Sarcoplasmic Reticulum Ca\(^{2+}\) Release by 4-Chloro-\(m\)-Cresol (4-CmC) in Intact and Chemically Skinned Ferret Cardiac Ventricular Fibers

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ABSTRACT

The purpose of this study was to determine whether 4-chloro-\(m\)-cresol (4-CmC) could generate caffeine-like responses in ferret cardiac muscle. The concentration dependence of 4-CmC-mediated release of Ca\(^{2+}\) from the sarcoplasmic reticulum was studied in intact cardiac trabeculae and saponin-skinned fibers in which the sarcoplasmic reticulum was loaded with Ca\(^{2+}\). In intact and saponin-skinned preparations isolated from right ventricle, the effect of 4-CmC on sarcoplasmic reticulum Ca\(^{2+}\) content was estimated by analysis of caffeine contracture after application of chlorocresol. In addition, the effects of 4-CmC on maximal Ca\(^{2+}\)-activated tension and the Ca\(^{2+}\) sensitivity of myofibrils were analyzed by using Triton-skinning cardiac fibers. The results show that 4-CmC generates a contractile response in saponin-skinned but not intact fibers. The sarcoplasmic reticulum is implicated in the 4-CmC response; more precisely, in Ca\(^{2+}\) release via the ryanodine receptor. Moreover, 4-CmC, like caffeine, has effects on maximal Ca\(^{2+}\)-activated tension and the Ca\(^{2+}\) sensitivity of myofibrils.

The release of Ca\(^{2+}\) by the sarcoplasmic reticulum is of critical importance to excitation-contraction coupling, and altered intracellular Ca\(^{2+}\) homeostasis has been implicated in heart disease (Sordahl et al., 1973; Baudet et al., 1992). The sarcoplasmic reticulum Ca\(^{2+}\) channel (or ryanodine receptor), a protein with a large cytoplasmic domain showing high affinity for ryanodine, is the main mechanism implicated in the release of Ca\(^{2+}\) from the sarcoplasmic reticulum in cardiac muscle (Ogawa, 1994; Sitsapesan et al., 1995; Franzini-Armstrong and Protasi, 1997). The ryanodine Ca\(^{2+}\)-release channel is activated by Ca\(^{2+}\) and by a large number of chemically diverse substances such as caffeine, halothane, and ryanodine (Lai et al., 1988; Rousseau et al., 1987, 1988; Rousseau and Meissner, 1989; Sitsapesan et al., 1995). Caffeine, which is used to study intracellular Ca\(^{2+}\) homeostasis in striated muscles, can determine the susceptibility of patients to malignant hyperthermia by the in vitro contracture test (Herrmann-Frank et al., 1996b). Effect of caffeine has been tested into planar lipid bilayers (Rousseau et al., 1988) and has shown that this substance that acts on the sarcoplasmic reticulum ryanodine Ca\(^{2+}\)-release channel also increases the number and duration of open events without changing the conductance of the channel. However, caffeine has been found to exert various side effects. In particular, caffeine increases the Ca\(^{2+}\) sensitivity of cardiac and skeletal contractile proteins and inhibits phosphodiesterases (Butcher and Sutherland, 1962; Wendt and Stephenson, 1983).

Chlorocresols, which are preservatives often added to commercial preparations of succinylcholine, recently have been shown to be strong stimulators of the Ca\(^{2+}\)-release channel in skeletal muscle and cerebellum (Zorzato et al., 1993). In heavy sarcoplasmic reticulum vesicles from rabbit skeletal back muscles, 4-chloro-\(m\)-cresol (4-CmC)-stimulated Ca\(^{2+}\) activated ([\(^{3}\)H]ryanodine binding with a half-maximal activation of about 100 \(\mu\)M, which suggests that it could be a potent tool in differentiating the Ca\(^{2+}\) release mechanism between normal muscles and those susceptible to malignant hyperthermia (Herrmann-Frank et al., 1996b).

In biopsies from muscle susceptible to malignant hyperthermia, 4-CmC evoked a caffeine-like contracture and has a concentration threshold lower than that in normal muscle (Herrmann-Frank et al., 1996b). Previous experiments have indicated that ferret heart is a good model for investigating excitation-contraction-coupling mechanisms (Huchet et al., 1992). Moreover, caffeine is a common tool for inducing contractile responses in intact and saponin-skinned cardiac fibers. For example, caffeine elicited a large transient contracture in isolated trabeculae from ferret heart by releasing Ca\(^{2+}\) from intracellular stores (Chapman and Leoty, 1976).

The purpose of this study was to determine whether
4-CmC could generate caffeine-like responses in ferret cardiac muscle. The concentration dependence of 4-CmC-mediated release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum was studied in intact cardiac trabeculae and saponin-skinned fibers in which the sarcoplasmic reticulum was loaded with Ca\textsuperscript{2+}. In both preparations, the effect of 4-CmC on sarcoplasmic reticulum Ca\textsuperscript{2+} content was estimated by analysis of caffeine contracture after application of chloroacetaldehyde. In addition, the effects of 4-CmC on maximal Ca\textsuperscript{2+}-activated tension and the Ca\textsuperscript{2+} sensitivity of myofibrils were analyzed by using Triton-skinned cardiac fibers. The results show that 4-CmC generates a contractile response in saponin-skinned but not intact fibers. The sarcoplasmic reticulum is implicated in 4-CmC response; more precisely, in Ca\textsuperscript{2+} release via the ryanodine receptor. Moreover, 4-CmC, like caffeine, has effects on maximal Ca\textsuperscript{2+}-activated tension and the Ca\textsuperscript{2+} sensitivity of myofibrils.

Materials and Methods

All procedures in this study were performed in accordance with the stipulations of the Helsinki Declarations for the care and use of laboratory animals. Adult male ferrets were anesthetized heavily by an ether vapor flow. After respiratory arrest, the heart was removed quickly and placed at room temperature in a physiological solution (see composition below).

Experiments in Intact Trabeculae. For contractile experiments, free-running trabeculae (50–250 μm in diameter; 5–8 mm in length) were isolated from the right ventricle. The cardiac preparation was placed on a coverslip in a drop of physiological solution, transferred to the experimental chamber, and mounted as described by Chapman and Le ôty (1976a). Briefly, both ends of the muscle were snared carefully by fine platinum wire loops, one fixed to the experimental dish and the other to the tip of a transducer (KD 2300 displacement measuring system; Kaman, Colorado Springs, CO). The preparation was perfused continuously with physiological solution at 20 ml/min, and the system of perfusion described by Chapman and Le ôty (1976a) allows a rapid change of the bathing solution in 0.2 s. A 10-mM caffeine-transient contracture was produced and the fiber was stretched until caffeine contracture amplitude was maximal. During the experiment, no significant change was observed in the characteristics of the transient contracture. The preparation then was perfused with 4-CmC solution at different concentrations for 2 min before application of the caffeine solution (2.5, 5, or 10 mM). The three concentrations of caffeine (2.5, 5, and 10 mM) used in the present experiments do not give identical responses. Moreover, the maximal response was obtained with 10 mM caffeine. Indeed, as shown by Baudet et al. (1992), the application of different concentrations of caffeine (0.1–10 mM) in ferret heart ventricular fibers evoked a transient contracture, whose strength showed a clear dependence on the drug concentration.

In normal Ringer’s solution, the concentration of caffeine that produced 50% of the maximal contracture was 3.71 mM in intact cardiac trabeculae. Consequently, 2.5- and 5-mM caffeine concentrations were chosen as values close to the concentration that produced 50% of the maximal contracture and that induced responses that amplitude allowed to estimate the inhibitory effect of 4-CmC. Furthermore, a concentration of 10 mM caffeine also was tested to study the effect of 4-CmC on maximal responses. The same concentrations of caffeine were used in saponin-skinned fibers to have a satisfactory estimate of the 4-CmC effects on caffeine contractures and also to compare the results with those obtained in intact preparations.

The variation in the amplitude (mN/mm\textsuperscript{2}) of caffeine contracture was normalized to the amplitude of the caffeine contracture before application of 4-CmC (0.1, 0.2, 0.5, 0.4, 0.5, 1, and 2 mM). All experiments were performed at 20°C.

Chemically Skinned Ventricular Fibers. Short, cut bundles (150–300 μm in diameter; 2.0–2.5 mm in length) from papillary muscles of adult ferret heart were dissected and placed in a relaxing solution of pCa 9.0 (pCa = −log Ca\textsuperscript{2+}). Bundles were treated for 30 min in pCa 9.0 solution containing 50 μg/ml saponin (Endo and Iino, 1980). This treatment disrupts the sarcolemma but does not affect the ability of the sarcoplasmic reticulum to accumulate and release Ca\textsuperscript{2+}. The preservation of sarcoplasmic reticulum function is indicated by the ability of caffeine to evoke contractures (Endo and Kitazawa, 1979). For Triton-skinned fibers, preparations were placed for 1 h in a relaxing solution (pCa 9.0) containing 1% (v/v) Triton X-100. After this skinning procedure, the fibers were transferred in a relaxing solution (pCa 9.0) that did not contain Triton X-100 before being mounted in the experimental chamber. This treatment permeabilizes the sarcolemma and the sarcoplasmic reticulum without affecting the biochemical and structural properties of the myofibrils, thereby allowing measurement of the Ca\textsuperscript{2+} sensitivity of contractile proteins and maximal Ca\textsuperscript{2+}-activated tension. The saponin- or Triton-skinned bundles were transferred and mounted in an experimental system, as described by Huchet and Le ôty (1993). This system allowed measurements of the tension developed by the preparation immersed in 2.5-m1 tubes (Nalge Nunc Int., Roskilde, Denmark). These tubes were placed on a rotary plate fixed on a disc placed on a magnetic stirrer (Rotamag 10; Prolabo, Paris, France), and the solutions were mixed continuously with stir bars. Fibers were mounted between two stainless steel tubes. One end of a fiber was snared in a loop of fine hair pulled into a tube glued to a fixed rod that was part of the transducer (KD 2300; 0.5 unshielded; Kaman). The other end of the preparation was snared similarly to a tube glued to a rod that formed the arm of the transducer. The diameter and length of the skinned muscle fibers were measured under a binocular microscope. The preparation was adjusted to slack length and then stretched step by step until the tension developed at pCa 4.5 became maximal. Maximal tension (Tmax) generally was reached when resting length was increased by 20%. All experiments were performed at 22°C.

Ca\textsuperscript{2+} Uptake and Release in Sarcoplasmic Reticulum of Saponin-Skinned Cardiac Muscle Fibers. For the experiments on saponin-skinned fibers, the preparations were immersed successively in five different solutions. This protocol allows the loading of the sarcoplasmic reticulum with Ca\textsuperscript{2+} and then the release of Ca\textsuperscript{2+} from sarcoplasmic reticulum through application of caffeine, which generates a transient contracture (Su and Hasselbach, 1984). The ionic composition of these solutions and the variations in EGTA, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} concentrations are indicated below. The saponin-skinned preparation was placed first in solution 1 (pCa 9.0, 10 mM EGTA, 1 mM Mg\textsuperscript{2+}, 25 mM caffeine), which depleted the sarcoplasmic reticulum of Ca\textsuperscript{2+}. Solution 2 (pCa 9.0, 10 mM EGTA, 1 mM Mg\textsuperscript{2+}) was used to wash out caffeine. Solution 3 (pCa 6.5, 10 mM EGTA, 1 mM Mg\textsuperscript{2+}) was a sarcoplasmic reticulum Ca\textsuperscript{2+}-loading solution obtained by mixing pCa 9.0, 10 mM EGTA, and 1 mM Mg\textsuperscript{2+} with pCa 4.5, 10 mM EGTA, and 1 mM Mg\textsuperscript{2+} in appropriate proportions, and has the same composition in ATP (3.16 mM) and in all other components as pCa 9.0 and pCa 4.5. Solution 4 (pCa 7.0 or 6.5, 0.1 mM EGTA, 0.1 mM Mg\textsuperscript{2+}) was used to wash out caffeine. Solution 5 (pCa 7.0 or 6.5, 0.1 mM EGTA, 0.1 mM Mg\textsuperscript{2+}) was similar to solution 4, but contained different caffeine concentrations (2.5, 5, or 10 mM) added to induce Ca\textsuperscript{2+} release from the sarcoplasmic reticulum. Saponin-skinned fibers were incubated for 2 min in each solution, except in solution 5, for which incubation time was based on contracture duration. Each preparation was run sequentially through load-release cycles. At the beginning of the experiment, two cycles were performed with caffeine. 4-CmC-induced release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum was investigated by using different concentrations of chloroacetaldehyde (0.1, 0.5, 1, or 2 mM) in solution 5 instead of caffeine. Immediately after application of 4-CmC, a fiber was immersed in 10 mM caffeine solution (solution 5) to estimate sarcoplasmic-
mic reticulum Ca\(^{2+}\) content. The reversibility of the effects of 4-CmC was tested by a control cycle without 4-CmC. For each contracture (caffeine or 4-CmC), amplitude (mN/mm\(^2\)), time to peak (s), and half-relaxation time (s) were measured.

**Triton X-100-Skinned Cardiac Muscle Fibers.** Tension-pCa relationships (pCa = \(-\log([Ca^{2+}])\)) were obtained by exposing Triton-skinned fibers sequentially to solutions of decreasing pCa. These intermediate solutions were obtained by mixing pCa 9.0 and pCa 4.5 solutions in appropriate quantities. At the beginning of each experiment, a full set of solutions containing different concentrations of Ca\(^{2+}\) was prepared, and each Ca\(^{2+}\) concentration was duplicated, one serving as the control and the other containing 4-CmC (0.05, 0.1, 0.5, 1, or 2 mM). Isometric tension was recorded continuously on chart paper (Linear Bioblock 1200; Linear Instruments, Reno, NV), and baseline tension was established at the steady state measured in a relaxing solution (pCa 9.0). Data for relative tensions were fitted by using a modified Hill equation (Huchet and Leoty, 1993):

\[
\text{Relative tension} = T/T_{\text{max}} = \frac{[Ca^{2+}]^{nH}/K^{nH}}{1 + [Ca^{2+}]^{nH}}.
\]

The Hill coefficient, nH, and the pCa for half-maximal activation, pCa\(_{50}\) = \(-\log([K]/nH)\), were calculated for each experiment by using linear regression analysis. pCa corresponds to the Ca\(^{2+}\) concentration (M) that induced half-maximal activation: K = 10\(^{-\text{pCa}_{50}}\). The Hill coefficient for each fiber was calculated as the slope of the fitted straight lines. Resting tension was that for pCa 9.0, and \(T_{\text{max}}\) tension was obtained in pCa 4.5. pCa\(_{50}\) expressed the apparent Ca\(^{2+}\) sensitivity of contractile proteins, and nH indicated the cooperativity (Ashley et al., 1991).

**Skinned Fiber Solutions.** The composition of the solutions, i.e., the Ca\(^{2+}\) concentration, was calculated by using the computer program of Godt and Nosek (1986). The basic solutions (pCa 9.0, 4.5, 6.5, and 7.0) used contained: 10 mM (pCa 9.0 and 4.5) or 0.1 mM EGTA (pCa 7.0 or 6.5), 30 mM imidazole, 30.6 mM Na\(^+\), 1 mM (pCa 9.0 and 4.5) or 0.1 mM Mg\(^{2+}\) (pCa 7.0 or 6.5), 3.16 mM Mg-ATP, 12 mM phosphocreatine, and 0.3 mM dithiothreitol. Ionic strength was adjusted to 160 mM with KCl, and the pH was adjusted to 7.1 with HCl or KOH. In saponin-skinned fiber experiments, solutions also contained phosphocreatine kinase (17.5 UI/ml) and sodium azide (1 mM). EGTA and phosphocreatine were obtained from Sigma Chemical Co. (St. Louis, MO), and 4-CmC was purchased from Fluka (Neu Ulm, Germany) and prepared as a stock solution (0.25 M) in dimethyl sulfoxide.

**Physiological Solutions.** The control physiological solution contained: 140 mM NaCl, 6 mM KCl, 3 mM CaCl\(_2\), 5 mM glucose, and 5 mM HEPES. The pH was adjusted to 7.35 with Tris base.

**Fitting of Inhibition Curves.** For the test of inhibition of caffeine contracture, the percentage of decrease of response amplitude was estimated as compared with caffeine contracture in control conditions. The points obtained at various concentrations of 4-CmC were fitted by a sigmoid equation. IC\(_{50}\) was the 4-CmC concentration producing half-maximal inhibition of caffeine contracture amplitude, and n was the slope of the linear section of the curves.

**Statistical Analysis.** All values are expressed as means ± S.E.M. Student’s unpaired t test was used to compare the different parameters among groups. Statistical significance was reached when \(P \leq .05\).

**Results**

**Effects of 4-CmC on Resting Tension and Caffeine Contracture in Intact Trabeculae from Ferret Heart.** The application of different concentrations of 4-CmC (0.1–2 mM) to quiescent isolated trabeculae produced no detectable change in resting tension (Fig. 1), possibly because of a lack of effect on the Ca\(^{2+}\)-release mechanism of sarcoplasmic reticulum. To test this possibility, caffeine contractures were elicited after application of 4-CmC on intact cardiac trabeculae. Three concentrations of caffeine were chosen (2.5, 5, and 10 mM) to see whether the possible effect of 4-CmC was related to the concentration of caffeine. Figure 1 shows an example of the transient responses elicited by 2.5 mM caffeine in control conditions (first trace) and then after application of different concentrations of 4-CmC for 2 min. Treatment of trabeculae with 4-CmC reduced the amplitude of caffeine contracture and accelerated the relaxation phase of caffeine contracture. These results strongly suggest that 4-CmC decreases sarcoplasmic reticulum Ca\(^{2+}\) content in a dose-dependent manner in intact trabeculae without causing a rise in tension. The decrease in the amplitude of caffeine contracture (2.5, 5, or 10 mM) was plotted as a function of 4-CmC concentrations. The fitted curves obtained (Fig. 2) were similar for all three concentrations of caffeine. In the presence of 4-CmC, whatever caffeine concentrations were tested (2.5, 5, or 10 mM), the IC\(_{50}\) was not significantly different, i.e., \((P \geq .05)\): 0.27 ± 0.01 (n = 5), 0.29 ± 0.04 (n = 6), and 0.29 ± 0.03 (n = 5) mM 4-CmC for 2.5, 5, and 10 mM caffeine, respectively. Moreover, the slopes of the caffeine inhibition curves (n) were not significantly different, i.e., \((P \geq .05)\): 0.27 ± 0.01 (n = 5), 0.29 ± 0.04 (n = 6), and 0.29 ± 0.03 (n = 5) mM 4-CmC for 2.5, 5, and 10 mM caffeine, respectively.
sigmoid. The slope of the curves (n) and the IC50 are indicated for each
compared with the control. Points are connected by a curve fitting a
caffeine contracture amplitudes for each concentration of 4-CmC, as
relaxation time of the contracture induced by 1 mM 4-CmC or
contractions but not significantly different. For example, the half-
half-relaxation time, was faster than for caffeine contrac-
tures (for 0.1, 0.5, and 1 mM), i.e., time to peak and
concentration: 5 mM (Fig. 4B). The kinetics of 4-CmC con-
amplitude of caffeine contracture was obtained for a higher
was obtained for 2 mM 4-CmC. On the contrary, maximal
percentage of inhibition of 2.5 (\(n = 5\)), 5 (\(n = 6\)); and 10 (\(n = 5\)) mM
results showed that the IC50 was similar for the three con-
centrations of caffeine tested. To analyze the effect of 4-CmC
at the sarcoplasmic reticulum level, experiments then were
performed on saponin-skinned fibers in which functional sar-
coplasmic reticulum was preserved and sarcolemma was dis-
rupted.

Effects of 4-CmC on Sarcoplasmic Reticulum Ca2+-
Release in Saponin-Skinned Cardiac Fibers of Adult
Ferret. Comparison with Caffeine. In saponin-skinned
fibers in which functional sarcoplasmic reticulum has been
preserved, caffeine induces a transient contracture that in-
dicates the presence of a definite amount of Ca2+ released
by the sarcoplasmic reticulum. In our experiment, the caffeine
in the Ca2+ releasing solution (solution 5) was replaced with
4-CmC at different concentrations (0.1, 0.5, 1, and 2 mM).
4-CmC induced a transient contracture in a dose-dependent
manner (Fig. 3), yielding the dose-response curve plotted in
Fig. 4A. The concentration threshold for 4-CmC (0.1 mM) was
similar to that obtained with caffeine. At pCa 7.0, 4-CmC
concentrations greater than 2 mM have been tested. Five and
10 mM 4-CmC induced smaller contractures than that ob-
tained for 2 mM. For example, 2 mM 4-CmC contracture
amplitude represented 28.0 ± 4.0% of \(T_{\text{max}}\) (n = 5) whereas
5 and 10 mM 4-CmC induced contractile responses of 27.0 ±
2.0 and 17.0 ± 3.5% of \(T_{\text{max}}\) (n = 4), respectively. According
to the concentrations of 4-CmC tested, the maximal response
was obtained for 2 mM 4-CmC. On the contrary, maximal
amplitude of caffeine contracture was obtained for a higher
concentration: 5 mM (Fig. 4B). The kinetics of 4-CmC con-
tactures (for 0.1, 0.5, and 1 mM), i.e., time to peak and
half-relaxation time, was faster than for caffeine contrac-
tures but not significantly different. For example, the half-
relaxation time of the contracture induced by 1 mM 4-CmC or
caffeine was 14.5 ± 2.9 s (n = 5) and 22.7 ± 7.0 s (n = 6),
respectively. However, 4-CmC contractures at a given
concentration were always smaller than those of caffeine. For 1
mM 4-CmC and caffeine, the relative amplitude of the con-
tructure (amplitude/\(T_{\text{max}}\) control) was 0.14 ± 0.04 (n = 5) and
0.38 ± 0.07 (n = 5), respectively (Fig. 4). This could be
explained if less Ca2+ was released by 4-CmC than by caf-
feine.

Furthermore, if 4-CmC affected the contractile apparatus,
the tension developed by the application of 4-CmC on sapo-
in-skinned fibers would be an underestimated image of the
Ca2+ released by this substance.

A similar protocol to the one described for intact fibers was
used to determine whether 4-CmC and caffeine were active
on the same Ca2+-release mechanism. When different con-
centrations of 4-CmC (0.1, 0.5, 1, and 2 mM) were applied for
1 min before caffeine solution, the caffeine contractures (2.5,
5, or 10 mM) were greatly reduced. For example, a 5-mM
caffeine contracture was decreased by about 72.8 ± 3.1% (n =
5) after application of 2 mM 4-CmC.

The decrease in the amplitude of caffeine contracture (2.5,
5, or 10 mM) was estimated as compared with control condi-
tions. The data plotted relative to the concentrations of 4-CmC showed a sigmoid relationship (Fig. 5). The IC50 values
of 2.5-, 5-, and 10-mM caffeine contractures were not
significantly different (\(P \geq .05\)): 0.89 ± 0.10 (n = 5), 0.86 ±
0.07 (n = 5), and 0.96 ± 0.23 (n = 7) mM 4-CmC. The slopes
of the inhibition curves (n) for the three caffeine concentra-

![Graph showing inhibition curves for 4-CmC](https://i.imgur.com/3o9.png)

**Fig. 2.** Inhibition curves of caffeine contracture for different concentrations of 4-CmC on ferret intact trabeculae. Data are shown as the percentage of inhibition of 2.5 (●, n = 5), 5 (○, n = 6), and 10 ▼, n = 5) mM caffeine contracture amplitudes for each concentration of 4-CmC, as compared with the control. Points are connected by a curve fitting a sigmoid. The slope of the curves (n) and the IC50 are indicated for each caffeine concentration in text. Vertical bars represent ±S.E.M.
tions (2.5, 5, and 10 mM) were not significantly different ($P < .05$): $1.48 \pm 0.04$ ($n = 5$), $1.19 \pm 0.16$ ($n = 5$), and $1.24 \pm 0.10$ ($n = 7$). Thus, 4-CmC decreased sarcoplasmic reticulum Ca$^{2+}$ content in saponin-skinned cardiac fibers by releasing Ca$^{2+}$, which suggests that the effect of 4-CmC might be related to the activation of sarcoplasmic reticulum Ca$^{2+}$-release channels, as reported for skeletal muscle (Herrmann-Frank et al., 1996a).

Previous experiments on cardiac muscle investigated whether caffeine acts on the ryanodine receptor in ferret cardiac fibers (Bers, 1987). It is known that high concentrations of ryanodine (i.e., $\geq 10 \mu M$) place the ryanodine receptor in a low-conductance state or inhibit its activation (Meissner, 1986; Lattanzio et al., 1987; Nagasaki and Fleischer, 1988; Sitsapesan et al., 1995). In our experiment, ryanodine was added to a caffeine solution, and this final solution was applied during many challenges until caffeine contracture disappeared. Indeed, Alderson and Feher (1987) reported that ryanodine can produce an effect only if its receptor is activated.

Because our results seemed to indicate that the ryanodine receptor was activated by millimolar concentrations of 4-CmC, we wanted to confirm this hypothesis. Ryanodine ($100 \mu M$) was added to a 10-mM caffeine solution and applied successively (three challenges) until caffeine contracture disappeared [2.1 $\pm$ 0.6 mN/mm$^2$ ($n = 6$) after ryanodine treatment is significantly different from 14.6 $\pm$ 2.6 mN/mm$^2$ ($n = 6$) in control conditions]. After this ryanodine treatment, the contracture induced by 1 mM 4-CmC [4.4 $\pm$ 0.3 mN/mm$^2$ ($n = 6$) in control conditions] was reduced strongly and decreased significantly by 97.3 $\pm$ 1.3% (Fig. 6). In another experiment, ryanodine ($100 \mu M$) was applied in the presence of 4-CmC (1 mM) until the 4-CmC contracture disappeared (three challenges were necessary). In this case, caffeine contracture after the ryanodine treatment also was decreased strongly. These different results indicate that 4-CmC, as caffeine, acts on the ryanodine receptor.

Effects of Ca$^{2+}$ Concentration on Ca$^{2+}$ Release Induced by Caffeine and 4-CmC. It has been shown that Ca$^{2+}$ plays the major role in regulating cardiac sarcoplasmic reticulum Ca$^{2+}$-release channel activity (Xu et al., 1996). Moreover, it has been shown that Ca$^{2+}$ shifts dose-response curves to lower 4-CmC concentrations in sarcoplasmic reticulum vesicles of skeletal muscle when pCa 7 and pCa 6 are used (Herrmann-Frank et al., 1996a). Nevertheless, Györke et al. (1994) demonstrated that the Ca$^{2+}$ sensitivity and Ca$^{2+}$ activation rate of single cardiac and skeletal sarcoplasmic reticulum Ca$^{2+}$ channels are similar when reconstituted in artificial planar bilayers, but in the cell there may be other factors that regulate calcium-induced calcium release. In this context, we tested the effects of cytoplasmic Ca$^{2+}$ concentration on Ca$^{2+}$ release induced by 4-CmC (0.1, 0.5, 1, and 2
mM) and compared the results with those obtained at pCa 7.0. The same experiments as those reported previously and carried out at pCa 7.0 ($10^{-7}$ M of intracellular Ca$^{2+}$) were realized by using a modified intracellular Ca$^{2+}$ concentration of $10^{-6.5}$ M (pCa 6.5). Up to 1 mM, 4-CmC induced similar responses for the two pCa tested (Fig. 4A). In the presence of 1 mM 4-CmC, contracture amplitude was $2.9 \pm 0.5$ mN/mm$^2$ ($n = 5$) and $2.0 \pm 0.7$ mN/mm$^2$ ($n = 5$) at pCa 7.0 and 6.5, respectively.

These results suggest that the increase of Ca$^{2+}$ concentration (pCa 7.0–6.5) in saponin-skinned cardiac fibers does not change the amplitude of the contracture induced by 4-CmC. At Ca$^{2+}$ concentrations lower than $10^{-7}$ M, 4-CmC produces no contractile response. Moreover, at Ca$^{2+}$ concentrations higher than $10^{-6.5}$ M, it was impossible to dissociate 4-CmC response from spontaneous oscillations. However, it would be interesting to test a larger range of Ca$^{2+}$ concentrations by using another approach, such as activation of calcium-release channels on sarcoplasmic reticulum vesicles, to see whether 4-CmC response might be a result of a Ca$^{2+}$-dependent mechanism.

Effects of 4-CmC on Properties of Contractile Proteins. The maximal Ca$^{2+}$-activated tension and apparent Ca$^{2+}$ sensitivity of contractile proteins were analyzed in the absence and presence of different concentrations of 4-CmC (0.05, 0.1, 0.5, 1, and 2 mM) in cardiac Triton-skinned fibers (Fig. 7). In heart fibers, a significant decrease in maximal Ca$^{2+}$-activated tension was observed in the presence of 4-CmC (Table 1), i.e., $16.9 \pm 3.3\%$ for 0.1 mM 4-CmC ($n = 5$) as compared with control fibers. This effect was enhanced with increasing concentrations. For each concentration of 4-CmC, the dose-response curves obtained with saponin-skinned fibers were recalculated by reporting the amplitude of 4-CmC contracture (mN/mm$^2$) to the T$_{max}$ developed in the presence of 4-CmC (Fig. 9). The results showed that up to 2 mM, 4-CmC contractures were lower than caffeine but not significantly different.

For concentrations of 4-CmC lower than 0.5 mM, the pCa$_{50}$ was not decreased significantly, whereas the Hill coefficient was increased significantly above control values. On the contrary, for the tension-pCa relationship (Fig. 8), a significant change was found in heart fibers in the presence of 4-CmC concentrations above 0.5 mM. Compared with the control, tension-pCa curves were shifted leftward, i.e., apparent Ca$^{2+}$ sensitivity increased in a dose-dependent manner from 0.5 mM 4-CmC. As shown in Fig. 8, the tension-pCa relationship

![Fig. 6](image1.png) 4-CmC contracture (1 mM) after ryanodine treatment (100 μM) in cardiac saponin-skinned fibers at pCa 7.0. The experiments were conducted as indicated: cycles 1, 2, 3, and then 4. The cycle consists of the saponin-skinned fibers protocol that leads to a contracture. The first two traces correspond to the contractures obtained with 10 mM caffeine (1) and 1 mM 4-CmC (2) in control conditions, i.e., before ryanodine treatment. The last traces (3 and 4) were obtained after ryanodine treatment. Trace 3 represents the tension developed in the presence of 10 mM caffeine plus 100 μM ryanodine after three running challenges. Trace 4 is the 1-mM 4-CmC response after ryanodine treatment.

![Fig. 7](image2.png) Effect of 4-CmC on Ca$^{2+}$-activated tension and maximal Ca$^{2+}$-activated tension (pCa 4.5) in cardiac Triton-skinned fibers. Representative traces of Triton-skinned fibers were obtained from cardiac muscle. Tension was induced by soaking fibers in solutions of decreasing pCa not containing (a–f) or containing (a’–f’) 2 mM 4-CmC. Values for pCa were: a, a’ = 6.5; b, b’ = 6.25; c, c’ = 6; d, d’ = 5.875; e, e’ = 5.75; f, f’ = 4.5. Then, fiber was returned in pCa 9.0, not containing (g) or containing (g’) 2 mM 4-CmC.
TABLE 1
Effect of 4-CmC on maximal Ca$^{2+}$-activated tension (pCa 4.5), pCa$_{50}$, and the Hill coefficient in Triton X-100-skinned cardiac fibers

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{max}}$</th>
<th>pCa$_{50}$</th>
<th>nH</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.0 ± 6.0</td>
<td>6.02 ± 0.03</td>
<td>1.91 ± 0.26</td>
<td>10</td>
</tr>
<tr>
<td>4-CmC (i): 0.05 mM</td>
<td>36.1 ± 3.8</td>
<td>5.99 ± 0.01</td>
<td>2.52 ± 0.06$^a$</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>34.1 ± 2.3$^a$</td>
<td>6.00 ± 0.02</td>
<td>2.36 ± 0.08$^a$</td>
<td>5</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>29.3 ± 3.8$^a$</td>
<td>6.00 ± 0.02</td>
<td>2.32 ± 0.26$^a$</td>
<td>5</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>24.8 ± 4.6$^a$</td>
<td>6.07 ± 0.02$^a$</td>
<td>2.16 ± 0.16$^a$</td>
<td>10</td>
</tr>
<tr>
<td>1 mM</td>
<td>20.9 ± 3.3$^a$</td>
<td>6.13 ± 0.02$^a$</td>
<td>1.75 ± 0.08$^a$</td>
<td>8</td>
</tr>
</tbody>
</table>

The means (±S.E.M.) for pCa$_{50}$ were obtained by fitting the curves to the Hill equation. The means (±S.E.M.) of the Hill coefficient (nH) were obtained from the Hill plot curve; n represents the number of fibers.

$^a$ Significant difference from the value obtained in control conditions at $P \leq .05$.

Discussion

Our results show that 4-CmC (0.1, 0.5, 1, and 2 mM) applied to saponin-skinned fibers induced a variation of tension related to Ca$^{2+}$ release from the sarcoplasmic reticulum mediated mainly by the ryanodine receptor.

4-CmC recently has been described as a specific, potent activator of ryanodine receptor in skeletal muscle cells, and could replace caffeine as a useful pharmacological tool for the detection of malignant hyperthermia (Herrmann-Frank et al., 1996b). Moreover, 4-CmC can be used at concentrations 10-fold lower than those of caffeine. Caffeine is known to induce contractile responses in a dose-dependent manner in cardiac muscle (Chapman and Léoty, 1976b), whereas 4-CmC failed to produce a contractile response on intact fibers in the present study. In intact fibers, the absence of responses resulting from the application of 4-CmC could be due to a slow diffusion of 4-CmC across the sarcolemma, and then the calcium would not be released rapidly enough to result in a contracture. Such a possibility was not supported by the experiments conducted in skeletal muscle, where 4-CmC produces a contractile response (Herrmann-Frank et al., 1996a). Another possibility would be a lack of Ca$^{2+}$ release from the sarcoplasmic reticulum. However, the decrease of caffeine contracture in a dose-dependent manner after treatment with 4-CmC indicated that sarcoplasmic reticulum Ca$^{2+}$ content was reduced by 4-CmC. Moreover, in saponin-skinned fibers in which the sarcolemma was disrupted and the sarcoplasmic reticulum was preserved, 4-CmC induced a contractile response. It has been shown previously that 4-CmC had no effect on the sarcoplasmic reticulum Ca$^{2+}$-ATPase in PC12 cell vesicles (Zorzato et al., 1993) and in intact skeletal muscle (Westerblad et al., 1998). Then it could be proposed that the absence of 4-CmC contracture was related to a Ca$^{2+}$ extrusion by sarcolemmal Ca$^{2+}$ mechanisms. Particularly, it has been demonstrated that in ferret heart the relaxation of caffeine contracture was mainly a result of sarcolemmal Ca$^{2+}$-ATPase and Na$^+/Ca^{2+}$ exchanger (Bassani et al., 1995). A possible stimulation of these mechanisms by 4-CmC would support further the absence of 4-CmC contracture in intact ferret heart. In saponin-skinned fibers, the disappearance of 4-CmC contracture after application of 100 μM ryanodine associated with caffeine and the activation of ryanodine receptor by 4-CmC confirm that 4-CmC releases Ca$^{2+}$ from the sarcoplasmic reticulum, probably by direct activation of the ryanodine receptor. 4-CmC, which is considered to be a specific activator of the skeletal ryanodine receptor (Herrmann-Frank et al., 1996a), also appears to be an activator of the cardiac ryanodine receptor. Our results indicate that inhibition of caffeine contracture by 4-CmC was not dependent on the concentration of caffeine used in intact and saponin-skinned fibers; this suggests that the potential site of 4-CmC action on the ryanodine receptor is different from that of caffeine. These results are consistent with those of Herrmann-Frank et al. (1996a), who suggested that 4-CmC acts on a different binding site than caffeine on sarcoplasmic reticulum vesicles. Moreover, their experiments indicated that 4-CmC activated the isolated channel more strongly when applied from luminal binding sites. These authors have shown that 4-CmC acts preferentially on the trans face of sarcoplasmic reticulum vesicles of skeletal muscle whereas caffeine acts mainly on the cis face as reported by Sitsapesan et al. (1995).
One reason for using 4-CmC on saponin-skinned fibers of adult ferret would be to activate Ca\(^{2+}\)-release channels without producing side effects on contractile proteins. The use of Triton-skinned fibers made it possible to determine the effects of 4-CmC on the myofibrillar Ca\(^{2+}\) responsiveness of mammalian cardiac muscle. At concentrations above 0.5 mM, 4-CmC increased the Ca\(^{2+}\) sensitivity of the contractile apparatus. With 1 mM 4-CmC, the increase was significant for the pCa\(_{50}\) control, showing a direct effect on sensitivity to the Ca\(^{2+}\) of contractile proteins. This effect could not be attributed to any action on ryanodine Ca\(^{2+}\)-release channels in sarcoplasmic reticulum because the skinned fibers were treated with 1% Triton X-100 to solubilize sarcoplasmic reticulum membranes. Moreover, because highly buffered EGTA-active solutions were used, any calcium release from remaining active sarcoplasmic reticulum would have been buffered by this solution.

Our study showed a clear decrease in maximal activated force in the presence of 4-CmC, which was dose-dependent and particularly apparent. This may explain why the contractile responses produced with 4-CmC, as compared with caffeine, were less in saponin-skinned fibers and absent in intact fibers. It is likely that 4-CmC affects the biochemical states of cross-bridges during the working cycle, causing a reduction in their number and/or generating force.

However, the tension-pCa curve was shifted to lower concentrations in heart muscle. Because pCa-tension curves generally are assumed to reflect the calcium-binding properties of Troponin C, the effect of 4-CmC may have been due to a direct action on contractile proteins, more particularly, Troponin C. Further research is needed to determine how 4-CmC might affect myofilaments. In terms of our results, it is likely that 4-CmC at concentrations above 0.5 mM exerts a dose-dependent sensitizing effect on the contractile apparatus and, from 0.1 mM, decreases maximal Ca\(^{2+}\)-activated tension for cardiac fibers. This could explain why 4-CmC on saponin-skinned fibers could induce lower contractures than with caffeine. Indeed, when using up to 10 mM caffeine, no variation in the T\(_{\text{max}}\) was observed. In contrast to the curves plotted in Fig. 4, the amplitude of the 4-CmC contractures were larger when effects of 4-CmC on the T\(_{\text{max}}\) were taken into account (Fig. 9). The results showed that with up to 2 mM, 4-CmC contractures were not significantly different from caffeine.

Interestingly, the effect of 4-CmC on myofibrillar responsiveness is reminiscent of that of caffeine in ferret and rat cardiac muscle (Wendt and Stephenson, 1983; Baudet and Ventura-Clapier, 1990). 4-CmC and caffeine both increase the Ca\(^{2+}\) sensitivity of the contractile apparatus. For example, 2 mM 4-CmC increases Ca\(^{2+}\) sensitivity from 6.02 ± 0.03 (n = 10) to 6.13 ± 0.02 (n = 8), whereas a 5-mM concentration of caffeine increases it from 5.80 ± 0.03 (n = 14) to 5.94 ± 0.01 (n = 14), as shown by Baudet and Ventura-Clapier (1990). Consequently, the contractile apparatus is more sensitive to 4-CmC.

In summary, 4-CmC induced tension variations in Ca\(^{2+}\)-loaded fibers due to Ca\(^{2+}\) release from the sarcoplasmic reticulum in association with an effect on the contractile machinery. Experiments with caffeine and ryanodine suggested that 4-CmC releases Ca\(^{2+}\) by acting on the ryanodine receptor, which is as sensitive to 4-CmC as caffeine. With 4-CmC, as with caffeine, care must be taken in interpreting results.

Xu L, Mann G and Meissner G (1996) Regulation of cardiac release channel (ryanodine receptor) by Ca²⁺, H⁺, Mg²⁺, and adenine nucleotides under normal and simulated ischemic conditions. Circ Res 78:1100–1109.

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