Peeled Mammalian Skeletal Muscle Fibers

Possible Stimulation of \( \text{Ca}^{2+} \) Release via a Transverse Tubule–Sarcoplasmic Reticulum Mechanism

SUE K. BOLITHO DONALDSON

From the Department of Physiology and the Department of Medical Nursing, Rush University, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612

ABSTRACT Single muscle fibers from rabbit soleus and adductor magnus and from semitendinosus muscles were peeled to remove the sarcolemma and then stimulated to release \( \text{Ca}^{2+} \) by (a) caffeine application or (b) ionic depolarization accomplished via substitution of choline chloride for potassium propionate at constant \([K^+] \times [Cl^-]\) in the bathing solution. Each stimulus, ionic or caffeine, elicited an isometric tension transient that appeared to be due to \( \text{Ca}^{2+} \) released from the sarcoplasmic reticulum (SR). The peak magnitude of the ionic (Cl-induced) tension transient increased with increasing Cl concentration. The application of ouabain to fibers after peeling had no effect on either type of tension transient. However, soaking the fibers in a ouabain solution before peeling blocked the Cl-induced but not the caffeine-induced tension transient, which suggests that ouabain's site of action is extracellular, perhaps inside transverse tubules (TTs). Treating the peeled fibers with saponin, which should disrupt TTs to a greater extent than SR membrane, greatly reduced or eliminated the Cl-induced tension transient without significantly altering the caffeine-induced tension transient. These results suggest that the Cl-induced tension transient is elicited via stimulation of sealed, polarized TTs rather than via ionic depolarization of the SR.

INTRODUCTION

Skeletal muscle fibers contract in response to depolarization of their sarcolemma via an excitation-contraction coupling mechanism that has not been fully elucidated. Although it is well established that the entire excitation-contraction coupling process is under the control of surface membrane and transverse tubule (TT) membrane potential (Huxley and Taylor, 1958; Hodgkin and Horowicz, 1960; Constantin and Taylor, 1973; Constantin, 1975) and that \( \text{Ca}^{2+} \) released internally from the sarcoplasmic reticulum (SR) is the ultimate trigger for the...
contractile apparatus, the steps from plasma membrane depolarization to Ca\(^{2+}\) release from the SR are not known (see Stephenson, 1981).

Perhaps the most versatile preparation for the study of the SR Ca\(^{2+}\) release mechanism is the skinned, or broken sarcolemma, fiber preparation originally developed by Natori (1954). Skinning can be accomplished mechanically by (a) peeling the fiber to remove the entire sarcolemma (Natori, 1954), or (b) splitting the fiber longitudinally (Nakajima and Endo, 1973) to remove only a portion of the surface membrane. Both peeled and split fibers allow access to their interiors without disruption of normal intracellular SR architecture and TT-SR junctions (Franzini-Armstrong, 1971). Other methods of disrupting the sarcolemma of single fibers, such as chemical or detergent treatments, alter internal membrane systems as well as sarcolemma.

Mechanisms of Ca\(^{2+}\) release from peeled and split frog skeletal fibers have been studied extensively. Ca\(^{2+}\) release can be elicited in this preparation by the application of Ca\(^{2+}\) (Ca\(^{2+}\)-induced release), caffeine, or Cl\(^{-}\) (Cl\(^{-}\)-induced release). Ca\(^{2+}\) and caffeine appear to release Ca\(^{2+}\) as a result of direct stimulation of the SR. Furthermore, Ca\(^{2+}\)- and caffeine-induced releases appear to occur via a common mechanism in peeled skeletal fibers, with caffeine acting by lowering the threshold for the Ca\(^{2+}\)-induced release. However, the normally high threshold for Ca\(^{2+}\)-induced release in skeletal muscle fibers makes it unlikely that the Ca\(^{2+}\)-induced mechanism is the primary one in vivo (see Endo, 1977).

Although Cl\(^{-}\) stimulation also elicits skinned fiber Ca\(^{2+}\) release, the Cl\(^{-}\) mechanism has properties that are distinct from the Ca\(^{2+}\)-induced one (see Endo, 1977; Stephenson, 1981). An abrupt increase in the bathing solution Cl\(^{-}\) concentration, in the absence of other permeant anions, would be expected to depolarize all of the Cl\(^{-}\)-permeable, sealed membrane systems within the skinned fibers. If the \([K^+] \times [Cl^-]\) of the bathing solutions increases as a result of the Cl\(^{-}\) substitution, the stimulus would include osmotic swelling of internal membrane spaces (Boyle and Conway, 1941; Mobley, 1979). Since Cl\(^{-}\) substitution at constant \([K^+] \times [Cl^-]\) yields a sizable tension transient in frog split (Mobley, 1979) and peeled fibers (Stephenson, 1983), the depolarizing diffusion potential per se probably triggers the Ca\(^{2+}\) release. However, it is impossible to know whether membrane depolarization actually occurs during Cl\(^{-}\) stimulation since a direct measurement of membrane potential is technically impossible.

All of the ionic stimuli that elicit Ca\(^{2+}\) release from skinned skeletal fibers might act via depolarization of sealed TTs as well as the SR (see Stephenson, 1981). Nakajima and Endo (1973) attempted to clarify this issue by splitting rather than peeling Xenopus laevis fibers, so as to prevent sealing of TTs and the TT contribution to Cl\(^{-}\)-induced releases. Although they could elicit Cl\(^{-}\)-induced Ca\(^{2+}\) releases in split fibers, they did not show that (a) all of the TTs in a Cl\(^{-}\)-responsive split fiber were open and depolarized, or (b) the Cl\(^{-}\)-induced Ca\(^{2+}\) release in the split fibers was due to ionic rather than osmotic stimulation as a result of the increase in \([K^+] \times [Cl^-]\) inherent in their Cl\(^{-}\) stimulus.

Other investigators have not taken adequate precautions to eliminate the contribution of sealed, polarized TTs to skinned fiber Cl\(^{-}\)-induced Ca\(^{2+}\) releases. As a result, depolarization of the SR per se has not been conclusively established
as the mechanism triggering Cl\textsuperscript{−}-induced Ca\textsuperscript{2+} release in skinned skeletal fibers (see Stephenson, 1981). Similarly, in intact fibers, SR depolarization has not been conclusively demonstrated to be the trigger for Ca\textsuperscript{2+} release during the twitch (Oetliker, 1982).

The purpose of this study was to characterize Ca\textsuperscript{2+} release properties of single peeled skeletal fibers taken from adductor magnus and soleus muscles of the rabbit under conditions that should have either allowed or eliminated the effects of polarized TTs. The emphasis was placed upon fibers from the adductor magnus, which is composed almost exclusively of fast-twitch fibers. Selected data were also collected from frog semitendinosus fibers to check for species dependence of the findings. The contribution of polarized TTs was assessed by determining the effect of ouabain and saponin on Ca\textsuperscript{2+} releases induced by either Cl\textsuperscript{−} or caffeine. Brief reports have been published previously (Donaldson, 1982, 1983).

**METHODS**

**Preparation of Single Peeled Skeletal Fibers**

Adult New Zealand white female rabbits were killed by an overdose of Nembutal and their adductor magnus and soleus muscles were immediately removed. Skeletal fibers from frog (*Rana pipiens*) semitendinosus muscles were also studied for comparison. The details of removing the muscles and isolating single fibers were as reported earlier (Donaldson and Hermansen, 1978; Donaldson and Kerrick, 1975), except that in this study the excised muscles were first placed in a modified Ringer's solution (0 added Ca with 0.05 mM EGTA, Na\textsuperscript{+} and propionate substitution of K\textsuperscript{+} and Cl\textsuperscript{−}, respectively, and imidazole as H\textsuperscript{+} buffer).

Individual fibers were peeled in a relaxing solution by pulling on a myofibrillar branch, which caused the sarcolemma to roll off as a cuff. The relaxing solution (see *Bathing Solutions*) mimics, to the extent that they are known, important intracellular ionic constituents of the sarcoplasm in a relaxed fiber.

**Measurement of Ca\textsuperscript{2+} Release as Tension Transients**

Ca\textsuperscript{2+} release was monitored as an isometric tension transient elicited as a result of Ca\textsuperscript{2+} activation of the fiber's contractile apparatus. Each peeled fiber was mounted in the forceps of a photodiode force transducer, as in earlier studies (Donaldson and Hermansen, 1978; Donaldson and Kerrick, 1975). Isometric tension was monitored continuously, with a given fiber being stimulated repeatedly in the protocols described below. Baseline or zero force was established with the mounted fiber immersed in a relaxing solution with very low Ca\textsuperscript{2+} activity.

**Quantitative Analysis of Tension Transients**

The peak magnitude of each Cl\textsuperscript{−}-induced tension transient (measured from the baseline force trace in relaxing solution) was normalized by expressing it as the fraction of peak force elicited by a representative maximum caffeine-induced tension transient for the same fiber. Maximum caffeine contractures were those that released saturating amounts of Ca\textsuperscript{2+} and thus elicited maximum force generation for a given fiber. This method of normalizing the peak magnitudes of the Cl\textsuperscript{−}-induced tension transients corrected for a change in the fiber's maximum force-generating capacity. As long as other factors affecting the magnitude of the Cl\textsuperscript{−}-induced tension transient, such as the SR Ca\textsuperscript{2+} uptake rate,
diffusion properties, contractile-apparatus Ca\(^{2+}\) sensitivity, and SR Ca\(^{2+}\) release capacity, remain constant for a given fiber, quantitative analysis of self-paired data is valid. Data from different fibers types could not be pooled since the contractile-apparatus Ca\(^{2+}\) sensitivity of rabbit soleus and adductor fibers varies (Donaldson and Hermansen, 1978). Similarly, because Mg\(^{2+}\) alters the contractile-apparatus Ca\(^{2+}\) sensitivity of skeletal fibers used in the study, even self-paired data could not be used to assess the effect of [Mg\(^{2+}\)] on Cl-induced Ca\(^{2+}\) release.

**Bathing Solutions**

**GENERAL** The composition of the aqueous bathing solutions determined the ionic environment of each fiber's internal membrane systems and contractile apparatus. Alterations in the bathing solution composition were the independent variables in this study. The bathing solution composition was changed by moving the mounted fiber between 1-ml bathing solution slots of a spring-form Plexiglas tray. The slots were covered when not in use to prevent evaporation of water. To limit the transfer of solution between slots by the fiber itself or the transducer clamps, the fibers were transferred through a series of relaxing solutions or duplicate solutions, the final one being "clean."

Although several different types of bathing solutions were used in this study, certain parameters were constant in all. All bathing solutions contained 2 mM MgATP\(^{2-}\), 66 mM K\(^+\) plus choline, 4 mM Na\(^+\), 15 mM CP\(^{2-}\) (creatine phosphate added as Tris\(\text{CP}\) and Na\(_{2}\)CP), 15 U/ml creatine phosphokinase (CPK), pH 7.0, and variable imidazole concentrations to make the ionic strength 0.15 M. The high-energy phosphate compounds and CPK enzyme assured maintenance of a constant MgATP\(^{2-}\) concentration for force generation and membrane ATPases. Experiments were performed at room temperature, 22 ± 1 °C. To avoid loading of mitochondria with Ca\(^{2+}\) and their possible contribution to Ca\(^{2+}\) releases, 1 mg/ml oligomycin was added to every bathing solution (Lardy et al., 1975). The ionic variables were [Ca\(^{2+}\)], [Mg\(^{2+}\)], total [EGTA], Cl\(^-\) vs. propionate or methanesulfonate concentrations, and K\(^+\) vs. choline concentrations. Other bathing solution variables were the presence or absence of ouabain, caffeine, procaine, and saponin. The particular combination of variables is discussed below under each specific type of solution. The solution-mixing procedure, the computer program method of solving each complex equilibrium, and the assays for accuracy are described in detail elsewhere (Donaldson and Kerrick, 1975; Donaldson and Hermansen, 1978).

**RELAXING SOLUTIONS** Relaxing solutions contained 10⁻³ M Mg\(^{2+}\) (pMg 3) and no added Ca\(^{2+}\), with 0.05 mM total EGTA to lower free [Ca\(^{2+}\)] to below 10⁻⁶ M. All peeled rabbit skeletal fibers are relaxed under these ionic conditions (Donaldson and Hermansen, 1978). Single fibers were isolated, soaked, and peeled in relaxing solutions before being mounted in the force transducer in the same solution.

The composition of the relaxing solution was selected very carefully since it would be the solution initially trapped in any sealed TTs within the peeled fibers. For this reason, Cl\(^-\) and K\(^+\) were eliminated. The Na\(^+\) concentration of the relaxing solution was 70 mM and propionate was the major anion. Thus, when the fiber was later exposed to solutions with either high [K\(^+\)] or high [Cl\(^-\)], the polarization of any sealed TTs or the TT [Cl\(^-\)] gradient, respectively, should have been enhanced.

**CAFFEINE RELEASE SOLUTION** The caffeine release solution consisted of a modified relaxing solution (0.1–0.05 mM EGTA, pMg 3 or 4) to which 10 mM caffeine was added. The usual caffeine release was at pMg 4 and served to lower the internal fiber Ca\(^{2+}\) content to a constant level for a given fiber.

**LOAD SOLUTION** To ensure that the peeled fibers contained sufficient Ca\(^{2+}\) for release, they were loaded in a solution containing 4 mM total EGTA and sufficient total
Ca\(^{2+}\) for pCa 5.6, [Mg\(^{2+}\)] was either 10\(^{-4}\) or 10\(^{-5}\) M. The choice of millimolar total Ca with low free [Ca\(^{2+}\)] was intended to simulate physiologic loading conditions and to avoid inadvertent loading of Ca\(^{2+}\) into the mitochondria (Somlyo et al., 1981). Fibers generated low levels of force at pCa 5.6, with greater force at pMg 4 than at pMg 3, as was expected from prior work (Donaldson and Hermansen, 1978).

The load solution had high K\(^{+}\) and low Cl\(^{-}\) concentrations, as indicated in Table I. It was expected that the internal SR composition would equilibrate with the loading solution since the diffusible ion compositions of the SR and the cytoplasm have been shown to be similar in intact fibers (Somlyo et al., 1977). In contrast, sealed TTs in the peeled fiber should be polarized by the transfer from relaxing to load solution and Na,K-ATPase should maintain the polarization in the face of passive K\(^{+}\) and Na\(^{+}\) leakage across the TT membrane. The 4 mM Na\(^{+}\) on the sarcoplasmic side of TTs provided a constant stimulus for any Na,K-ATPase.

**Table I**

| Cl\(^{-}\)-induced Ca\(^{2+}\) Release: Concentrations of the Monovalent Ionic Variables in the Bathing Solutions |
| Solution type | Monovalent ionic variable composition (mM) |
|---------------|------------------------------------------|
|               | K\(^{+}\) | Cl\(^{-}\) | Choline | Propionate | Na\(^{+}\) |
| Load          | 66       | 4       | 0       | 66\(^{*}\) | 4       |
| Rinse         | 66       | 4       | 0       | 66\(^{*}\) | 4       |
| Cl\(^{-}\) release (De) | 66 | 4 | 66 | 62 | * | 4 |
| 40            | 6.6      | 40      | 59.4    | 26\(^{*}\) | 4       |
| 20            | 13.2     | 20      | 52.8    | 46\(^{*}\) | 4       |
| Cl\(^{-}\) release (Cl) | 66 | 66 | 0 | * | 4 |

* The propionate concentration exceeded this amount slightly since it was the major monovalent anion for all cations in solution.

**RINSE SOLUTION** Fibers are at baseline tension in the rinse solution, just as they are in the relaxing solution in which they were skinned. However, the rinse solution differs in monovalent ion and EGTA concentration from the relaxing solution in that rinse solutions had 66 mM K\(^{+}\), 4 mM Cl\(^{-}\), 4 mM Na\(^{+}\), and only 0.02 mM EGTA (see Table I). Fibers were immersed in the rinse solution to remove the load solution Ca and to lower the EGTA buffer concentration to 0.02 mM EGTA without altering the [K\(^{+}\)] x [Cl\(^{-}\)], [Na\(^{+}\)], or [Mg\(^{2+}\)] from that of the load solution.

**Cl\(^{-}\) RELEASE SOLUTIONS** The Cl\(^{-}\) release solutions were identical in EGTA concentration to the rinse solutions. Thus, the free [Ca\(^{2+}\)] was constant as Cl\(^{-}\) was applied by transferring the fiber from rinse to the Cl\(^{-}\) release solution. A total EGTA concentration of 0.02 mM was ideal for producing measurable Cl\(^{-}\)-induced tension transients. An EGTA concentration of 0.05 mM eliminated tension transients associated with Cl\(^{-}\) stimulation in the peeled mammalian fibers, presumably by buffering the internally released Ca\(^{2+}\). Two types of Cl\(^{-}\) stimulation were used, as indicated in Table I. In the first type, choline and Cl\(^{-}\) replaced varying amounts of K\(^{+}\) and propionate according to the desired level of Cl\(^{-}\) stimulation; the [K\(^{+}\)] x [Cl\(^{-}\)] was identical in load, rinse, and Cl\(^{-}\) release solutions in order to avoid volume changes in internal membrane systems during Cl\(^{-}\) stimulation (Boyle and Conway, 1941; Mobley, 1979). This type of Cl\(^{-}\) release solution is called depolarizing ("De" in figures and tables) since the stimulus should be a membrane potential change.
The second type of Cl\(^-\) stimulation is in part osmotic (labeled "Cl") since only anion substitution was used and \([K^+] \times [Cl^-]\) increased during ionic depolarization.

**Protocol for Cl\(^-\) and Caffeine Stimulation**

Fig. 1 illustrates the protocol used for eliciting a Cl\(^-\)-induced tension transient (constant \([K^+] \times [Cl^-]\), 66 mM Cl\(^-\)) from a peeled soleus muscle fiber at pMg 4. The baseline was established with the mounted fiber in a relaxing solution. The fiber was then placed for 2 min in a load solution, where it generated measurable isometric tension at pCa 5.6. Next, the fiber was placed for 1.5 min in a rinse solution, in which it relaxed to baseline; subsequent transfer into the 66 mM Cl\(^-\) solution elicited a tension transient. The fiber was then stimulated with a 10 mM caffeine solution to bring the internal Ca content back to baseline. In Fig. 1, beginning again with the relaxing solution (i.e., transfer through a

![Figure 1](image-url)
series of identical relaxing solutions to limit cross-contamination), the protocol was repeated with little change in the fiber's responses. In the trace in Fig. 2, the same protocol is illustrated at time marks 1–4, for a peeled adductor fiber at pMg 3.

**Protocol for Procaine Application**

The local anesthetic procaine has been shown to block Ca\(^{2+}\)-induced and caffeine-induced Ca\(^{2+}\) releases in mechanically skinned frog skeletal fibers (Endo, 1977). In this study, procaine was used in an attempt to eliminate any Ca\(^{2+}\)-induced Ca\(^{2+}\) release contributing to the tension transient elicited by ionic depolarization with Cl\(^-\). Procaine's effectiveness as a blocker of Ca\(^{2+}\)-induced Ca\(^{2+}\) release was judged individually in each fiber in terms of its ability to completely block the caffeine (10 mM) tension transient at the same Mg\(^{2+}\) concentration. Since procaine may also block SR loading of Ca\(^{2+}\) (Almers, 1978), it was applied in the rinse solution after loading. From pilot studies, it was found that a 1-min soak was sufficient for a maximum effect of the procaine and that its effect on the tension transients was eliminated by a 1-min wash in relaxing solution. Therefore, 1.5 min was always allowed for wash-in and wash-out of the procaine.

![Figure 2](image-url)

**FIGURE 2.** Cl\(^-\)-induced and caffeine-induced tension transients of a single peeled adductor magnus muscle fiber (80 μm diam) in the presence and absence of procaine at 10\(^{-3}\) M Mg\(^{2+}\). The trace shows isometric tension (ordinate) in time (abscissa) with bathing solution changes given at the time marks. Bathing solution changes are a repetitive pattern of four, as labeled for the first four time marks, except as noted otherwise. The fiber was loaded with Ca\(^{2+}\), rinsed (Ri), ionically depolarized with 66 mM Cl\(^-\) at constant [K\(^+\)] \times [Cl\(^-\)] (De), and then exposed to 10 mM caffeine (Caf). Unlike the soleus fiber of Fig. 1, the adductor fiber did not generate observable tension in the load solution because of the high [Mg\(^{2+}\)] and the lower Ca\(^{2+}\) sensitivity of adductor fibers (Donaldson and Hermansen, 1978). The fiber responded initially to Cl\(^-\) and caffeine with tension transients (the asterisk indicates a fiber placed in 0.05 mM EGTA relaxing solution to speed relaxation). After the second load, 10 mM procaine was added to the rinse and the ionic depolarization solutions. See the legend to Fig. 1 and the Methods for greater detail regarding bathing solution changes. The second Cl\(^-\)-induced tension transient, elicited in the presence of procaine, was only slightly smaller. Ca\(^{2+}\)-induced release was completely suppressed by the procaine, as evidenced by the inability of caffeine (pMg 3) to elicit a contracture until after the procaine was removed in a wash (Wa) relaxing solution (0.05 mM EGTA). The final four time marks are identical to the first set.
Fig. 2 illustrates the protocol for procaine blockade during Cl⁻ stimulation. After the second load, the rinse solution contained procaine, as did the Cl⁻ release solution. The Cl⁻-induced tension transient was lengthened in the presence of procaine, perhaps because of reduced SR reaccumulation of Ca²⁺. Next, the completeness of the procaine blockade was checked with a 10 mM caffeine (pMg 3) challenge in the presence of procaine; this caffeine stimulation elicited no response. It was essential that the Ca²⁺ content of the fiber be assayed by caffeine stimulation after removal of the procaine, as illustrated in Fig. 2, to make sure that the procaine effects were not due to SR Ca²⁺ depletion. The effects of procaine were always completely reversible, as illustrated by the final Cl⁻-induced and caffeine-induced tension transients of Figs. 1 and 2.

Protocol for Ouabain Application
Ouabain was used to block Na,K-ATPase and thus prevent sustained polarization of sealed TTs that might have formed within the peeled fibers. Since the binding site for ouabain is on the outside of the sarcolemma (and thus the inside of the TTs) (Venosa and Horowicz, 1981) and ouabain is not very lipid-soluble (Erdmann et al., 1977; Schwartz et al., 1975), it was necessary to allow diffusion of ouabain into the TTs before peeling. Single unpeeled fibers were cut in half transversely in the relaxing solution. One half was soaked in an iced relaxing solution with 0.01–1.0 mM added ouabain for 2.5 h. Lower concentrations of ouabain and/or shorter soaking times yielded negative results. Ouabain inhibition should occur at 10⁻⁶ M, but higher concentrations were used to enhance diffusion of ouabain into TTs. The ionic composition (low [K⁺], pMg 3) of the relaxing solution should have enhanced the rate of ouabain binding (Hegyvary, 1976). Both the ouabain and non-ouabain halves of a given fiber were peeled and tested using the Cl⁻ and caffeine stimulation protocol described above; the same transducer and identical bathing solutions were used for halves of a single cell. In most experiments, 10⁻⁶ M ouabain was added to all of the bathing solutions and thus ouabain was applied to both halves of the fiber after peeling.

Protocol for Saponin Application
Saponin increases membrane permeability by combining with cholesterol molecules (Ohtsuki et al., 1978). Thus, saponin should disrupt TTs to a greater extent than SR in the peeled rabbit fibers, because the TT cholesterol content is significantly greater than that of SR (Rosemblatt et al., 1981); rabbit SR cholesterol content is <2% of the total lipid content (Meissner and Fleischer, 1971). Saponin has been shown to act specifically on the surface membrane of amphibian skeletal muscle fibers without altering the SR (Endo and Iino, 1980).

In order to assess the relative effects of saponin on the contractile apparatus and Cl⁻-induced and caffeine-induced tension transients, peeled fibers were first loaded, rinsed, and tested with Cl⁻ and caffeine, and were then exposed to saponin before repeating the same load-test protocols. Saponin was applied by soaking the peeled fiber in 0.5% saponin relaxing solution for 15 s and then immersing the fiber in relaxing solution to wash out saponin before reloading. Thus, each fiber was tested before and after saponin treatment and yielded self-paired data for analysis.

RESULTS

Characteristics of the Caffeine-induced Tension Transient
Caffeine elicited tension transients in the mammalian peeled skeletal fiber after loading of the fiber with Ca²⁺; infrequently, caffeine elicited a tension transient
without prior loading of a peeled fiber. The peak magnitude of the caffeine-induced tension transient was (a) reduced by increased EGTA concentration in the caffeine solution and (b) directly related to the loading time for a fiber up to some maximum that represented loading and releasing of sufficient Ca\(^{2+}\) to saturate the contractile apparatus. The threshold for caffeine stimulation appeared to be directly related to [Mg\(^{2+}\)]. After a fiber gave a caffeine-induced Ca\(^{2+}\) release at pMg 3, additional Ca\(^{2+}\) could be released by transferring the fiber to a 10 mM caffeine solution at pMg 4, but increasing the Mg\(^{2+}\) concentration in the presence of caffeine did not elicit additional release of Ca\(^{2+}\) (data not shown). The caffeine-induced tension transients were blocked by 10 mM procaine, as shown in Figs. 2 and 7.

Caffeine never completely emptied the peeled fiber Ca\(^{2+}\) stores. After a caffeine-induced tension transient at pMg 4, treatment of the peeled mammalian fibers with 1% Brij-58, a detergent that destroys all membranes (Orentlicher et al., 1974), caused additional release of Ca\(^{2+}\), which elicited a tension transient at low EGTA concentration. After Brij-58 treatment, the peeled mammalian fibers no longer responded with tension transients to caffeine or Cl\(^{-}\) stimulation (data not shown).

**Characteristics of the Cl\(^{-}\)-induced Tension Transient**

Although peeled mammalian skeletal fibers consistently gave sizable tension transients in response to caffeine, they did not always respond to Cl\(^{-}\) stimulation. The failure rate appeared to be animal-dependent in that fibers from a given muscle seemed to be either nonresponsive or responsive to Cl\(^{-}\) stimulation. The important point is that it was possible to elicit a sizable caffeine-induced tension transient in the absence of a detectable Cl\(^{-}\)-induced one.

When peeled fibers did respond to prolonged Cl\(^{-}\) application, the tension generation was always transient. The time course of the Cl\(^{-}\)-induced tension transient was quite slow (seconds to minutes) relative to known physiological activation times in intact fibers. It took seconds for the development of peak magnitude of the Cl\(^{-}\)-induced tension transient.

The mean normalized peak magnitude of the Cl-induced tension transient (De 66) at pMg 3 for 37 adductor magnus fibers was 0.66 ± 0.05 (mean ± SEM, one datum per fiber). However, the distribution of these data, shown as open bars in Fig. 5, was bimodal, with zero fibers exhibiting normalized peak Cl\(^{-}\)-induced tension transients in the range 0.43–0.65. The highest frequency (n = 13) was for normalized peak magnitudes in the 0.91–1.0 range, with the second highest frequency (n = 6) for 0.21–0.30. Repeated stimulation did not significantly alter the normalized peak magnitude of the Cl\(^{-}\)-induced tension transient, as shown in the self-paired data of Table II.

It was at times possible to detect a biphasic response in the Cl\(^{-}\)-induced tension transient, as illustrated in the Fig. 1 tension record of a peeled soleus fiber at pMg 4. The first and second Cl\(^{-}\)-induced tension transients (all at constant [K\(^{+}\)] × [Cl\(^{-}\)]) appear to have a primary component and a secondary overshoot of tension; the magnitude of the overshoot, or secondary component, was augmented at the low Mg\(^{2+}\) concentration (10\(^{-4}\) M) used in this experiment. The
FIGURE 3. Bar graph of normalized peak magnitude of adductor fiber Cl\(^-\)-induced tension transients stimulated by 66 mM Cl\(^-\) at constant [K\(^+\)] \(\times\) [Cl\(^-\)] and 10\(^{-3}\) M Mg\(^{2+}\). The ordinate shows the number of fibers with Cl\(^-\)-induced tension transients in each range of normalized magnitudes (x 100) on the abscissa. The peak magnitude of each Cl\(^-\)-induced tension transient was normalized by dividing it by the peak magnitude of a maximum caffeine contracture for the fiber. The open bars represent the data from the total sample of adductor fibers (n = 37) (one datum per fiber). The striped bars show a subset of the data (n = 20) representing the self-paired data reported in the first column of Table III.

The third Cl\(^-\)-induced tension transient for the peeled soleus fiber in Fig. 1 was elicited in the presence of procaine; procaine blocked the secondary component of the Cl\(^-\)-induced tension transient. The fiber was adequately loaded with Ca\(^{2+}\), despite the smaller release of Ca\(^{2+}\) when stimulated by Cl\(^-\), since the caffeine-induced transient elicited after washing off the procaine was large. The fourth Cl\(^-\)-induced and caffeine-induced tension transients in Fig. 1 are very similar to the ones preceding procaine application, which shows that the effects of the anesthetic were entirely reversible and were not due to deterioration of the fiber.

Procaine significantly reduced the peak magnitude of the Cl\(^-\)-induced tension transients at both pMg 3 and 4, as indicated in Table III. The data of Table III are self-paired at each Mg\(^{2+}\) concentration.

The distribution of the data in the first column of Table III is shown in Fig. 3 (striped bars); these data are not significantly different from the total of all such data represented by the open bars (P > 0.05, Mann-Whitney U test). For the subset of fibers that exhibited a normalized peak magnitude of <0.50 in the

| TABLE II |
| Normalized Peak Magnitude of Adductor First and Second Cl\(^-\)-induced Tension Transients at pMg 3* |
|---------------------|---------------------|
| First De 66         | Second De 66        |
| 0.58±0.08 (14)$     | 0.55±0.09 (14)$     |

* Mean ± SEM (n = number of fibers).
$ Not significantly different (P > 0.05) two-tailed t test for paired samples.
absence of procaine (subset of data in the first column of Table III, n = 8), the effect of procaine was not significant (P > 0.05, two-tailed t test for paired samples). This is illustrated in Fig. 2, where the peak magnitude of the initial Cl⁻-induced tension transient is <0.50 and procaine does not alter it significantly (middle De 66).

Ouabain Blockade of Cl⁻-stimulated Ca²⁺ Release

Perhaps the most interesting finding was that in many fibers the Cl⁻-induced tension transient (primary component) could be blocked by presoaking the fiber in relaxing solution containing ouabain. Out of 34 attempts to block the Cl⁻-induced tension transients with ouabain, 24 were successful (adductor = 19, soleus = 5) in that the Cl⁻-induced tension transient was eliminated, 4 were partial (reduced peak magnitude), and 6 failed.

Table III

|         | pMg 3       | pMg 4       |
|---------|-------------|-------------|
| Without procaine | 0.69±0.08 (20) | 0.29±0.04 (20) |
| With procaine   | 0.41±0.11 (4)  | 0.93±0.03 (4)  |

* Mean ± SEM (n = number of fibers).
1 Significantly different (P < 0.05) two-tailed t test for paired samples.

Fig. 4 shows the tension records for halves of a peeled adductor magnus fiber. The half that was not exposed to ouabain before peeling (upper trace) had sizable Cl⁻-induced and caffeine-induced tension transients at both pMg 3 and 4. Ouabain (10⁻⁵ M) was present in all bathing solutions of the stimulation protocol. However, for the fiber half that was soaked for 2.5 h in 10⁻⁵ M ouabain before peeling (lower trace, Fig. 4), the Cl⁻ response was eliminated at both pMg 3 and 4. Since the caffeine-induced tension transient was unaffected by the ouabain pretreatment, the SR itself appeared unaltered. The only variable for the halves in Fig. 4 was the exposure to 10⁻⁵ M ouabain in the relaxing solution before peeling. For some fibers (n = 5), each half was soaked for 2.5 h and the ouabain-treated half was tested first, yielding results identical to those of Fig. 4.

Saponin Elimination of Cl⁻-induced Tension Transient

Fig. 5 shows the effect of saponin on a peeled adductor magnus fiber. The fiber initially responded with a small Cl⁻-induced tension transient (at constant [K⁺] × [Cl⁻]), followed by a large caffeine-induced one. Soaking the fiber for only 15 s in a 0.5% saponin relaxing solution eliminated or significantly reduced (P < 0.05, two-tailed t test for paired samples, n = 10) the peak magnitude of the Cl⁻-induced tension transient, while not significantly affecting (P > 0.05, two-tailed t test for paired samples, n = 10) the caffeine-induced tension transient. The contractile apparatus was not affected by the saponin since the steady state force generation elicited by the Ca²⁺ in the load solution was the same before and after
saponin treatment. Thus, saponin selectively reduced or eliminated the Cl\(^-\)-induced tension transient.

**Gradation of Cl\(^-\)-induced Tension Transient**

The upper trace of Fig. 6 shows the gradation of the magnitude of the Cl\(^-\)-induced tension transient with variable Cl\(^-\) substitution at constant [K\(^+\)] \times [Cl\(^-\)]

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**FIGURE 4.** The effect of ouabain on the Cl\(^-\)-induced and caffeine-induced tension transients of a peeled adductor magnus muscle fiber (60 \(\mu\)m diam). The traces are for each half of the fiber and show isometric tension generation (ordinate) in time (abscissa) with bathing solution changes given at the time marks. Solution changes are a repetitive pattern of four, as labeled for the first four time marks below each trace. The fiber was loaded with Ca\(^{2+}\), rinsed (Ri), ionically depolarized with 66 mM Cl\(^-\) at constant [K\(^+\)] \times [Cl\(^-\)] (De), and then exposed to 10 mM caffeine (Caf). See the legend to Fig. 1 and the Methods for greater detail regarding bathing solution changes. The fiber halves were mounted in the same transducer and exposed to the same bathing solutions after peeling. Upper trace: this half of the fiber was exposed to 10\(^{-5}\) M ouabain in the bathing solutions only after peeling. Both Cl\(^-\) and caffeine elicited transient contractures at 10\(^{-3}\) and 10\(^{-4}\) M Mg\(^{2+}\). Lower trace: this half of the fiber was soaked in a relaxing solution containing 10\(^{-5}\) M ouabain for 2.5 h (0\(^{\circ}\)C) before peeling; 10\(^{-5}\) M ouabain was also present in all bathing solutions after peeling. The ouabain pretreatment eliminated the Cl\(^-\)-induced but not the caffeine-induced tension transient at 10\(^{-3}\) and 10\(^{-4}\) M Mg\(^{2+}\).
and at increased \([K^+] \times [Cl^-]\). The Cl\(^-\)-induced tension transient for 40 mM Cl\(^-\) (first and third De of Fig. 6) was significantly smaller than that for 66 mM Cl\(^-\) (second De, Fig. 6). The last Cl\(^-\) stimulation of the upper trace in Fig. 6 (Cl) was with 66 mM Cl but increased \([K^+] \times [Cl^-]\); the resulting tension transient was smaller than the preceding one for 66 mM Cl at constant \([K^+] \times [Cl^-]\). The unchanging caffeine-induced releases indicate that variations in the Cl\(^-\)-induced tension transients were not due to changes in SR loading or the contractile apparatus. The lower trace showing the record for the ouabain-pretreated half of this fiber demonstrates that the largest Cl\(^-\)-induced response was completely blocked by loading the fiber with ouabain before peeling; the caffeine-induced tension transient was not affected by the ouabain preloading. All fibers tested with 66 mM Cl\(^-\) stimulation showed a reduced peak magnitude of the Cl\(^-\)-induced tension transient at increased \([K^+] \times [Cl^-]\) relative to that at constant \([K^+] \times [Cl^-]\) (\(P < 0.05\), two-tailed \(t\) test for paired samples, \(n = 10\)).

The peak magnitudes of the Cl\(^-\) tension transients elicited by 40 mM and 66 mM Cl\(^-\) (constant \([K^+] \times [Cl^-]\)), although constant for a given fiber, varied

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{pCa} & 5.6 & 6.5 \\
\text{mM EGTA} & 4 & 0.02 & 0.01 & 0.05 \\
\text{pMg} & 3 & 4 \\
\text{% Saponin} & 0.5 \\
\hline
\end{array}
\]

**Figure 5.** Cl\(^-\)-induced and caffeine-induced tension transients in a single peeled adductor magnus muscle fiber (80 \(\mu\)m diam) before and after exposure to saponin. The trace shows isometric tension (ordinate) in time (abscissa) with bathing solution changes given at the time marks. Bathing solution changes are a repetitive pattern of four, as labeled for the first four time marks, except as noted otherwise. The fiber was loaded with Ca\(^{2+}\), rinsed (Ri), ionically depolarized with 66 mM Cl\(^-\) at constant \([K^+] \times [Cl^-]\) (De), and then exposed to 10 mM caffeine (Caf). See the legend to Fig. 1 and the Methods for greater detail regarding bathing solution changes. The fiber initially responded with both Cl\(^-\)-induced and caffeine-induced tension transients. Before the second load, the fiber was placed in a 0.05 mM EGTA relaxing solution containing 0.5% saponin for 15 s. The saponin was removed by rinsing in an otherwise identical bathing solution. After this saponin treatment, the original four bathing solution changes were repeated. The fiber's steady state tension generation in the load solution was unaltered, which indicates that the contractile apparatus was unaffected by saponin. However, the Cl\(^-\)-induced tension transient was selectively eliminated by the saponin treatment, since the caffeine-induced tension transient was only slightly diminished.
**Figure 6.** Cl\(^{-}\)-induced and caffeine-induced tension transients in halves of a single peeled adductor magnus muscle fiber (80 μm diam) at variable Cl\(^{-}\) concentrations and [K\(^{+}\)] × [Cl\(^{-}\)]. The trace shows isometric tension (ordinate) in time (abscissa) with bathing solution changes given at the time marks. Bathing solution changes are a repetitive pattern of four, as labeled for the first four time marks, except as noted otherwise. The fiber was first loaded with Ca\(^{2+}\), then rinsed (Ri), ionically depolarized with Cl\(^{-}\) at constant [K\(^{+}\)] × [Cl\(^{-}\)] (De 40 or 66 mM Cl\(^{-}\)) or with increased [K\(^{+}\)] × [Cl\(^{-}\)] (Cl), and then exposed to 10 mM caffeine (Caf). See the legend to Fig. 1 and the Methods for greater detail regarding bathing solution changes. Upper trace: this half of the fiber was exposed to 10\(^{-5}\) M ouabain in the bathing solutions only after peeling. The first three Cl\(^{-}\)-induced tension transients (time marks 1–12) are at constant [K\(^{+}\)] × [Cl\(^{-}\)] but varying [Cl\(^{-}\)]; Cl\(^{-}\)-induced tension transients 1 and 3 were elicited by 40 mM Cl\(^{-}\) and transient 2 was elicited by 66 mM Cl\(^{-}\). The 40 mM Cl\(^{-}\) tension transients were smaller than the 66 mM Cl\(^{-}\)-induced tension transient. The last Cl\(^{-}\)-induced tension transient is labeled “Cl” (see Table I) to signify the concurrent increase in [K\(^{+}\)] × [Cl\(^{-}\)] as a result of substituting only 66 mM Cl\(^{-}\) for the propionate. The Cl\(^{-}\) concentration of this last Cl\(^{-}\)-induced tension transient of the upper trace was equal to that of the second one (66 mM Cl\(^{-}\)) but the magnitude was smaller than for 66 mM Cl\(^{-}\) stimulation at constant [K\(^{+}\)] × [Cl\(^{-}\)] (i.e., with concurrent choline substitution for K\(^{+}\)). Lower trace: this half of the fiber was soaked in a relaxing solution containing 10\(^{-5}\) M ouabain for 2.5 h (0°C) before peeling. The ouabain pretreatment eliminated the largest Cl\(^{-}\)-induced tension transient (66 mM Cl\(^{-}\) at constant [K\(^{+}\)] × [Cl\(^{-}\)]) but not the caffeine-induced tension transient. The fiber halves were mounted in the same transducer and exposed to the same bathing solutions after peeling.
relative to each other between fibers. The ratio of the self-paired peak magnitude De 40 to the De 66 Cl\(^-\) induced tension transients for adductor fibers at pMg 3 ranged from 0.00 to 0.94 and the ratio of De 20 to De 66 ranged from 0.00 to 0.81. In some fibers (n = 2), the 40 mM Cl\(^-\) response was absent and the 66 mM Cl\(^-\) tension transient was small, which gave the appearance of an on-off response. However, self-paired data demonstrate that the peak magnitude of the Cl\(^-\) induced tension transient was graded by the Cl\(^-\) concentration of the ionic stimulus. Table IV shows means for the normalized data at the three [Cl\(^-\)] stimulation levels for adductor magnus fiber at pMg 3. Statistical analysis (two-tailed t test for paired samples) for appropriate self-paired subsets of the data of Table IV demonstrates that normalized peak magnitudes of De 20 vs. De 40, De 40 vs. De 66, and De 20 vs. De 66 are all significantly different (P ≤ 0.01). All of the fibers (n = 16) in Table IV showed an increased normalized peak magnitude of their Cl\(^-\) induced tension transient with at least one higher Cl\(^-\) concentration; seven fibers demonstrated gradation with all three Cl\(^-\) concentrations.

### Table IV

| [Cl\(^-\)] in De solution | 20 mM | 40 mM | 66 mM |
|---------------------------|-------|-------|-------|
| 0.32±0.10 (9)             | 0.48±0.09 (15) | 0.67±0.09 (16) |

* Mean ± SEM (n = number of fibers).

Fig. 7 shows the record of a peeled adductor magnus fiber that was not loaded or caffeine-stimulated between successive Cl\(^-\) stimuli at constant [K\(^+\)] × [Cl\(^-\)]; reloading was not essential since the fiber appeared to sequester the Ca\(^{2+}\) released during each Cl\(^-\) stimulation. The fiber was first loaded for 1 min, then rinsed for 1.5 min, and finally stimulated with 20 mM Cl\(^-\). Rather than having caffeine stimulate and reload the fiber as was done previously, the fiber was placed directly back into the rinse solution for a 2-min soak to re-establish resting ionic conditions (66 mM K\(^+\), 4 mM Cl\(^-\)). The next stimulation was 40 mM Cl\(^-\), and, after rinsing again for 2 min, 66 mM Cl\(^-\) was applied. The Cl\(^-\)-induced tension transients were definitely graded by the extent of Cl\(^-\) substitution in the bathing solutions. Even if Ca\(^{2+}\) had been lost from the fiber as a result of not reloading, Ca\(^{2+}\) depletion would not contribute to the observed gradation in magnitude, because of the order of Cl\(^-\) concentration changes.

Stimulating the fiber with 20 mM Cl\(^-\) and then 66 mM Cl\(^-\), without rinsing in between, caused a smaller tension transient for 66 mM Cl\(^-\) than the one elicited when the Cl\(^-\) concentration was abruptly increased from 4 to 66 mM. Cl\(^-\)-induced tension transients were not elicited in these same bathing solutions when the fiber was transferred through them in descending order of Cl\(^-\) concentration. Thus, the response was Cl\(^-\) gradient-specific and was not due to Ca\(^{2+}\) contamination of the bathing solutions.
Figure 7. Cl⁻-induced and caffeine-induced tension transients in a single peeled adductor magnus muscle fiber (50 µm diam) at variable Cl⁻ concentrations but constant [K⁺] × [Cl⁻] in the presence and absence of procaine. The trace shows isometric tension (ordinate) in time (abscissa). The pattern of bathing solution changes is unlike the records of the other figures. The fiber was loaded with Ca²⁺ and then rinsed, all at 4 mM Cl⁻ and 66 mM K⁺. The first Cl⁻-induced tension transient (De) was elicited by 20 mM Cl⁻ and was very small. The fiber was then transferred back to the 4 mM Cl⁻, 66 mM K⁺ rinse solution to repolarize the membranes. Caffeine contractures were not elicited and so reloading of the fiber was not necessary. The next two ionic depolarizations were elicited by 40 and 66 mM Cl⁻, with a rinse in between. The first three Cl⁻-induced tension transients show gradation according to the Cl⁻ concentration gradient; not reloading the fiber before each Cl⁻ stimulation biased against this gradation. After a repolarizing rinse, the fiber was stimulated by changing the Cl⁻ concentration in a stepwise fashion from 20 to 66 mM Cl⁻; although the 20 mM Cl⁻ transient was similar to the first one, the 66 mM Cl⁻ transient was smaller than the 66 mM Cl⁻ one preceding and following it. The fiber was then transferred through the 40 mM, 20 mM, and 40 mM Cl⁻ ionic depolarization solutions; none elicited tension transients when presented in this order after the 66 mM Cl⁻-induced transient. The asterisk marks a bump of the transducer as it was transferred into the rinse solution. The next 66 mM Cl⁻-induced tension transient shows that the full magnitude of the response was recovered by the repolarization in the rinse solution. The caffeine-induced tension transient at the end of this series of depolarizations is as large as the one generated directly after a load and rinse. The final load was followed by a rinse, 66 mM Cl⁻ depolarization, and caffeine stimulation in the presence of 10 mM procaine. The Cl⁻-induced tension transient was present, although reduced, whereas the caffeine-induced tension transient was eliminated by procaine. Washing the fiber in a 0.05 mM EGTA relaxing solution removed the procaine and restored the caffeine-induced tension transient.
As can be seen in the latter half of the Fig. 7 record, the caffeine transient at the end of the series of Cl\(^-\) stimuli is identical to a subsequent caffeine-induced tension transient elicited immediately after loading and rinsing of the fiber. Clearly, the fiber sequestered the Ca\(^{2+}\) released during the various Cl\(^-\)-induced tension transients. The last 66 mM Cl\(^-\) tension transient in Fig. 7 was elicited under conditions of procaine block. Since this tension transient is smaller than the previous Cl\(^-\)-induced ones, it appears that, without procaine, the Cl\(^-\)-induced transients included the secondary Ca\(^{2+}\)-induced component. Thus, the entire Cl\(^-\)-induced release, including the secondary component, was graded by the Cl\(^-\) concentration.

The Cl\(^-\) gradation of the Cl\(^-\)-induced tension transients might have been due to an effect on Ca\(^{2+}\) loading rather than release from the peeled fibers. To assess the effect of ionic depolarization (constant [K\(^+\)] \(\times\) [Cl\(^-\)]) on Ca\(^{2+}\) uptake, peeled adductor fibers were depolarized with 20 or 66 mM Cl\(^-\) during loading; the effect on loading was assayed by comparing peak magnitudes of submaximal caffeine-induced tension transients, elicited under standard conditions, after each load (self-paired data). All of the loading and caffeine release conditions for a given fiber were identical except for the amount of Cl\(^-\) (and choline) substitution during the loading time period. Care was also taken that the Ca\(^{2+}\) released by the caffeine was not saturating the contractile apparatus (i.e., submaximal), since this might have masked actual differences in Ca\(^{2+}\) loading. Rinse solutions with 0.02 or 0.05 mM EGTA were used for loading rather than the usual load solution, in order to better match loading conditions that would be occurring during Cl\(^-\)-induced tension transients. Of the six adductor fibers tested in this manner, the peak magnitude of the submaximal caffeine-induced tension transient after 20 mM stimulation during Ca\(^{2+}\) loading was not significantly smaller than that after 66 mM Cl\(^-\) stimulation (\(P > 0.05\), two-tailed t test for paired samples, \(n = 6\)). However, the Cl\(^-\)-induced tension transients of these fibers were graded by the Cl\(^-\) concentration. These data suggest that the magnitude of the Cl\(^-\) concentration gradient created by the ionic stimulus (constant [K\(^+\)] \(\times\) [Cl\(^-\)]) grades Ca\(^{2+}\) release without significantly affecting Ca\(^{2+}\) re-uptake.

*Frog Peeled Fibers*

A small sample of frog peeled semitendinosus fibers was studied to determine whether the ouabain block of the Cl\(^-\)-induced tension transient was species- or counteranion-specific. Fig. 8 shows tension traces for halves of a single frog fiber stimulated in bathing solutions with propionate as the Cl\(^-\) counterion. The Cl\(^-\)-induced tension transient is selectively blocked in the fiber half that was pre-soaked in ouabain (10\(^{-3}\) M, 2.5 h). Fig. 9 shows traces analogous to those of Fig. 8 for halves of a frog peeled fiber stimulated in bathing solutions with methanesulfonate as the Cl\(^-\) counterion. For both Figs. 8 and 9, the lower trace was recorded immediately after the upper one; thus, both fiber halves were soaked for \(>2\) h before peeling and testing. The data in Figs. 8 and 9 show that it is possible to selectively block the Cl\(^-\)-induced (constant [K\(^+\)] \(\times\) [Cl\(^-\)]) tension transient in frog skeletal fibers by exposing the fibers to ouabain before peeling and that the Cl\(^-\)-induced tension transient and ouabain block of it are not
FIGURE 8. Cl\(^-\)-induced (propionate counterion) and caffeine-induced tension transients in halves of a single peeled frog semitendinous fiber (50 μm diam). Both traces show isometric tension generation (ordinate) in time (abscissa) with bathing solutions changes given at the time marks. Solution changes are a repetitive pattern of five, as labeled for the first fiber marks of each trace. The fiber was loaded with Ca\(^{2+}\), rinsed (Ri) first at 0.05 mM EGTA and then at 0.02 mM EGTA, ionically depolarized with Cl\(^-\) at constant [K\(^+\)] × [Cl\(^-\)] (De), and then exposed to 10 mM caffeine (Caf). The asterisk marks the time at which the fiber was placed in relaxing solution before repeating the cycle. See the legend to Fig 1 and the Methods for greater detail on bathing solution changes. For this fiber, the Cl\(^-\) counterion was propionate. The fiber halves were mounted in the same transducer and exposed to the same bathing solutions containing 10\(^{-5}\) M ouabain after peeling. Upper trace: this half of the fiber was exposed to ouabain only after peeling and was soaked for almost 2.5 h (0°C) in relaxing solution before peeling. Both Cl\(^-\) (pMg 3) and caffeine elicited tension transients. The Cl\(^-\)-induced tension transient decreased in magnitude relative to the caffeine-induced tension transient with the second stimulation. Lower trace: this half of the fiber was soaked in a relaxing solution containing 10\(^{-5}\) M ouabain for 2.5 h (0°C) before peeling. The lower trace was obtained immediately after the upper one. The ouabain pretreatment eliminated the Cl\(^-\)-induced but not the caffeine-induced tension transient.
counteranion-dependent. Interestingly, for the non-ouabain-pretreated half of each frog fiber in Figs. 8 and 9 (upper traces), the Cl\textsuperscript{−}-induced tension transient declines in peak magnitude relative to the caffeine tension transient with each ionic depolarization (De 66).

**Figure 9.** Cl\textsuperscript{−}-induced (methanesulfonate counterion) and caffeine-induced tension transients in halves of a single peeled frog semitendinosus fiber (40 μm diam). Both traces show isometric tension generation (ordinate) in time (abscissa) with bathing solutions changes given at the time marks. Solution changes are a repetitive pattern of five, as labeled for the first fiber marks of each trace. The fiber was loaded with Ca\textsuperscript{2+}, rinsed (Ri) first at 0.05 mM EGTA and then at 0.02 mM EGTA, ionically depolarized with Cl\textsuperscript{−} at constant [K\textsuperscript{+}] \times [Cl\textsuperscript{−}] (De), and then exposed to 10 mM caffeine (Caf). The asterisk marks the time at which the fiber was placed in relaxing solution before repeating the cycle. See the legend to Fig. 1 and the Methods for greater detail regarding bathing solution changes. For this fiber, the Cl\textsuperscript{−} counterion was methanesulfonate. The fiber halves were mounted in the same transducer and exposed to the same bathing solutions containing 10\textsuperscript{−3} M ouabain after peeling. Upper trace: this half of the fiber was exposed to ouabain only after peeling and was soaked for almost 2.5 h (0°C) in relaxing solution before peeling. Both Cl\textsuperscript{−} (pMg 3) and caffeine elicited tension transients. The Cl\textsuperscript{−}-induced tension transient decreased in magnitude relative to the caffeine-induced tension transient with the second stimulation. Lower trace: this half was soaked in a relaxing solution containing 10\textsuperscript{−3} M ouabain for 2.5 h (0°C) before peeling. The lower trace was obtained immediately after the upper one. The ouabain pretreatment eliminated the Cl\textsuperscript{−}-induced but not the caffeine-induced tension transient.


DISCUSSION

Mechanisms of Ca$^{2+}$ Release from Peeled Fibers

The Cl$^-$-induced and caffeine-induced tension transients appear to be due to Ca$^{2+}$ released within the interior of the peeled fibers, since their magnitudes were reduced by increasing the EGTA concentration. Preliminary experiments using $^{45}$Ca as a tracer also show that Cl$^-$- and caffeine-induced tension transients are associated with Ca$^{2+}$ release from the peeled fibers (Donaldson, S. K. B., unpublished data).

Both the Cl$^-$-induced and caffeine-induced tension transients appear to be dependent upon membraneous mechanisms since they are completely eliminated by soaking the peeled fibers in the detergent Brij-58, which should disrupt all membranes (Orentlicher et al., 1974). The source of Ca$^{2+}$ released by Cl$^-$ stimulation appears to be the SR, since eliciting a caffeine-induced tension transient before stimulation with Cl$^-$, which should reduce only the SR Ca$^{2+}$ content, eliminates the Cl$^-$-induced tension transient.

The characteristics of the mammalian caffeine-induced tension transient are similar to those of amphibian skinned fibers and indicate that the mammalian peeled fiber caffeine responses are probably the result of a Ca$^{2+}$-induced Ca$^{2+}$ release mechanism (see Endo, 1977). The Cl$^-$-induced tension transient does not appear to be triggered by a Ca$^{2+}$-induced release mechanism, since (a) a primary component of the Cl$^-$-induced tension transient was not eliminated by procaine, even under conditions where procaine eliminated the entire response to 10 mM caffeine, and (b) the Cl$^-$ response was selectively eliminated by treatments that did not significantly affect the Ca$^{2+}$-induced mechanism of the caffeine response (ouabain presoak, saponin).

However, the secondary, or overshoot, component of the Cl$^-$-induced tension transient did appear to be due to a Ca$^{2+}$-induced mechanism in that it was blocked by procaine (see Endo, 1977). The secondary component was probably initiated by the abrupt rise of [Ca$^{2+}$] of the primary component of the Cl$^-$-induced tension transient. The presence of the secondary Ca$^{2+}$-induced component in some, rather than all, fibers probably accounts for the bimodal distribution of the normalized peak magnitude of the Cl$^-$-induced tension transient (Fig. 3). The fibers having a normalized peak magnitude of their Cl$^-$-induced tension transient in the upper range of values (>0.50) appear to be the ones displaying a secondary component since (a) procaine has a significant effect only on Cl$^-$-induced tension transients in the upper range of peak magnitudes of Fig. 3, and (b) the mean value of the adductor normalized peak magnitude of the Cl$^-$-induced tension transient in the presence of procaine (Table III, column 2) corresponds to that of the lower mode of the bimodal distribution of all such data in the absence of procaine (Fig. 3).

The transient nature of the Cl$^-$-induced Ca$^{2+}$ release might plausibly be due to inactivation of a release process or to depletion of a localized pool of Ca$^{2+}$ or chemical transmitter. Either possibility would also explain the lesser Ca$^{2+}$ release for a stepwise vs. an abrupt change in Cl$^-$ concentration. The transient nature of the Cl$^-$-induced tension transient is not due to generalized SR depletion of Ca$^{2+}$, however, since (a) the fibers clearly had large amounts of Ca$^{2+}$ in the SR
that were releasable by caffeine at the end of each Cl\textsuperscript{−}-induced tension transient, and (b) rinsing the fiber at low Cl\textsuperscript{−} concentration without reloading Ca stores yielded complete recovery of the Cl\textsuperscript{−}-induced tension transient. Although the recovery time at low [Cl\textsuperscript{−}] clearly restored the responsiveness of the adductor fibers to Cl stimulation (see the data of Table II), the normalized peak magnitude of Cl-induced tension transients for frog peeled fibers declined (Figs. 8 and 9) with repeated Cl\textsuperscript{−} stimulation. This decline for frog was not due to deterioration of the SR function or the contractile apparatus since the caffeine-induced tension transients remained relatively stable. It is not known whether this was due to insufficient recovery time at low [Cl\textsuperscript{−}] (load and rinse solutions) or to a selective loss of responsiveness to Cl\textsuperscript{−} in frog peeled fibers.

**Site of Cl\textsuperscript{−} Stimulation**

The Cl\textsuperscript{−}-induced tension transient was selectively eliminated by treatments that should have prevented the polarization of TTs in peeled mammalian fibers. Soaking the fibers in ouabain before peeling them eliminated the Cl\textsuperscript{−}-induced tension transient but not the caffeine response. Ouabain would be expected to block the polarization of any sealed TTs within the peeled fiber, and if the TT membrane is not polarized it should not respond to ionic depolarization (Hodgkin and Horowicz, 1960; Nakajima and Endo, 1973). The finding that ouabain application after peeling did not affect the Cl\textsuperscript{−}-induced Ca\textsuperscript{2+} release is consistent with ouabain’s lipid insolubility (Erdmann et al., 1977; Schwartz et al., 1975) and with the location of the ouabain binding sites on the interior (extracellular side) of any sealed TTs (Venosa and Horowicz, 1981). The failure to block the Cl\textsuperscript{−} induced tension transient by ouabain presoak in some fibers may have been due to damage that impaired ouabain entry into TTs.

The selective reduction or elimination of the Cl\textsuperscript{−}-induced tension transient by saponin is further evidence that the TTs are the locus of Cl\textsuperscript{−} stimulation and ionic depolarization, although saponin may act at the TT-SR junction rather than at TTs per se. The polarization of TTs within the amphibious peeled fibers is plausible, even though TTs have only 5% of the total plasmalemmal Na,K-ATPase sites (Venosa and Horowicz, 1981), because of the high surface-to-volume ratio of the TTs (Mobley and Eisenberg, 1975).

The SR appeared to be unaffected by ouabain and saponin in that it could still sequester and release Ca\textsuperscript{2+} upon caffeine stimulation, although its response to the Cl\textsuperscript{−} stimulation was eliminated or significantly reduced after these treatments. A TT-SR coupling step must have been impaired by the ouabain and saponin.

**Nature of the Cl\textsuperscript{−} Stimulus**

The depolarizing effect of the Cl\textsuperscript{−} stimulus used in this study depends upon the membranes being highly permeable to Cl\textsuperscript{−} and K\textsuperscript{+} and relatively impermeable to propionate and choline. Mammalian TTs are permeable to K\textsuperscript{+} and Cl\textsuperscript{−} (Dulhunty, 1979), and probably impermeable to propionate and choline by analogy with surface membrane properties. The selective inhibition of the Cl\textsuperscript{−}-induced tension transient by ouabain suggests that polarization of TTs is required for the Cl\textsuperscript{−} response. Thus, the Cl\textsuperscript{−} stimulus in this study probably depolarized sealed TTs in the peeled fibers.
The nature of the Cl\textsuperscript{−} stimulation of the SR in this study is perhaps debatable. On the basis of studies of fragmented SR vesicles, the SR is very permeable to Cl\textsuperscript{−}. Cl\textsuperscript{−} is ~50 times more permeant than K\textsuperscript{+}, and choline is relatively impermeant to the SR (Kometani and Kasai, 1978). The propionate permeability of SR vesicles appears to be somewhere between that of K\textsuperscript{+} and Cl\textsuperscript{−} according to the data of Kometani and Kasai (1978), but their measurements suffer from heterogeneous vesicle properties (see Meissner, 1983) and the inherent alterations created by disruption of the SR. However, if the SR propionate permeability were significant, then the substitution of choline Cl for K propionate used in this study would have caused a decrease in SR volume concurrent with depolarization because of the K\textsuperscript{+} gradient.

To clarify this issue, this laboratory has conducted some studies on rabbit peeled fibers using choline Cl substitution of K methanesulfonate. Methanesulfonate is relatively impermeant to the SR (Kometani and Kasai, 1978) and thus the SR should be ionically depolarized without a change in volume when stimulated by choline Cl substitution of K methanesulfonate at constant [K\textsuperscript{+}] × [Cl\textsuperscript{−}]. The preliminary results indicate that peeled adductor fibers with large caffeine contractures that do not respond to Cl\textsuperscript{−} stimulation with propionate as the counterion also fail to respond to the same Cl\textsuperscript{−} stimulus with methanesulfonate as the counterion. Thus, the absence of the Cl\textsuperscript{−}-induced tension transient in peeled rabbit fibers with large caffeine contractures cannot be explained by the nature of the ionic depolarization or the concurrent SR volume change that might have occurred when propionate was used as the Cl\textsuperscript{−} counterion.

The large variation in normalized peak magnitudes of the Cl\textsuperscript{−}-induced (constant [K\textsuperscript{+}] × [Cl\textsuperscript{−}]) tension transients for 20, 40, and 66 mM Cl\textsuperscript{−} is perhaps explained by (a) the variable initial polarization of sealed TTs and (b) the variable size of secondary Ca\textsuperscript{2+}-induced components. The peak tension–membrane potential relationship is quite steep from −58 to −46 mV for K\textsuperscript{+} contractures in intact frog fibers (Hodgkin and Horowicz, 1960) and thus variations in resting membrane potential between fibers would be expected to alter their relative responsiveness to a given ionic depolarization stimulus. Analysis of self-paired data, which should take into account each fiber’s responsiveness and the degree of TT polarization, revealed a significant gradation in Ca\textsuperscript{2+} release stimulated by the various Cl\textsuperscript{−} concentrations.

**SR Depolarization as a Trigger for Ca\textsuperscript{2+} Release**

The failure of Cl\textsuperscript{−} to induce tension transients in the ouabain-presoaked and saponin-treated fibers makes SR depolarization an unlikely trigger mechanism for SR Ca\textsuperscript{2+} release. The SR should have been capable of being ionically depolarized (i.e., Cl\textsuperscript{−}-stimulated) since it was still capable of sequestering and releasing Ca\textsuperscript{2+} in response to caffeine stimulation. Ionic depolarization of the SR also fails to elicit Ca\textsuperscript{2+} release in peeled mammalian cardiac fibers (Fabiato and Fabiato, 1977). However, since the SR membrane potential could not be measured directly, it is not clear whether the SR was in fact ionically depolarized and unresponsive, or the Cl\textsuperscript{−} stimulus did not ionically depolarize the SR. In either
case, it appears that SR depolarization is not an underlying mechanism for the peeled-fiber, Cl\(^-\)-induced tension transient.

**Species Dependence**

The data of Figs. 8 and 9 demonstrate that the major findings of this study are applicable to frog as well as mammalian skeletal fibers. Thus, the Cl\(^-\)-induced tension transient in frog peeled fibers appears to be elicited via a TT-SR coupling step and not via ionic depolarization of the SR.

It is interesting to note that other investigators studying peeled frog skeletal fibers have made observations consistent with TT contribution to Ca\(^{2+}\) releases. Constantin and Podolsky (1967), using electrical stimulation to elicit graded localized contractions of peeled frog fibers in oil, found that high [K\(^+\)] of the bathing solution before peeling the fibers delayed the responses to the current pulses for an interval consistent with TT Na,K-ATPase activity, and, in addition, pretreatment of the fibers with cardiac glycoside abolished the response. Stephenson and Podolsky (1977) noted that the responsiveness of peeled frog skeletal fibers to Cl\(^-\) stimulation was larger when the bathing solution used before peeling had a reduced Cl\(^-\) concentration. The Cl\(^-\) concentration of solutions bathing intact fibers should not affect SR [Cl\(^-\)] but would determine the [Cl\(^-\)] inside sealed TTs and thus the TT [Cl\(^-\)] gradient during Cl\(^-\) stimulation (see Stephenson, 1981).

One difference between the mammalian peeled and frog skinned (split) (Mobley, 1979) and frog peeled (Stephenson, 1983) fiber data is that the tension transient elicited in response to Cl\(^-\) at increased [K\(^+\)] × [Cl\(^-\)] (no cation substitution), which should swell the SR concurrent with the ionic depolarization, was always smaller in rabbit fibers than the Cl\(^-\)-induced tension transient at constant [K\(^+\)] × [Cl\(^-\)]. However, the frog studies used methanesulfonate as the Cl\(^-\) counterion rather than propionate as used here, which may account for or contribute to this apparent difference.

**Physiological Significance of Cl\(^-\)-induced Tension Transient**

Although the Cl\(^-\) stimulation of mammalian peeled fibers appears to involve a TT-SR coupling mechanism, it may not be the physiologic one. The Cl\(^-\) stimulus used in this study is perhaps more akin to that of a K\(^+\) contracture of intact fibers than the TT depolarization eliciting a twitch. The Cl\(^-\)-induced tension transient is far too slow, relative to the known intact fiber twitch time course, to be considered physiological. It is not known to what extent the slow time course of the Cl\(^-\)-induced tension transient is due to an inherently slow mechanism of release vs. other factors resulting from the method of stimulation and monitoring of Ca\(^{2+}\) release. The lowered temperature used in these experiments, along with slow equilibration of Cl\(^-\) within the fibers (Stephenson, 1981) and probable spatial nonuniformities in the distribution of polarized TTs, undoubtedly contributed to the slow time course of the Cl\(^-\)-induced tension transients but would not explain the difference between the twitch and Cl\(^-\)-induced tension transient time courses. Thus, the physiological significance of the TT-SR mechanism
identified in this study of mammalian peeled skeletal muscle fibers remains to be demonstrated.

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REFERENCES

Almers, W. 1978. Gating currents and charge movements in excitable membranes. Rev. Physiol. Biochem. Pharmacol. 82:96–190.

Boyle, P. J., and E. J. Conway. 1941. Potassium accumulation in muscle and associated changes. J. Physiol. (Lond.) 100:1–65.

Costantin, L. L. 1975. Contractile activation in skeletal muscle. Prog. Biophys. Mol. Biol. 29:197–224.

Costantin, L. L., and R. J. Podolsky. 1967. Depolarization of the internal membrane system in the activation of frog skeletal muscle. J. Gen. Physiol. 50:1101–1124.

Costantin, L. L., and S. R. Taylor. 1973. Graded activation in frog muscle fibers. J. Gen. Physiol. 61:424–443.

Donaldson, S. K. 1982. Mammalian skinned muscle fibers: evidence of an ouabain-sensitive component of Cl−-stimulated Ca2+ release. Biophys. J. 37:23a. (Abstr.)

Donaldson, S. K. 1983. Mammalian peeled fibers: Ca2+ release is graded by Cl− and blocked by ouabain. Biophys. J. 41:251a. (Abstr.)

Donaldson, S. K. B., and L. Hermansen. 1978. Differential direct effects of H+ on Ca2+-activated tension generation of skinned fibers from the soleus, cardiac, and adductor magnus muscles of rabbits. Pflügers Arch. Eur. J. Physiol. 376:55–65.

Donaldson, S. K. B., and W. G. L. Kerrick. 1975. Characterization of the effects of Mg2+ on Ca2+ and Sr2+-activated tension generation of skinned skeletal muscle fibers. J. Gen. Physiol. 66:427–444.

Dulhunty, A. F. 1979. Distribution of potassium and chloride permeability over the surface and T-tubule membranes of mammalian skeletal muscle. J. Membr. Biol. 45:293–310.

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. Physiol. Rev. 57:71–108.

Endo, M., and M. Iino. 1980. Specific perforation of muscle cell membranes with preserved SR functions by saponin treatment. J. Muscle Res. Cell Motil. 1:89–100.

Erdmann, E., W. Krawietz, and P. Presek. 1977. Receptor for cardiac glycosides. In Myocardial Failure. G. Riecker, A. Weber, and J. Goodwin, editors. Springer-Verlag, New York. 120–131.

Fabiato, A., and F. Fabiato. 1977. Calcium release from the sarcoplasmic reticulum. Circ. Res. 40:119–129.

Franzini-Armstrong, C. 1971. Studies of the triad. II. Penetration of tracers into the junctional gap. J. Cell Biol. 49:196–203.

Hegyvary, C. 1976. Ouabain-binding and phosphorylation of (Na+ + K+) ATPase treated with N-ethylmaleimide or oligomycin. Biochim. Biophys. Acta. 422:365–379.

Hodgkin, A. L., and P. Horowicz. 1960. Potassium contractures in single muscle fibers. J. Physiol. (Lond.). 153:386–403.
Huxley, A. F., and R. E. Taylor. 1958. Local activation of striated muscle fibres. J. Physiol. (Lond.). 144:426-441.
Lardy, H., P. Reed, and H. C. Lin. 1975. Antibiotic inhibitors of mitochondrial ATP synthesis. Fed. Proc. 34:1707-1710.
Kometani, T., and M. Kasai. 1978. Ionic permeability of sarcoplasmic reticulum vesicles measured by light scattering method. J. Membr. Biol. 41:295-308.
Meissner, G. 1983. Monovalent ion and calcium fluxes in sarcoplasmic reticulum. Mol. Cell. Biochem. 55:65-82.
Meissner, G., and S. Fleischer. 1971. Characterization of sarcoplasmic reticulum from skeletal muscle. Biochim. Biophys. Acta. 241:356-378.
Mobley, B. A. 1979. Chloride and osmotic contractures in skinned frog muscle fibers. J. Membr. Biol. 46:315-329.
Mobley, B. A., and B. R. Eisenberg. 1975. Sizes of components in frog skeletal muscle measured by methods of stereology. J. Gen. Physiol. 66:31-45.
Nakajima, Y., and M. Endo. 1973. Release of calcium induced by ‘depolarisation’ of the sarcoplasmic reticulum membrane. Nat. New Biol. 246:216-218.
Natori, R. 1954. The property and contraction process of isolated myofibrils. Jikeikai Med. J. 1:119-126.
Oetliker, H. 1982. An appraisal of the evidence for a sarcoplasmic reticulum membrane potential and its relation to calcium release in skeletal muscle. J. Muscle Res. Cell Motil. 3:247-272.
Ohtsuki, I., R. M. Manzi, G. E. Palade, and J. D. Jamieson. 1978. Entry of macromolecular tracers into cells fixed with low concentration of aldehydes. Biol. Cell. 31:119-126.
Orentlicher, M., J. P. Reuben, H. Grundfest, and P. W. Brandt. 1974. Calcium binding and tension development in detergent-treated muscle fibers. J. Gen. Physiol. 63:168-186.
Rosemblatt, M., C. Hidalgo, C. Vergara, and N. Ikemoto. 1981. Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle. J. Biol. Chem. 256:8140-8148.
Schwartz, A., G. E. Lindenmayer, and J. C. Allen. 1975. The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. Pharmacol. Rev. 27:3-134.
Somlyo, A. V., H. Shuman, and A. P. Somlyo. 1977. The composition of sarcoplasmic reticulum in situ: electron probe x-ray microanalysis of cryo sections. Nature (Lond.). 268:556-558.
Somlyo, A. V., A. P. Somlyo, H. Gonzalez-Serratos, H. Shuman, and G. McCllelan. 1981. Sarcoplasmic reticulum and mitochondria in excitation-contraction (E-C) coupling in smooth and striated muscle. In The Regulation of Muscle Contraction: E-C Coupling. A. D. Grinnell and M. A. B. Brazier, editors. Academic Press, Inc., New York. 199-214.
Stephenson, E. W. 1981. Activation of fast skeletal muscle: contributions of studies on skinned fibers. Am. J. Physiol. 240:C1-C19.
Stephenson, E. W. 1983. Stimulation of $^{40}$Ca release in skinned muscle fibers by sustained ion gradients. Biophys. J. 41:231a. (Abstr.)
Stephenson, E. W., and R. J. Podolsky. 1977. Influence on magnesium on chloride-induced calcium release in skinned muscle fibers. J. Gen. Physiol. 69:17-35.
Venosa, R., and P. Horowicz. 1981. Density and apparent location of the sodium pump in frog sartorius muscle. J. Membr. Biol. 59:225-232.