cl loss in propionate solutions; a positively charged luminal side would reduce the electrochemical driving force for outward Cl movement. A transmembrane potential difference also helps to explain the effectiveness of the Cl stimulus when the ratio of applied [Cl] to Ringer [Cl] is about 1. In addition, luminal [Cl] may be less than Ringer [Cl] in the intact fiber, due to fixed negative charges (Rapoport, 1969).

Reduction of extracellular [Cl] has a secondary effect on frog muscle fibers: swelling of the T-tubule system (Foulks et al., 1965; Rapoport et al., 1969). This swelling does not seem to play a primary role in the Cl response of skinned fibers, because the response occurred when the Ringer [Cl] had been normal. The swelling does not potentiate the ability of the SR to release Ca, because the Mg dependence of Ca-induced Ca release was not altered by the pretreatment (see Results). The swelling appears maximal by about 50 mM Cl (Rapoport et al., 1969), while the Cl responses probably increase further between 12 and 6 mM Cl in the Ringer, although this grading was not statistically significant with the present data. The swelling might potentiate, by an unknown mechanism, a specific response produced by a different mechanism. In contrast, the primary effect of reduced [Cl], an increase in the transmembrane [Cl] gradient during stimulation, accounts for potentiation by the same plausible mechanism as the basic response.

Implication of the Cl Responses for the Ca-Induced Release Mechanism

The observations on Cl responses relate to the role of Ca-induced Ca release in two ways. First, Ca dependence of Cl-induced ^{45}Ca release suggests some interaction between the two modes of stimulation, and raises the possibility either that Ca is involved in the pathway by which Cl increases efflux, and/or that Ca-induced release can result from Cl-induced release and contribute significantly to the total response. Second, the responses to a weak Cl stimulus may reflect the

properties of Ca-induced release; as discussed above, the large response in low Mg at 18–20°C probably has a large component of Ca-induced release amplifying a small Cl effect. If this interpretation is correct, the weak Cl stimulus provides a means of analyzing Ca-induced release when the stimulus is distributed throughout the fiber by Cl diffusion. In the case of an applied Ca stimulus, it is difficult to evaluate Mg inhibition of the efflux mechanism proper because increased Ca uptake near the fiber surface could interfere with the inward spread of the Ca stimulus (Stephenson and Podolsky, 1977). In the case of the weak Cl stimulus, the presence of a large response in high Mg at low temperature suggests that Mg does not block Ca-stimulated efflux. From these observations on Cl responses, it seems that Ca could play a role in Ca release even at the Mg^{++} levels believed to exist in intact fibers, but the nature and extent of its influence require clarification.

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