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Chloride-induced Ca^{2+} Release from the Sarcoplasmic Reticulum of Chemically Skinned Rabbit Psoas Fibers and Isolated Vesicles of Terminal Cisternae

J.R. Patel, M. Sukhareva, R. Coronado, R. Moss
Department of Physiology, University of Wisconsin School of Medicine, Madison, WI 53706

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Abstract. There is increasing evidence that Ca^{2+} release from sarcoplasmic reticulum (SR) of mammalian skeletal muscle is regulated or modified by several factors including ionic composition of the myoplasm. We have studied the effect of Cl^- on the release of Ca^{2+} from the SR of rabbit skeletal muscle in both skinned psoas fibers and in isolated terminal cisternae vesicles. Ca^{2+} release from the SR in skinned fibers was inferred from increases in isometric tension and the amount of release was assessed by integrating the area under each tension transient. Ca^{2+} release from isolated SR was measured by rapid filtration of vesicles passively loaded with $^{45}Ca^{2+}$. Ca^{2+} release from SR was stimulated in both preparations by exposure to a solution containing 191 mM choline-Cl, following pre-equilibration in Ca^{2+}-loading solution that had propionate as the major anion. Controls using saponin (50 μg/ml), indicated that the release of Ca^{2+} was due to direct action of Cl^- on the SR rather than via depolarization of T-tubules. Procaine (10 mM) totally blocked Cl^-- and caffeine-elicited tension transients recorded using loading and release solutions having $([Na^+] + [K^+]) \times [Cl^-]$ product of 6487.69 mM^2 and 12361.52 mM^2, respectively, and blocked 60% of Ca^{2+} release in isolated SR vesicles. Surprisingly, procaine had only a minor effect on tension transients elicited by Cl^- and caffeine together. The data from both preparations suggests that Cl^- induces a relatively small amount of Ca^{2+} release from the SR by activating receptors other than RYR-1. In addition, Cl^- may increase the Ca^{2+} sensitivity of RYR-1, which would then allow the small initial release of Ca^{2+} to facilitate further release of Ca^{2+} from the SR by Ca^{2+}-induced Ca^{2+} release.

Key words: Sarcoplasmic reticulum — Skeletal muscle — Ca^{2+} release — Chloride anion

Correspondence to: J.R. Patel

Introduction

During excitation-contraction coupling in skeletal muscle, depolarization of T-tubules initiates the release of Ca^{2+} from sarcoplasmic reticulum (SR). Once released, Ca^{2+} activates the myofilament and this accounts for increased tension during the rising phase of the twitch. Ca^{2+} release due to T-tubule depolarization has been demonstrated in mechanically skinned fibers in which the peripheral ends of the tubules close upon skinning, thereby forming sealed sacs. In such preparations, Ca^{2+} release can be elicited from the SR by pre-equilibration in a solution containing less-permeable anions such as propionate or methanesulfonate and subsequent transfer to a solution containing Cl^- (Donaldson, 1985; Lamb & Stephenson, 1990). Transfer to a solution containing a permeating anion rapidly dissipates T-tubule membrane potential (*reviewed* by Schneider, 1981 and by Rios & Pizarro, 1991). On the other hand, biochemical studies have suggested that Cl^- may have direct effects on the SR membrane. Using a vesicular preparation of SR with a low density of functional T-tubule-SR junctions, Kasai and Miyamoto (1976) demonstrated that Ca^{2+} could be released from SR as a result of direct exposure to Cl^-. However, because K-gluconate or K-methanesulfonate was replaced with KCl in these experiments, it was also argued that the observed release of Ca^{2+} from SR vesicles may have involved osmotic swelling and eventual bursting of SR vesicles (Meissner & McKinley, 1976).

Sukhareva, Morrissette and Coronado (1994) reexamined Cl^--induced Ca^{2+} release from heavy SR vesicles by applying Cl^- both symmetrically and asymmetrically across the SR membrane. These studies showed that external Cl^- was much more effective than internal Cl^- in increasing the SR Ca^{2+} permeability. Moreover, Cl^- stimulated Ca^{2+} release when it was equally distributed on both sides of the SR membrane. Cl^--induced release in symmetrical solutions suggested that Cl^- facilitated

Ca^{2+} release from SR occurred not as a consequence of depolarization of SR membrane but due to activation of a Ca^{2+} permeability pathway. Since the ligand dependence of Cl^- induced Ca^{2+} release was markedly different from that of ryanodine receptors, it was suggested that Cl^- induced Ca^{2+} release from SR was due to activation of channels other than the skeletal RYR-1 type ryanodine receptor present in this tissue.

The aim of the present study was to examine the direct role of Cl^- in triggering SR Ca^{2+} release in chemically skinned fibers from rabbit psoas muscle. A useful feature of this preparation is that the structure of the junction between T-tubules and the SR is deranged following chemical skinning with glycerol (Eastwood et al., 1979). Since the gap between T-tubules and SR is bridged by structures that are thought to conduct a mechanical excitatory stimuli from the voltage sensor in the T-tubules to Ca^{2+} release channels in the SR (McPherson & Campbell, 1993), the contribution of T-tubule depolarization to SR Ca^{2+} release is not present in these fibers. To corroborate results obtained in skinned fibers, we also measured time-resolved $^{45}Ca^{2+}$ fluxes under identical conditions in terminal cisternae from the same tissue source. Some of the results have been presented in preliminary form (Patel et al., 1995).

Methods and Materials

Skinning and Attaching of Psoas Fibers

Small bundles of fibers were removed from psoas muscle of New Zealand White rabbits, tied to glass capillary tubes and bathed for 24 hr at 4°C in skinning solution containing 50% glycerol (v/v), (in mM): 100 KCl, 10 imidazole, 1 $MgCl_2$, 2 EGTA and 4 ATP. The bundles were then placed in fresh solution and stored at −20°C for up to 3–4 weeks. On the day of an experiment, a bundle was transferred to glycerol-free solution and skinned single fibers were dissected free (Moss, 1979). The single fibers were then transferred to an experimental trough system and attached to a motor arm and a force transducer arm as described previously by Moss (1979). After attachment, the fibers were stretched to a sarcomere length of 2.3–2.4 μm and were allowed to equilibrate in relaxing solution (*see* Table 1) for 10–15 min before the start of an experiment. All experiments were done at 22–24°C.

Experimental Protocol for Caffeine and Cl^--induced Ca^{2+} Release from SR in Skinned Single Psoas Fibers

The test cycle used to examine caffeine- and Cl^--induced Ca^{2+} release from the SR consisted of five steps. The fibers were transferred from relaxing solution (R) to the following sequence of solutions: (1) a solution containing 100 μM EGTA (PA1, 1 min), (2) a solution containing 10 μM EGTA (PA2, 1 min), (3) a loading solution (L) containing 30 μM Ca^{2+} and 100 μM EGTA (free Ca^{2+} of pCa 7.15, 5 min), (4) a solution containing (in mM): 5 caffeine (Caff1) or 191 choline chloride (Cl1) or 5 caffeine plus 191 choline chloride (Caff2), and (5) finally back to R.

The ability of the fibers to generate maximum tension, was assessed by bathing the fibers sequentially in R, PA1, PA2 and finally in maximally activating solution (MA) in which the free Ca^{2+} in the presence of 5 mM EGTA was pCa 4.5.

Analysis of Tension Transients

Caffeine-induced tension transients are routinely used as an index of Ca^{2+} release from the SR and, hence, were used to assess SR function in skinned fibers prior to measurements of Cl^--induced Ca^{2+} release from the SR. Fibers that failed to developed caffeine-induced tensions greater than 75% of maximum Ca^{2+}-activated tension were discarded from the study.

The use of either peak tension or the integrated area under caffeine-induced tension transients to assess the amount of Ca^{2+} released from the SR in skinned fibers is well documented (Endo, Tanaka & Ogawa, 1970; Horiuti, 1986; Herland, Julian & Stephenson, 1990; Steele, Smith & Miller, 1990). In the present study, we measured integrated area under the tension transients induced by exposing the fibers to solutions containing either choline chloride, caffeine or caffeine plus choline chloride.

Isolation of Rabbit Skeletal Muscle Junctional SR

SR sedimenting in 36% sucrose (w/v) was prepared from leg and back muscle of New Zealand White rabbits by discontinuous sucrose density gradient centrifugation as described by Sukhareva, Morrissette & Coronado (1994). Isolated junctional (heavy) SR was stored at −80°C in 10% (w/v) sucrose, 5 mM NaPIPES, pH 6.8. A total of four preparations, each from a separate animal, were used in this study.

$^{45}Ca^{2+}$ Content of Heavy SR Equilibrated in Cl^--free and Cl^--containing Solutions

To remove sucrose, a thawed aliquot of heavy SR was diluted in 10 volumes of one of two basic solutions: solution A contained (in mM): 191 K-propionate, 70 BES, pH 7.2; solution C contained 133.88 K-propionate, 28.56 KCl, 28.56 TRIS-propionate, 70 BES, pH 7.2 (Table 2). The diluted preparations were kept on ice for ~60 min. Heavy SR was pelleted by centrifugation for 10 min in a benchtop centrifuge (Eppendorf-Brinkmann Instruments) at 12,000 rpm and was then resuspended to a final protein concentration of ~1.5 mg/ml in either solution A or C containing 5 mM $^{45}Ca(acetate)_2$ (2,000 cpm/nmol). At the end of 120 min of passive $^{45}Ca^{2+}$ loading of heavy SR at room temperature, $^{45}Ca^{2+}$ content was determined by rapid filtration of duplicate aliquots for a nominal time of 2 msec at 4 ml/sec filtration rate or by exposure of aliquots to reference solution in the presence of 20 μM ruthenium red (RR). Filters were rinsed under mild vacuum with 2 ml of washing solution containing solution A plus 6 mM $Mg(acetate)_2$ and 20 μM RR. The equilibrated $^{45}Ca^{2+}$ content of the SR was 109.9 ± 12.0 nmoles/mg ($n = 12$) in solution A and 95.9 ± 5.9 nmoles/mg ($n = 18$) in solution C.

Measurements of $^{45}Ca^{2+}$ Release

$^{45}Ca^{2+}$ release from SR was measured by a rapid filtration technique as described previously (Sukhareva, Morrissette & Coronado, 1994) and by manual filtration. Rapid filtration experiments were performed on a Biologic Rapid Filtration Apparatus (Biologic Instruments, Echirolles, France) at a constant filtration rate of 4 ml/sec using type AA 0.8 μm

Millipore filters (Badford, MA). Prior to each rapid filtration, extravesicular free Ca^{2+} concentration was lowered to an estimated nanomolar range by mixing 40 μl of $^{45}Ca^{2+}$-loaded sample (~6 μg protein) to 1 ml of solution A or C containing 5 mM $Mg(acetate)_2$ and 1 mM Na_2EGTA. The diluted SR was immediately spread on to the surface of a filter held in the filtration holder by mild vacuum, and rapid filtration was then done. Manual filtration experiments were done to extend filtration time to ~5 seconds. In these experiments, 40 μl of $^{45}Ca^{2+}$-loaded sample was diluted in 1 ml of rapid filtration solution 5 sec prior to filtration. The concentration of free Ca^{2+} in the rapid filtration solution was reduced from 200 μM to 10 nM by increasing the concentration of Na_2EGTA to 3.5 mM. The composition of filtration solution which induced $^{45}Ca^{2+}$ release in each experiment is described in the figure legends. After rapid and manual filtration, all filters were rinsed under mild vacuum with 2 ml of washing solution. Filters were counted for $^{45}Ca^{2+}$ content in 4 ml of scintillation fluid. Filtration was done in duplicate and then averaged. Background $^{45}Ca^{2+}$ was evaluated using ionophore A23187, as described by Valdivia et al. (1992), and was subtracted from the determination for each filter.

Solutions and Chemicals

Propionic acid, ruthenium red and $^{45}Ca^{2+}$ (1 mCi) was purchased from Fluka (Ronkonkoma, NY), Alpha Products (Andover, MA) and Du Pont-New England Nuclear (Wilmington, DE), respectively. All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). The compositions of solutions used for skinned fiber experiments and for flux experiments in isolated heavy SR are shown in Tables 1 and 2, respectively.

Abbreviations

BES (*N*,*N*-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid); EGTA (ethyleneglycol-bis-(-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetra acetic acid); PIPES (piperazine-*N*,*N*′-bis-2-ethanesulfonic acid).

Results

Increases in SR Ca^{2+} Permeability Induced by Cl^- as Inferred from Tension Transients

Examples of the tension responses of skinned fibers exposed to Cl^- and to caffeine are shown in Fig. 1. In the first protocol (*A*), a transient increase in tension was observed when the fiber, following preincubation in loading solution (L) containing propionate as the major anion, was exposed to a solution containing Cl^- (Cl1) as the major anion. This tension transient is referred to as Cl^--induced tension transient in the remainder of the text. Despite the large amount of Ca^{2+} available for release from the SR, as indicated by the caffeine-induced tension transient in the second protocol (*B*), the fiber did not develop tension when exposed to a test solution containing propionate instead of Cl^- as the major anion or when the loading solution contained Cl^- instead of propionate (*A*). These results indicate that the release of Ca^{2+} from the SR occurs only when propionate is replaced by Cl^-. After recording a Cl^--induced tension transient in the fiber, the integrated area under the caffeine-induced tension transient (record *A*) was smaller than the integrated area under the caffeine-induced transient (record *B*). This suggests that both Cl^- and caffeine can release Ca^{2+} from the SR and indicates that much of the Ca^{2+} released by Cl^- was not resequestered into the SR thereby leading to a smaller caffeine-induced transient. Furthermore, we were able to confirm that the Cl^-- and caffeine-induced tension transients were due to activation of myofilaments by Ca^{2+} released from the SR rather than contaminant Ca^{2+} in the release solutions. The transients were abolished by treating the fibers with Brij-58 (*data not shown*), a detergent which is known to destroy all intracellular membranes including SR (Orentlicher et al., 1974).

Cl^--induced tension transients recorded using the same solutions as in Fig. 1*A* were partially blocked by procaine (*data not shown*), a local anesthetic which is known to inhibit Ca^{2+} release from the SR mediated by activation of release channels (Endo, 1977; Xu et al., 1993). This result suggests that the Cl^--induced tension transient was elicited by the Ca^{2+} released from the SR as a result of activation of release channels (procaine-sensitive). In addition, a component of Cl^--induced release was procaine-insensitive. Because in these experiments, both the $([Na^+] + [K^+]) \times [Cl^-]$ (11872.56 mM^2) and $[K^+] \times [Cl^-]$ (4966 mM^2) product of release solution was greater than that of the loading solution (0.00 mM^2), it is likely that transfer of the fiber from loading to release solution resulted in swelling and some disruption of the SR. Thus some or all of the procaine-insensitive Ca^{2+} release is most likely due to non-specific release of Ca^{2+}.

Although structural linkages between the T-tubule and SR appear to be lost upon treatment with glycerol (Eastwood et al., 1979), we used saponin to ensure that the Cl^--induced tension transients were not due to release of Ca^{2+} from the SR resulting from depolarization of T-tubule. Saponin is a cholesterol precipitating agent that is known to disrupt the T-tubule membrane to a much greater extent than the SR membrane, presumably due to the greater cholesterol content of T-tubules (Rosemblatt et al., 1981). Following a 15-min incubation of the fiber in relaxing solution containing 50 μg/ml saponin, the integrated area of the Cl^-- and caffeine-induced tension transients in Fig. 1 were 105% (record *D*) and 89% (record *E*) of control, respectively, and maximum Ca^{2+} activated tension was 84% (record *F*) of control. These results indicate that, (i) Cl^--induced tension transients in skinned fibers were very unlikely to be due to Ca^{2+} release from the SR as a result of depolarization of T-tubules, (ii) saponin under these conditions of time and concentration does not alter the functional integrity of the SR, and (iii) saponin has no direct effect on the responsiveness of the myofilament to Ca^{2+}.

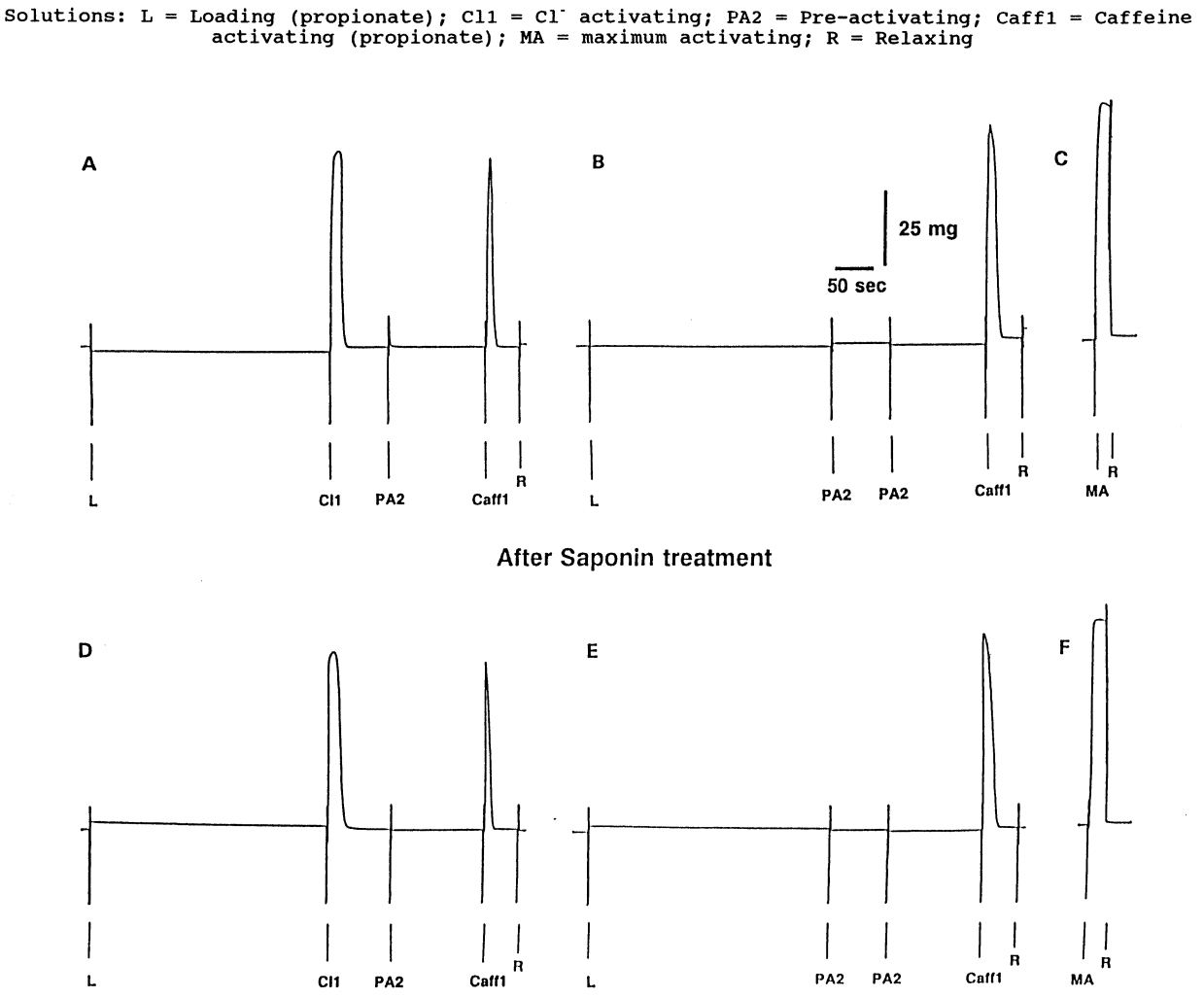

Fig. 1. Lack of effect of saponin on Cl^-- and caffeine-induced Ca^{2+} release from SR in skinned muscle fibers. The compositions of the experimental solutions were similar to those presented in Table 1 except pre-activating (PA2) and loading (L) solution contained 191 mM K-propionate and Tris-propionate and KCl were omitted. The $([Na^+] + [K^+]) \times [Cl^-]$ product of loading and Cl^--activating solution (Cl1) was 0.00 mM^2 and 11872.56 mM^2, respectively. (*A*) The fiber (84 μm diam.) was bathed for 5 min in propionate containing loading solution before exposing first to Cl^--activating solution (Cl1) then to pre-activating solution (10 μM EGTA) and finally to caffeine-activating solution (Caff1). After recording caffeine-induced tension transient, the fiber was returned to relaxing solution (R). In (*B*), the above steps were repeated except that Cl1 was replaced with PA2. In (*C*), maximum tension generating capacity was assessed by transferring the fiber from PA2 to maximum activating solution (MA) and back to R. *D–F* are the same protocols as *A–C* repeated after a 30-min incubation of the fiber in relaxing solution containing 50 μg/ml saponin.

The ability of Cl^- to induce Ca^{2+} release from the SR was corroborated by directly measuring Ca^{2+} permeability of isolated SR using the rapid filtration technique. Isolated heavy SR vesicles were passively loaded with $^{45}Ca^{2+}$ in loading solution (*see* Table 2) containing propionate as the major anion and K^+ as the major cation. Figure 2 shows the time course of $^{45}Ca^{2+}$ release from heavy SR in the presence of ~10 nM free Ca^{2+} when exposed to a release solution containing either K-propionate (curve 1) or a release solution in which propionate and K^+ were replaced by Cl^- and choline$^+$ (curve 2). The amount of Ca^{2+} released from heavy SR was significantly greater when the heavy SR was exposed to choline-Cl rather than K-propionate. In both cases, the release was essentially complete within 50 msec.

Cl^- Concentration-dependence of Tension Transients

Experiments were done to assess the Cl^- dose-dependence of Ca^{2+} release in skinned fibers (Fig. 3). Unlike the experiments described earlier, these experiments were done using loading and release solutions having $([Na^+] + [K^+]) \times [Cl^-]$ product of 6487.69 mM^2 and 12361.52 mM^2, respectively (Table 1). This minimized

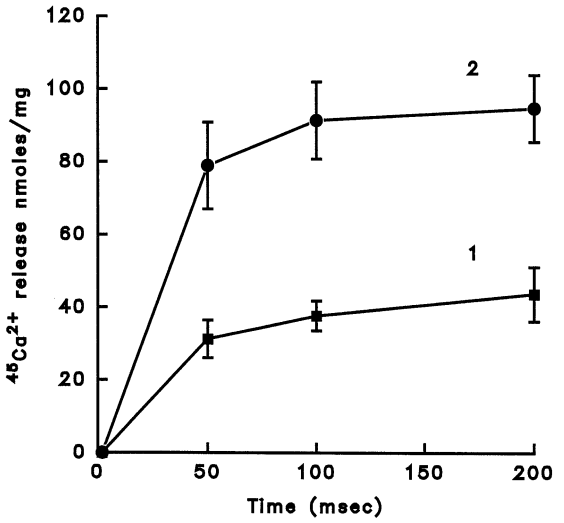

Fig. 2. The time course of Cl^--induced Ca^{2+} release from isolated heavy SR vesicles. Heavy SR vesicles were equilibrated in solution containing 191 mM K-propionate, 70 mM BES, and 5 mM $^{45}Ca(acetate)_2$ (2,000 cpm/nmol). Release of $^{45}Ca^{2+}$ from heavy SR was initiated by rapid filtration in a release solution containing 191 mM K-propionate, 70 mM BES, 10 μM Na_2EGTA (curve 1) or 191 mM choline-Cl, 70 mM BES, 10 μM Na_2EGTA (curve 2). Each data point is the mean ± SEM ($n = 4$).

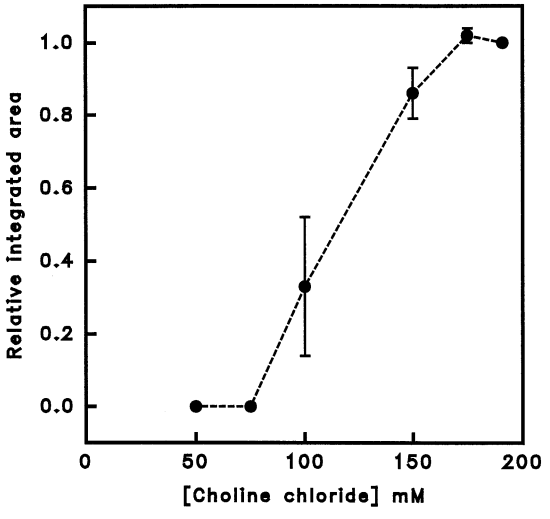

Fig. 3. Cl^- dose-dependent release of Ca^{2+} from the SR in skinned fibers. The compositions of experimental solutions used are shown in Table 1. After incubating the fibers in loading solution for 5 min, the fibers were randomly exposed to solutions (Table 1) containing different concentrations of choline-Cl (50–191 mM). The integrated area under each tension transient was expressed as a fraction of the integrated area of the transient elicited by 191 mM choline-Cl. Each data point is the mean ± SEM ($n = 4$).

the effects on Ca^{2+} release due to osmotic effects discussed above. After incubation in loading solution for 5 min, the skinned fibers were randomly exposed to six concentrations of choline-Cl between 50 and 191 mM. The integrated areas of the tension transients elicited by different concentrations of choline-Cl were expressed as a fraction of the integrated area of the tension transient elicited by 191 mM choline-Cl. The amount of Ca^{2+} released was zero at 50 and 75 mM choline-Cl, increasing to 30–40% at 100 mM, 80–90% at 140 mM, and essentially 100% at 175 mM choline-Cl. These data indicate that Cl^--induced Ca^{2+} release from the SR in skinned fibers is graded and not an all-or-none phenomenon. Most likely, increasing the concentration of Cl^- induces an increase in the number of Ca^{2+} release channels of the SR that are open or available to open, which in turn increases the efflux of Ca^{2+} from the SR. Similar Cl^- concentration-dependence of Ca^{2+} release was observed when isolated SR vesicles were used instead of skinned fibers (*data not shown*).

Effects of Procaine on Cl^--, Caffeine- and Caffeine Plus Cl^--induced Tension Transients

Procaine reversibly blocks ryanodine sensitive Ca^{2+} release channels and prevents activation of these channels by Ca^{2+} and caffeine (Endo, 1977; Xu et al., 1993). Thus, procaine was used in the present study to investigate the contribution of ryanodine sensitive Ca^{2+} release channels to the Cl^--induced tension transients.

Figure 4 illustrates the effect of procaine on 5 mM caffeine-, 191 mM Cl^-, and 5 mM caffeine plus 191 mM Cl^--induced tension transients recorded using loading and release solutions having $([Na^+] + [K^+]) \times [Cl^-]$ product of 6487.69 mM^2 and 12361.52 mM^2, respectively (Table 1). The integrated areas under the Cl^-- (record *B*) and caffeine plus Cl^--induced tension transients (record *C*) were 157% (mean of 152 ± 4%, $n = 7$) and 230% (mean of 235 ± 7%, $n = 7$), respectively, of the area of the caffeine-induced transient (record *A*). Thus, the integrated area of the tension transient elicited by Cl^- alone was somewhat greater than that elicited by caffeine alone. This may be due to either a different potency in triggering Ca^{2+} release from the SR or a lower buffering of released Ca^{2+}, since there was 10 μM rather than 50 μM EGTA in the Cl^--containing release solution. Whichever is the case, the integrated area of the caffeine plus Cl^--elicited tension transient was at least twice that elicited by caffeine alone. Procaine (10 mM) totally inhibited caffeine- (record *D*) and Cl^-- (record *E*) elicited tension transients, but only partially inhibited the transient elicited by caffeine plus Cl^- (record *F*). In this case, the integrated area decreased from 230% of control in record *C* (mean of 235 ± 7%, $n = 7$) to 121% of control in record *F* (mean of 123 ± 12%, $n = 7$). The inhibitory effects of procaine on the caffeine-, Cl^--, and caffeine plus Cl^--induced tension transients were totally

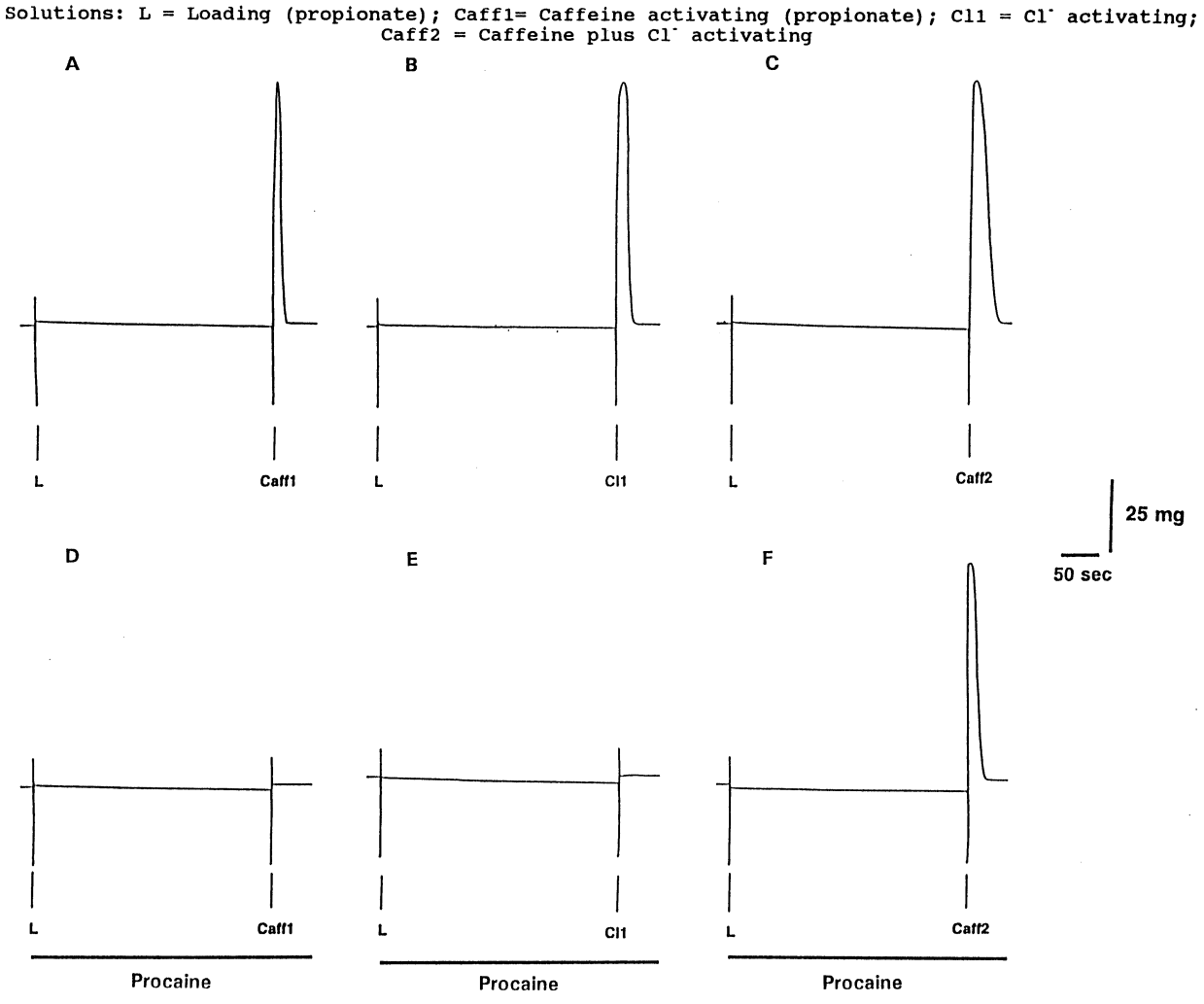

Fig. 4. Effects of procaine on tension transients elicited by caffeine, Cl^- and caffeine plus Cl^-. The compositions of experimental solutions used are shown in Table 1. After incubation in loading solution (L), the fiber (97 μm diam.) was exposed to caffeine solution (Caff1) in the first protocol (*A*), to Cl^- solution (Cl1) in the second protocol (*B*), and to caffeine plus Cl^- solution (Caff2) in the third protocol (*C*). These protocols were repeated with 10 mM procaine in both the loading and releasing solutions (*D*, *E* and *F*, respectively).

reversed by a 15-min incubation of the fiber in relaxing solution. This result suggests that caffeine and Cl^- when applied separately but not together induce Ca^{2+} release by activating procaine-sensitive SR Ca^{2+} channels.

The inhibitory effect of procaine on Cl^--induced Ca^{2+} release was further investigated using isolated vesicles of heavy SR (Fig. 5). Aliquots of SR vesicles were equilibrated in solution containing 5 mM $^{45}Ca^{2+}$ plus solution C (28.56 mM Cl^-). The release of $^{45}Ca^{2+}$ was initiated by a 5-sec exposure to solution B (191 mM Cl^-) containing 10 nM free Ca^{2+}. Figure 5 shows that 10 mM procaine had an inhibitory effect on Ca^{2+} release from isolated SR vesicles, reducing the amount of Cl^--induced Ca^{2+} release by ~60% of control. Thus, the effect of procaine on Ca^{2+} release is qualitatively similar in isolated vesicles and in skinned fibers, but the effect is more nearly complete in fibers.

Discussion

Cl^--induced Ca^{2+} Release from the SR

In the present study, glycerol treatment of rabbit psoas muscles yielded skinned fibers with functional SR Ca^{2+} uptake and release mechanisms. The skinning protocol had no evident deleterious effects on the Ca^{2+} pump or on caffeine-sensitive Ca^{2+} release channels, in as much as the SR accumulated Ca^{2+} when the fibers were incubated in loading solution, and released Ca^{2+} when the fibers were exposed to solution containing caffeine.

Previous studies on mechanically skinned fibers from rabbit soleus muscles indicated that Cl^- could elicit tension transients before but not after treatment with saponin (500 μg/ml for 15-sec incubation, Donaldson,

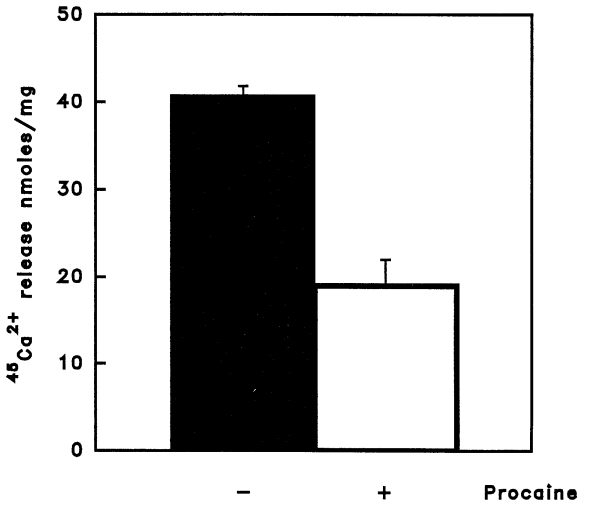

Fig. 5. Effect of procaine on Cl^--induced Ca^{2+} release from isolated vesicles of heavy SR. Vesicles were separately equilibrated in 5 mM $^{45}Ca(acetate)_2$ loading solutions containing 133.88 mM K-propionate, 28.56 mM KCl, 28.56 mM TRIS-propionate, 70 mM BES. Release was initiated by a 5-sec exposure of each sample to solution containing (in mM): 191 choline-Cl, 70 BES, 3.5 Na_2EGTA, 5.83 Na_2ATP, 5 $Mg(acetate)_2$. Bar on the left (-) is data obtained with no procaine in the loading solution and release solutions and bar on the right (+) is data obtained with 10 mM procaine in loading and release solutions. Each bar is the mean plus SEM ($n = 4$).

1985). Based on this observation, it was concluded that Cl^--induced tension transients in mechanically skinned fibers were due to release of Ca^{2+} from SR as a consequence of depolarization of the T-tubules. Using slightly different conditions (50 μg saponin/ml for 30 min, Endo & Inno, 1980), we showed that saponin had no noticeable effect on Cl^--induced tension transients, even though this protocol should disrupt surface membranes including T-tubules. Therefore, in our experiments Cl^- must have activated Ca^{2+} release by directly increasing SR permeability to Ca^{2+} rather than by depolarizing T-tubules. This conclusion was supported by the fact that identical solutions elicited the release of a substantial amount of Ca^{2+} from isolated vesicles of terminal cisternae. Since isolated terminal cisternae SR has a very low density of triads containing T-tubule-SR junctions, and since the Ca^{2+} loading protocol was done in the absence of ATP thereby preventing any polarization of T-tubules, the observed release could only be explained by a direct increase in Ca^{2+} permeability of the SR. Furthermore, the threshold concentration of Cl^- (40–75 mM) for inducing Ca^{2+} release appeared to be higher than the Cl^- concentration (~10 mM) required to depolarize T-tubules (Donaldson, 1985). Thus, while our results do not eliminate a role of T-tubule depolarization in eliciting Ca^{2+} release, they strongly suggest that Cl^- can effect Ca^{2+} release by a mechanism other than T-tubule depolarization.

A swelling of the SR is more likely to occur when the product of permeable cations and anions of the release solution is greater than that of the loading solution. On the basis of the ionic product of all the permeable ionic species of loading and release solutions in Table 1, one would expect the SR to swell and cause Ca^{2+} release. This is because the product $([Na^+] + [K^+]) \times ([Cl^-])$ of the release solution (12361.52 mM^2) was greater than that of the loading solution (6487.69 mM^2), in spite of the fact that the $[K^+] \times [Cl^-]$ product was kept constant (5454.96 mM^2). Thus, using solutions in Table 1, Cl^--induced tension transients may be elicited, at least in principle, by the Ca^{2+} released from the SR as a result of both activation of release channels and a nonspecific disruption of SR membrane due to swelling. Since procaine inhibits Ca^{2+} release as a result of channel activation (Endo, 1977; Xu et al., 1993) but is unlikely to affect release due to swelling of the SR, then the Cl^--induced tension transients recorded using solutions in Table 1 should be comprised of procaine-sensitive and procaine-insensitive components. We have shown (Fig. 4, record *E*) that procaine totally inhibits Cl^--induced tension transients recorded using solutions in Table 1. This result suggested that there may have been a very small, if any, release of Ca^{2+} due to osmotic swelling of the SR arising from the osmotic imbalance caused by the presence of $[Na^+]$ ions in both loading and release solutions. This is consistent with the absence of an effect of Na^+ ion on Cl^--induced tension transients recorded in split fibers from frog semitendinosus muscle (Mobley, 1979). Furthermore, there was neither any loss of $[^3H]$-inulin nor an increase in Ca^{2+} release from isolated SR vesicles when Ca^{2+} efflux from isolated SR vesicles was studied using solutions in Table 2. One plausible explanation for this difference is that the skinned fibers were exposed for a longer duration to release solution than the preparation of isolated SR vesicles.

Neither caffeine (5 mM) nor Cl^- (191 mM) would be expected to release all the SR Ca^{2+} available for release. This is because caffeine in excess of 5 mM is required for maximal release (Fryer & Neering, 1989) and probably because Cl^-, compared to other ligands, is a weak stimulant for Ca^{2+} release. Consistent with this idea, the integrated area of the caffeine plus Cl^--induced tension transients was almost twice that of the integrated area of the Cl^--induced tension transients. It is, however, intriguing that procaine inhibited caffeine$^-$ and Cl^--induced tension transients, but only partially inhibited the response to caffeine plus Cl^-. This suggests that either caffeine- or Cl^--induced Ca^{2+} release persisted in the presence of procaine.

A substantial amount of Ca^{2+} was also released when isolated SR vesicles were pre-incubated in loading solution containing propionate as the major anion and then exposed to solution containing Cl^- as the major

Table 1. Composition of experimental solutions used in skinned fiber experiments (mM)

Solution	EGTA	K-prop.	Tris prop.	Ch.Cl	KCl	Na_2ATP	Mg-acetate	Ca-acetate
Relax	5.00	176.00				5.82	6.25	0.03
PA1	0.10	133.88	28.56		28.56	5.83	6.00	
PA2	0.01	133.88	28.56		28.56	5.83	6.00	
L	0.10	133.88	28.56		28.56	5.83	6.00	0.03
Cl1	0.01			191.0		5.83	6.00	
Caff1	0.05	133.88	28.56		28.56	5.83	6.00	
MA	5.00	171.00				5.88	6.00	5.08

In addition all solutions contained 70 mM BES. Caffeine-activating solution (Caff1 and Caff2) contained 5 mM caffeine. Caff2 contained 191 mM choline chloride (Ch.Cl) instead of potassium propionate (K-prop.), Tris-propionate (Tris prop.) and KCl. Ionic strength was 250 mM and pH of all the solutions was adjusted to 7.2 with sodium hydroxide at 22°C. The free Ca^{2+} in loading solution (L) and maximum activating (MA) solution was pCa 7.15 and 4.5, respectively. ($[Na^+]$ + $[K^+]$) x $[Cl^-]$ product of L and release solution was 6487.69 mM^2 and 12361.52 mM^2, respectively. To maintain ionic strength and the product of ($[Na^+]$ $[K^+]$) × $[Cl^-]$ constant, the changes in the concentration of choline chloride were accompanied by changes in the concentration of K-propionate and Tris-propionate.

Table 2. Composition of solutions in $^{45}Ca^{2+}$ flux experiments (mM)

Solution	K-prop	KCl	TRIS-	Choline-
A	191			
B				191
C	133.88	28.56	28.56	

In addition all solutions contained 70 mM BES and 28.56 mM K^+ (contribution by buffer). Ionic strength was 250 mM and pH of all the solutions was adjusted to 7.2 with sodium hydroxide at 22°C. Solution A or C containing (a) 5 mM Ca (acetate)$_2$ plus ≈2,000 cpm/nmole $^{45}CaCl_2$ and (b) 5 mM Mg(acetate)$_2$ and 1 mM Na_2EGTA were referred to as loading and dilution solution. Solution A, B and C containing 10 μM or 3.5 mM Na_2EGTA were referred to as release solution. In most experiments, the release solution was also supplemented with 5 mM Mg(acetate)$_2$ and 5.83 mM Na_2ATP or 10 mM procaine. Solution A containing 6 mM Mg(Acetate)$_2$ and 20 μM Ruthenium red was referred to as filter rinse solution. $[K^+]$ × $[Cl^-]$ product in solution A was 0.00, and in solution B and C was 5454.96 mM^2.

anion. This is consistent with earlier observations reported by Sukhareva et al. (1994), who used gluconate instead of propionate in the loading solution. A major difference between the present results and those of Sukhareva et al. (1994) was our finding that procaine inhibited 60% of the Ca^{2+} release induced by Cl^- when recorded at long times. This difference may be explained if the rate of procaine binding to RYR1 Ca^{2+} release channels is slower than the rate of Ca^{2+} release via these channels—in such a case Ca^{2+} release via RYR1 would be prevented only by pre-incubating the preparation in procaine, as was the case in the present study.

Possible Release of Ca^{2+} from SR via Two Ca^{2+} Release Channels

Sukhareva et al. (1994) hypothesized that there are at least two Ca^{2+} release channels, i.e., RYR1 channels and Cl^--sensitive channels, in the terminal cisternae of the SR and that Cl^- facilitates Ca^{2+} release from the SR by activating Cl^--sensitive channels rather than RYR1 channels. This hypothesis may be sufficient to explain the data from skinned fiber experiments presented here. In the absence of Cl^-, caffeine activates RYR1 channels and induces Ca^{2+} release from the SR, which in turn activates the myofilament (Fig. 4, test protocol *A*). Once Cl^- is added to the release solution, caffeine not only activates RYR1 channels but also activates Cl^--sensitive release channels, thereby increasing the amount of Ca^{2+} release from the SR, which in turn elicits larger transients than those recorded in the absence of Cl^- (Fig. 4, compare records *C* with *A*). Furthermore, activation of Cl^--sensitive release channels by caffeine persists in the presence of procaine (Fig. 4, record F). The ability of caffeine to cause Ca^{2+} release from the SR by activating channels other than RYR1 is also in agreement with the finding that caffeine induces Ca^{2+} release from SR of mutant mice lacking skeletal muscle RYR1 receptors (Takeshima et al., 1994).

To fully account for our results we further propose that Cl^- is an activator of RYR1 channels, presumably by increasing their sensitivity to Ca^{2+}. This would then explain why in the presence of procaine, a significant proportion (~40% of the total Ca^{2+} released in Fig. 5) of Ca^{2+} was still released when isolated SR vesicles were exposed to Cl^- solution. In skinned fibers, however, the amount of Ca^{2+} released from the SR as a result of activation of the Cl^--sensitive channels must have been below the threshold required for activating the myofilament (Fig. 4*E*). Because of the apparent Cl-induced increase in Ca^{2+} sensitivity of RYR1 channels, the Ca^{2+} released as a result of Cl^--induced Ca^{2+} release then facilitates significant release of Ca^{2+} from the SR via

RYR1 channels, and subsequent activation of the myofilaments (Fig. 4*B*).

In summary, the data from skinned fiber experiments supports the hypothesis (Sukhareva et al., 1994) that there are at least two Ca^{2+} release pathways which when activated facilitate Ca^{2+} release from the SR. Cl^- not only facilitates a small release of Ca^{2+} from the SR by activating Cl^--sensitive Ca^{2+} release channels in the terminal cisternae but also sensitizes RYR1 to Ca^{2+}. As a consequence, the small amount of Ca^{2+} released via Cl^--sensitive Ca^{2+} release channels now activates RYR1 and facilitates a much larger release of Ca^{2+} from the SR by the process of Ca^{2+}-induced Ca^{2+} release. The Ca^{2+} released, as a result of both Cl^--induced Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release, accounts for the full blown tension transients recorded when the skinned fibers, preequilibrated in loading solution containing propionate ions, were then exposed to release solution containing Cl^- ions. Caffeine can activate both the RYR1 and Cl^--sensitive Ca^{2+} release channels in the presence of Cl^- but not propionate ions. This may be because the Cl^--sensitive Ca^{2+} release channels are blocked by propionate ions (Sukhareva et al., 1994).

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