Differential Effects of 4-Chloro-\textit{m}-cresol and Caffeine on Skinned Fibers from Rat Fast and Slow Skeletal Muscles\textsuperscript{1}

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\begin{abstract}
Contractile responses to 4-chloro-\textit{m}-cresol (4-CmC) were tested in saponin- and Triton X-100-skinned fibers from soleus and edl (extensor digitorum longus) muscles of adult rats and compared with those to caffeine. The testing of different concentrations of 4-CmC on saponin-skinned fibers showed that 4-CmC induced a dose-dependent caffeine-like transient contractile response in edl and soleus due to an activation of the ryanodine receptor. Both types of skeletal muscles showed a 10 to 20 times lower 4-CmC threshold concentration and EC\textsubscript{50} value (concentration providing 50\% of the maximal 4-CmC contracture) than for caffeine. The results indicate that edl is more sensitive than soleus to 4-CmC and that this difference in sensitivity is more marked than with caffeine. Furthermore, an increase in cytosolic Ca\textsuperscript{2+} activity induced a more marked shift of dose-response curves toward lower concentrations for 4-CmC than caffeine. Experiments conducted on Triton X-100-skinned fibers showed that in both muscles, 4-CmC decreased in a dose-dependent manner the Ca\textsuperscript{2+}-activated force of contractile apparatus, particularly in edl. Furthermore, the tension pCa curves indicated that 4-CmC induced a dose-dependent sensitizing (soleus) or desensitizing (edl) effect on the Ca\textsuperscript{2+} sensitivity of myofibrils. These results indicate that edl and soleus contractile responses can be discriminated with 4-CmC instead of caffeine and that care must be taken in interpreting results because muscular pathology could be due in part to an increase in intracellular Ca\textsuperscript{2+}.

Molecular and functional differences between fast-twitch [extensor digitorum longus (edl)] and slow-twitch (soleus) skeletal muscles have been well documented in recent years in relation to contractile proteins (Danielli-Bettolo et al., 1990), pumping mechanisms of the sarcoplasmic reticulum (Brandl et al., 1986), intracellular proteins, or calcium-release mechanisms from the sarcoplasmic reticulum (Jorgensen and Jones, 1986; Damiani and Margreth, 1994; Delbono and Meissner, 1996). In mammalian skeletal muscle, the main source of Ca\textsuperscript{2+} is the sarcoplasmic reticulum, from which Ca\textsuperscript{2+} is released mainly through the ryanodine receptor RyR1 (Takeishiama et al., 1989; Ogawa, 1994; Franzini-Armstrong and Protasi, 1997). The mechanisms of Ca\textsuperscript{2+} release in fast- and slow-twitch fibers have been analyzed in sarcoplasmic reticulum vesicles (Lee et al., 1991) and in intact (Delbono and Meissner, 1996) and skinned (Salviati and Volpe, 1988) fibers through the use of different drugs and calcium-release modulators of ryanodine receptor (caffeine, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, ryanodine, doxorubicin). Although caffeine has been widely used as a Ca\textsuperscript{2+}-releasing agent in intact and skinned fibers (Rousseau et al., 1988; Salviati and Volpe, 1988; Fryer and Neering, 1989; Su and Chang, 1995; Pagala and Taylor, 1998), it is known to exert various side effects, particularly inhibition of phosphodiesterases and increase in Ca\textsuperscript{2+} sensitivity of cardiac and skeletal contractile proteins (Butcher and Sutherland, 1962; Wendt and Stephenson, 1983).

Chlorocresols, especially 4-chloro-\textit{m}-cresol (4-CmC), have recently been reported to be strong stimulators of ryanodine receptors in cerebellum, intact skeletal muscle, and cardiac skinned fibers (Zorzato et al., 1993; Herrmann-Frank et al., 1996a,b; Westerblad et al., 1998; Choisy et al., 1999). In particular, 4-CmC stimulated Ca\textsuperscript{2+}-activated \textsuperscript{3}H\textsubscript{ryanodine binding on heavy sarcoplasmic reticulum vesicles from rabbit back muscles, producing a half-maximal activation at about 100 \textmu M (Herrmann-Frank et al., 1996a,b). Moreover, 4-CmC increased the affinity of \textsuperscript{3}H\textsubscript{ryanodine binding on sarcoplasmic reticulum vesicles from malignant hyperthermia-susceptible muscle compared with normal muscle. Consequently, it has been proposed that 4-CmC could replace caffeine in the test for muscle susceptibility to malignant hyperthermia (Herrmann-Frank et al., 1996a,b). Furthermore, 4-CmC induced a caffeine-like transient contracture in intact fibers at concentrations 10 times less than that with caffeine (Herrmann-Frank et al., 1996a,b). Thus, the results in the literature indicate that slow- and fast-twitch muscles have dif-

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\textbf{ABBREVIATIONS:} edl, extensor digitorum longus; 4-CmC, 4-chloro-\textit{m}-cresol; RyR, ryanodine receptor.
ferent sensitivities to caffeine (Salviati and Volpe, 1988) and that 4-CmC is a more sensitive tool than caffeine (Herrmann-Frank et al., 1996a,b). In this context, the aim of this work was to compare the effect of 4-CmC on edl and soleus muscles and to investigate whether 4-CmC induces a more specific contractile response than caffeine. The experiments were conducted on saponin-skinned fibers (in which the sarcoplasmic reticulum and contractile apparatus were functional and the sarcolemma disrupted) of edl and soleus rat muscles. The effect of 4-CmC on the Ca$^{2+}$ content of sarcoplasmic reticulum was estimated by analysis of caffeine contracture after the application of different concentrations of chlorocresol. The cytosolic Ca$^{2+}$ concentration dependence of the 4-CmC response was also investigated. Moreover, the effects of 4-CmC on the contractile apparatus of edl and soleus Triton X-100-skinned fibers were tested.

**Materials and Methods**

All procedures in this study were performed according to a university committee and to the stipulations of the Helsinki Declarations for the care and use of laboratory animals.

Adult male rats were heavily anesthetized by an ether vapor flow. After respiratory arrest, the heart was quickly excised, and fast- and slow-twitch skeletal muscles (edl and soleus) were removed and placed at room temperature in a physiological solution that contained 140 mM NaCl, 6 mM KCl, 3 mM CaCl$_2$, 5 mM glucose, and 5 mM HEPES. The pH was adjusted to 7.4 with Tris-base. All experiments were conducted on chemically skinned preparations of hind-limb muscles.

**Chemically Skinned Skeletal Fibers.** Small bundles (100- to 250-µm diameter and 1.5–2.5 mm in length) of soleus and edl muscles were dissected and placed in a relaxing solution of pCa 9.0 (pCa = –log$_{10}$(Ca$^{2+}$)), of a composition that is reported in Table 1, for subsequent chemical skinned treatments (saponin or Triton X-100). Saponin 50 µg/ml (Endo and Iino, 1980) was prepared in a pCa 9.0 solution in which the preparations were immersed for 30 min under constant stirring. This treatment disrupts the sarcolemma but does not affect the ability of the sarcoplasmic reticulum to accumulate and release Ca$^{2+}$. The preservation of the sarcoplasmic reticulum function is indicated by the capacity of caffeine to induce contractures (Endo and Kitazawa, 1978).

For Triton-skinned fibers, preparations were placed for 1 h in a relaxing solution (pCa 9.0) containing Triton X-100 1% (v/v) under constant stirring and then transferred into pCa 9.0 not containing Triton X-100. This treatment permeabilizes the sarcolemma without affecting the biochemical and structural properties of myofibrils (Stephenson et al., 1981), so that measurement of the Ca$^{2+}$ sensitivity of contractile proteins and maximal Ca$^{2+}$-activated tension ($T_{\text{max}}$) can be performed.

Saponin- or Triton-skinned fibers were transferred and mounted in an experimental system, as described by Huchet and Leoty (1993). This system allowed measurements of the tension development by the preparation immersed in 2.5-mL tubes (Nalge Nune International, Roskilde, Denmark). The tubes were placed on a rotative plate fixed on a disk positioned on a magnetic stirrer (Rotamag 10, Prolabo, Paris, France), which allowed the solutions to be continuously mixed. The measurement system was composed of two stainless steel tubes fixed to an assembly. One end of the fiber bundle was snared in a loop of fine hair pulled into a tube glued to a fixed rod. The second end of the preparation was snared in identical manner to a tube glued to a fixed rod. The second end of the tube was placed on a rotative plate fixed on a disk positioned on a magnetic stirrer (Rotamag 10, Prolabo, Roskilde, Denmark). The tubes were placed on a rotative plate fixed on a magnetic stirrer (Rotamag 10, Prolabo, Roskilde, Denmark). The tubes were placed on a rotative plate fixed on a magnetic stirrer (Rotamag 10, Prolabo, Roskilde, Denmark). The tubes were placed on a rotative plate fixed on a magnetic stirrer (Rotamag 10, Prolabo, Roskilde, Denmark).

A single fiber was successively immersed in five different solutions (Table 1). This protocol makes it possible to load the sarcoplasmic reticulum with Ca$^{2+}$ and then release it by applying caffeine (Su and Hasselbach, 1984). EGTA, Mg$^{2+}$, and caffeine concentrations varied with the solutions. Solution 1 (pCa 9.0), consisting of 10 mM EGTA, 1 mM Mg$^{2+}$, and 25 mM caffeine, was used to deplete the sarcoplasmic reticulum of Ca$^{2+}$. Solution 2 was a caffeine-free washing solution and was similar to solution 1. Solution 3 (pCa 7.0), consisting of 10 mM EGTA and 1 mM Mg$^{2+}$, allowed Ca$^{2+}$ loading of the sarcoplasmic reticulum and was obtained by mixing pCa 9.0 and pCa 4.5, (10 mM EGTA, 1 mM Mg$^{2+}$), in appropriate proportions. Solution 4 (pCa 7.0 or 7.5), consisting of 0.4 mM EGTA and 0.1 mM Mg$^{2+}$, was used to wash out solution 3 and to prepare the fiber for the next solution. Solution 5 was similar to solution 4 but contained different concentrations of caffeine (0.1–25 mM) or 4-CmC (2.5 µM to 2 mM) added to induce transient contracture. At the beginning of the experiments, two or three challenges were performed with caffeine (10 mM). The experimental protocol consisted of a test cycle of 4-CmC contracture using different 4-CmC concentrations (2.5 µM to 2 mM) added to solution 5 in the place of caffeine. Immediately after the application of 4-CmC, the fiber was immersed in a 10 mM (or 2.5 mM) caffeine solution to estimate the decrease of sarcoplasmic reticulum Ca$^{2+}$ content. Skinned fibers were incubated for 2 min in all solutions except solution 5, in which fiber was immersed until the end of contracture. The experiments were conducted in slow- and fast-twitch skeletal muscles. For caffeine or 4-CmC response, contracture amplitude (mN/mm$^2$), time to peak (s), and time of half-relaxation (s) were measured. Contracture amplitudes were related to the maximal tension developed in the presence of 4-CmC (or caffeine), and the dose-response curves were fitted for each fiber.

The reversibility of 4-CmC effects was tested by performing a subsequent control (10 mM caffeine) every other test cycle. Isometric tension was recorded on chart paper (Linear Bioblock), and baseline tension was established at the steady state measured in relaxing solution (pCa 9.0).

**Triton X-100-Skinned Skeletal Muscles Fibers.** Tension-pCa relationships were obtained by exposing Triton-skinned fibers of slow- and fast-twitch skeletal muscles sequentially to solutions of decreasing pCa. The intermediate solutions were obtained by mixing pCa 9.0 and pCa 4.5 (10 mM EGTA, 1 mM Mg$^{2+}$) solutions (Table 1) in appropriate quantities. Solutions containing different concentrations of Ca$^{2+}$ were prepared. For each concentration, one solution served as a control and the other contained 4-CmC (0.01, 0.5, 1, or 2 mM). Isometric tension was recorded, as for saponin-skinned fibers. Baseline tension was established at the steady state measured in the relaxing solution pCa 9.0.

For each tested fiber, data for relative tension above 10% and below 90% were fitted using a modified Hill equation (Huchet and Leoty, 1993). Relative tension:

\[
\frac{T}{T_{\text{max}}} = \frac{[\text{Ca}^{2+}]^{n_H} + [\text{Ca}^{2+}]^{n_i}}{[\text{Ca}^{2+}]^{n_H}}
\]

where, tension was expressed in millinewtons per square millimeter.

The Hill coefficient, $n_H$, and the pCa for the half-maximal activation, pCa$_{50} = –\log_{10}(K_{H})$, were calculated for each experiment using linear regression analysis. K corresponds to the calcium concentration (M) that induced half-maximal activation:

\[
K = 10^{-pCa_{50}}
\]

The Hill coefficient for each type of fiber was calculated as the slope of the fitted straight lines. Baseline tension was the same as that for pCa 9.0, and $T_{\text{max}}$ was obtained for pCa 4.5. pCa$_{50}$ expressed the
apparent Ca$^{2+}$ sensitivity of contractile proteins, and $n_H$ indicated the cooperativity (Ashley et al., 1991).

**Skinned Fiber Solutions.** The composition of the solutions (i.e., the Ca$^{2+}$ concentrations used for saponin or Triton X-100 protocols) was calculated using the computer program of Godt and Nosek (1986). The composition of basic solutions (pCa 9.0, 4.5) was reported in Table 1. For each solution, ionic strength was adjusted to 160 mM with KCl and pH was adjusted to 7.1 with HCl or KOH. In saponin-skinned fiber experiments, solutions also contained phosphocreatine kinase (17.5 I.U./ml) and sodium azide (1 mM). Chemical products were obtained from Sigma Chemical Co. (St. Louis, MO). 4-CmC was purchased from Fluka (New Ulm, Germany).

### Table 1

Composition of solutions used for saponin and Triton X-100 experiments

For saponin experiments, solution 1 contained 25 mM caffeine and solution 5 contained 0.1 to 25 mM caffeine or 2.5 μM to 2 mM 4-CmC.

<table>
<thead>
<tr>
<th>Solution: Function (pCa)</th>
<th>EGTA</th>
<th>Imidazole</th>
<th>Na$^+$</th>
<th>Mg$^{2+}$</th>
<th>Mg$^{2+}$ ATP</th>
<th>Phosphocreatine</th>
<th>Dithiothreitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic solutions: relaxing and maximal activating (9.0 and 4.5)</td>
<td>10</td>
<td>30</td>
<td>30.6</td>
<td>1</td>
<td>3.16</td>
<td>12</td>
<td>0.3</td>
</tr>
<tr>
<td>1: depleting (9.0)</td>
<td>10</td>
<td>30</td>
<td>30.6</td>
<td>1</td>
<td>3.16</td>
<td>12</td>
<td>0.3</td>
</tr>
<tr>
<td>2: washing (9.0)</td>
<td>10</td>
<td>30</td>
<td>30.6</td>
<td>1</td>
<td>3.16</td>
<td>12</td>
<td>0.3</td>
</tr>
<tr>
<td>3: loading (7.0)</td>
<td>0.4</td>
<td>30</td>
<td>30.6</td>
<td>0.1</td>
<td>3.16</td>
<td>12</td>
<td>0.3</td>
</tr>
<tr>
<td>4: washing (7.0 or 7.5)</td>
<td>0.4</td>
<td>30</td>
<td>30.6</td>
<td>0.1</td>
<td>3.16</td>
<td>12</td>
<td>0.3</td>
</tr>
<tr>
<td>5: releasing (7.0 or 7.5)</td>
<td>0.4</td>
<td>30</td>
<td>30.6</td>
<td>0.1</td>
<td>3.16</td>
<td>12</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Fig. 1.** 4-CmC contractures in soleus (A) and edl (B) saponin-skinned fibers at pCa 7.5. The first trace corresponds to the application of 10 mM caffeine, and the last four traces represent responses obtained for 0.05, 0.1, 0.5, and 1 mM 4-CmC. The “squared” variation in tension occurring before the contracture is due to the change of solutions. Experiments were performed at 22°C.
prepared as a stock solution of 0.25 M in dimethyl sulfoxide (dimethyl sulfoxide had a maximal final concentration of 0.8%), and diluted for further use to obtain a final concentration of 1 µM to 2 mM.

**Analysis of Fitted Curves for Saponin-Skinned Fibers.** For each experimented fiber, 4-CmC (or caffeine) contracture amplitudes were related to the maximal tension developed in the presence of 4-CmC (or caffeine), and results were fitted using a modified Hill equation (Huchet and Leoty, 1993), which gave an EC, and a Hill coefficient. Mean values calculated for these two parameters were used to plot the dose-response curves on which mean values of each concentration of 4-CmC (or caffeine) were reported.

For each experimented fiber, the amplitude of the caffeine response obtained after 4-CmC pretreatment was compared with the control response (without 4-CmC pretreatment), and the percentage of decrease of the caffeine response was related to the various 4-CmC concentrations tested. As the points fitted a sigmoid curve, the modified Hill equation was used to estimate the IC, (i.e., the 4-CmC concentration that induced half-maximal inhibition of caffeine contracture).

**Statistical Analysis.** All values are expressed as mean ± S.E.M. Student’s unpaired t test was used to compare the different parameters between edl and soleus. Statistical significance was reached when P < .05.

**Results**

**4-CmC Effects on Sarcoplasmic Reticulum Ca, Release Mechanisms and Comparison with Caffeine.** Different 4-CmC concentrations (2.5 µM to 2 mM) were added to the Ca, release solution (pCa 7.5). After an identical loading procedure in saponin-skinned fibers of edl and soleus muscles, the application of 4-CmC produced a caffeine-like transient contracture in a dose-dependent manner (Fig. 1, A and B). In edl fibers, a contracture threshold was found at 10 µM 4-CmC, and in soleus fibers, a threshold was found at larger concentrations of 25 to 50 µM (Fig. 2A). The maximal amplitude of 4-CmC response (Table 2) was also obtained at lower concentrations in edl (0.5 mM) than in soleus (2 mM). The 4-CmC-EC, (i.e., the 4-CmC concentration providing 50% of the maximal 4-CmC contracture) was significantly lower in edl (70 ± 10 µM, n = 9) than in soleus (180 ± 40 µM, n = 8; P < .05). The results for different concentrations of 4-CmC (0.5, 1 and 2 mM) showed that the time to peak (s) of the edl contracture was significantly shorter than for soleus (Table 3). At 1 mM 4-CmC, the time to peak of edl was 38% shorter than that observed for soleus. The time of half-relaxation of 4-CmC contracture was different for edl and soleus. Indeed, an increase in the 4-CmC concentration induced a decrease of the time of half-relaxation for soleus but increased it for edl (Table 3).

The effects of caffeine (0.1 to 25 mM) were also tested in saponin-skinned fibers. Slow-twitch fibers exhibited a threshold contractile response at a lower caffeine concentration (0.75 mM, n = 5) than fast-twitch fibers (1 mM, n = 6) at pCa 7.5 (Fig. 3A). However, as for 4-CmC, the caffeine EC, found in edl (1.72 ± 0.17 mM, n = 6) was lower than in soleus (2.18 ± 0.25 mM, n = 5) but not significantly different (P ≥ .05). Contrary to 4-CmC, no significant differences in contracture kinetics were found between the two types of skeletal muscle at all caffeine concentrations tested.

Both types of skeletal muscle showed a 4-CmC transient contracture, with an EC, value and a threshold concentration 10 to 20 times lower than for caffeine. The amplitude obtained for the maximal response developed in the presence of caffeine or 4-CmC was similar for edl and soleus muscle. For example, contracture amplitude for edl was 80.8 ± 7.0 mN/mm² (n = 15) in the presence of 0.5 mM 4-CmC (Table 2) and 80.3 ± 8.4 mN/mm² (n = 6) with 10 mM caffeine.

These results indicate that edl is more sensitive than soleus to 4-CmC and that this difference in sensitivity is more marked than with caffeine. The similarity between 4-CmC and caffeine contractile responses suggested that 4-CmC produces Ca, release from the sarcoplasmic reticulum by activating the ryanodine receptor. This possibility was investigated further on the two types of skeletal muscle.

**Fig. 2.** 4-CmC dose-response curves for saponin-skinned fibers at pCa 7.5 (A) and pCa 7.0 (B). Amplitudes of the contractures obtained for soleus (n = 8, A; n = 6, B) and edl (n = 9, A; n = 8, B) are expressed as a percentage of maximal response. Curves were fitted using the modified Hill equation.
These results showed that like caffeine, 4-CmC induced Ca\(^{2+}\) releases from the sarcoplasmic reticulum in edl and soleus fibers, the influence of cytosolic (cis) Ca\(^{2+}\) concentrations (31 and 100 mM) on 4-CmC contractile responses was tested in both types of fibers. 4-CmC (2.5 μM to 2 mM) was applied to the Ca\(^{2+}\)-release solution at pCa 7.0 (100 nM Ca\(^{2+}\)), and contractile responses were compared with those obtained at pCa 7.5 (31 nM Ca\(^{2+}\)). 4-CmC dose–response curves plotted for pCa 7.0 showed that the threshold concentrations in both muscles (2.5–5 μM, n = 8 for edl; 10–50 μM, n = 6 for soleus) release channel is inhibited by concentrations of ryanodine ≥10 μM only if this receptor is activated (Alderson and Feher, 1987). Caffeine is an activator of the ryanodine receptors. Caffeine (10 mM) was associated with ryanodine (100 μM), and after three challenges, the caffeine contracture disappeared (Fig. 5, traces 1 and 2). In these conditions, to see whether 4-CmC was acting as caffeine, we applied 4-CmC (1 mM) with ryanodine (100 μM): after three challenges, 4-CmC contracture was totally suppressed (Fig. 5, traces 3 and 4). Then, because 4-CmC and caffeine associated with ryanodine had the same effect, it suggested that 4-CmC was acting on the same Ca\(^{2+}\)-release mechanism as caffeine (i.e., the ryanodine receptor).

### Effect of 4-CmC on Sarcoplasmic Reticulum Ca\(^{2+}\) Content

Caffeine contracture is commonly used to study sarcoplasmic reticulum Ca\(^{2+}\) content. In an attempt to determine whether 4-CmC affected sarcoplasmic reticulum Ca\(^{2+}\) content, caffeine contractures were elicited after application of different concentrations of 4-CmC to saponin-skinned slow- and fast-twitch muscles. Experiments conducted at pCa 7.5 consistently in applying different concentrations of 4-CmC (0.01–2 mM) for 1 min followed by the application of caffeine. To assess the effects of 4-CmC, 2.5 mM caffeine was selected as the concentration producing approximately 50% of maximal contracture and 10 mM as that producing maximal response.

4-CmC induced a dose-dependent decrease of caffeine contracture in edl and soleus fibers. As illustrated in Fig. 4, A and B, the decrease in 10 mM caffeine contracture was greater for low concentrations of 4-CmC in edl than soleus muscle. The concentrations of 4-CmC that gave 50% inhibition of 10 and 2.5 mM caffeine contractures (IC\(_{50}\)) did not differ significantly (p ≥ .05) in edl, 100 ± 20 μM (n = 10) and 90 ± 20 μM (n = 6), respectively; whereas for soleus, the 4-CmC concentrations were significantly different (p < .05), 270 ± 10 μM (n = 5) and 160 ± 30 μM (n = 5), respectively. These results showed that like caffeine, 4-CmC induced Ca\(^{2+}\) release from the sarcoplasmic reticulum in edl and soleus muscles.

### Action of 4-CmC and Caffeine on Ryanodine Receptor

It is well known that the sarcoplasmic reticulum Ca\(^{2+}\)-release channel is inhibited by concentrations of ryanodine ≥10 μM only if this receptor is activated (Alderson and Feher, 1987). Caffeine is an activator of the ryanodine receptors. Caffeine (10 mM) was associated with ryanodine (100 μM), and after three challenges, the caffeine contracture disappeared (Fig. 5, traces 1 and 2). In these conditions, to see whether 4-CmC was acting as caffeine, we applied 4-CmC (1 mM) with ryanodine (100 μM): after three challenges, 4-CmC contracture was totally suppressed (Fig. 5, traces 3 and 4). Then, because 4-CmC and caffeine associated with ryanodine had the same effect, it suggested that 4-CmC was acting on the same Ca\(^{2+}\)-release mechanism as caffeine (i.e., the ryanodine receptor).

### Cytosolic Ca\(^{2+}\) Modulation of Ca\(^{2+}\) Release Induced by 4-CmC

It has been shown that the effectiveness of caffeine in increasing the rate of Ca\(^{2+}\) release is dependent on the free Ca\(^{2+}\) concentration in the release medium (Rousseau et al., 1988); to further compare the two substances, the effect of an increase in free Ca\(^{2+}\) concentration on the Ca\(^{2+}\)-release rate by caffeine and 4-CmC was tested. In saponin-skinned fibers, the influence of cytosolic (cis) Ca\(^{2+}\) concentrations (31 and 100 nM) on 4-CmC contractile responses was tested in both types of fibers. 4-CmC (2.5 μM to 2 mM) was applied to the Ca\(^{2+}\)-release solution at pCa 7.0 (100 nM Ca\(^{2+}\)), and contractile responses were compared with those obtained at pCa 7.5 (31 nM Ca\(^{2+}\)). 4-CmC dose–response curves plotted for pCa 7.0 showed that the threshold concentrations in both muscles (2.5–5 μM, n = 8 for edl; 10–50 μM, n = 6 for soleus)
and the EC$_{50}$ values were shifted to lower values than those observed at pCa 7.5 (Fig. 2, A and B). Thus, edl saponin-skinned fibers showed a 7- to 10-fold lower 4-CmC-EC$_{50}$ when sarcoplasmic reticulum Ca$^{2+}$-release channels were activated by 4-CmC in the presence of 100 nM Ca$^{2+}$. For example, in edl, the EC$_{50}$ values were significantly different ($P < .05$) at pCa 7.5 and pCa 7.0: 70 ± 10 μM (n = 6) and 10 ± 2 μM (n = 8) of 4-CmC, respectively. Moreover, 4-CmC-EC$_{50}$ found at pCa 7.0 was significantly different between edl and soleus. An increase in intracellular Ca$^{2+}$ induced a more reduced shift in the 4-CmC dose-response curves of soleus than edl. The EC$_{50}$ value was significantly potentiated ($P < .05$) more than twice as much when cis Ca$^{2+}$ was increased by 69 nM in soleus muscle [i.e., 70 ± 10 μM (n = 6) compared with 180 ± 40 μM, n = 8, at pCa 7.5].

Similar experiments were conducted with caffeine at pCa 7.0 in edl and soleus muscles. As illustrated by the dose-response curves plotted for caffeine (Fig. 3, A and B), there was a shift in the concentration threshold (to 0.1 mM) and in EC$_{50}$ values toward lower values in soleus and edl. The EC$_{50}$ value was significantly reduced ($P < .05$) to 0.72 ± 0.24 mM (n = 7) (i.e., by a ratio of 2.5 in edl) and to 0.41 ± 0.05 mM (n = 3) (i.e., by a ratio of 5.0 in soleus) compared with values obtained at pCa 7.5. Nevertheless, at pCa 7.0, caffeine EC$_{50}$ was not significantly different between edl and soleus. 4-CmC exhibited a greater sensitivity than caffeine to an increase in cytosolic Ca$^{2+}$ activity.

Because caffeine exerts side effects on the contractile apparatus, we conducted tests on Triton X-100-skinned fibers to determine whether similar effects were produced with 4-CmC.

**Effects of 4-CmC on Properties of Contractile Proteins.** Maximal Ca$^{2+}$-activated tension ($T_{max}$) and apparent Ca$^{2+}$ sensitivity of contractile proteins (pCa$_{50}$) were analyzed in the absence and presence of different concentrations of 4-CmC (0.01, 0.5, 1, or 2 mM) in skeletal Triton X-100-skinned fibers. Tension in soleus and edl muscle fibers was measured in control conditions and in the presence of 4-CmC (Fig. 6). The relationships between steady-state Ca$^{2+}$-activated tension and free Ca$^{2+}$ concentrations in the presence and absence of 1 and 2 mM 4-CmC are illustrated in Fig. 7. In both edl and soleus fibers, the capacity of contractile proteins to develop force when maximally activated by Ca$^{2+}$ (pCa 4.5) was reduced in the presence of increasing concentrations of 4-CmC (Table 4). The decrease in maximal tension was more pronounced in edl than in soleus (Table 4). With 2 mM 4-CmC, $T_{max}$ was decreased by 59.4% in edl fibers (n = 9) compared with only 28.4% in soleus fibers (n = 12). The effect of large concentrations of 4-CmC (1 and 2 mM) on maximal Ca$^{2+}$-activated tension was not reversible.

Under control conditions, the values for pCa$_{50}$ (the Ca$^{2+}$ concentration producing 50% maximal tension) and the Hill coefficient ($n_{H}$, representing cooperativity) were significantly different between edl and soleus and similar to those usually found for these two typical fast- and slow-twitch muscles ( Stephenson and Williams, 1981). As shown in Table 4, slow-twitch muscle was more sensitive to Ca$^{2+}$, and cooperativity was lower than that for fast-twitch fibers. The addition of 4-CmC to the Ca$^{2+}$-buffered solutions (10 mM EGTA) in which isometric tension was measured led to changes in apparent Ca$^{2+}$ sensitivity in both types of skeletal fibers. The $n_{H}$ coefficients were changed to a lesser degree in soleus than edl (Table 4). The application of 4-CmC induced a different shift in force-pCa relationships for edl and soleus (Fig. 7). The increase in 4-CmC concentrations produced a progressive shift of the pCa-tension curve for edl to the right, indicative of a significant decrease of the Ca$^{2+}$ sensitivity of contractile proteins beginning at 0.01 mM 4-CmC. For example, application of 2 mM 4-CmC decreased the pCa$_{50}$ control (Table 4) by ΔpCa$_{50}$ = 0.167 (n = 9). Soleus muscle showed a reduced and nonsignificant decrease in Ca$^{2+}$ sensitivity for low concentrations of 4-CmC and a significant shift ($P < .05$) of control pCa$_{50}$ to higher pCa for concentrations of
0.5 mM (Table 4). Contrary to edl, a significant increase of Ca\textsuperscript{2+} sensitivity occurred in soleus at high 4-CmC concentrations (0.5–2 mM).

**Discussion**

This study shows that 4-CmC induces caffeine-like transient contractures in a dose-dependent manner in saponin-skinned fibers isolated from fast- and slow-twitch skeletal muscles of the rat and that the muscles show differences in sensitivity (Fig. 1, A and B). Previous studies have reported that 4-CmC is a potent activator of skeletal (Herrmann-Frank et al., 1996a,b; Westerblad et al., 1998) and cardiac (Choisy et al., 1999) ryanodine receptors. Unlike caffeine, which induces contractures with millimolar concentrations (Salviati and Volpe, 1988; Herrmann-Frank et al., 1996a,b), 4-CmC proved efficient with micromolar concentrations. This result is consistent with that of Herrmann-Frank et al. (1996b), who found a threshold activity for 75 \( \mu \)M 4-CmC on intact human skeletal fibers isolated from malignant hyperthermia nonsusceptible muscle. Moreover, other authors have shown that 4-CmC was efficient at micromolar concentrations on PC12 cells (Zorzato et al., 1993), intact mouse skeletal muscle (Westerblad et al., 1998), and frog skeletal fibers (Struk and Melzer, 1999). The difference in sensitivity to 4-CmC and caffeine of skeletal muscles could be explained by the presence of distinct site or sites of action of these two substances on the ryanodine receptor. Indeed, it has been reported that caffeine acts preferentially on the cytosolic side.

**Fig. 4.** Inhibition curves of caffeine contracture for different concentrations of 4-CmC in soleus (A) and edl (B) saponin-skinned fibers at pCa 7.5. Data are the percentages of inhibition of 2.5 (\( n = 5 \), A; \( n = 6 \), B) and 10 (\( n = 5 \), A; \( n = 10 \), B) mM caffeine contractures for each concentration of 4-CmC compared with the control. Points were fitted using a sigmoid equation, which yielded the slope of the curve (n) and the IC\textsubscript{50}. Vertical bars represent ±S.E.M.

**Fig. 5.** 4-CmC (1 mM) and caffeine contracture (10 mM) after ryanodine treatment (100 \( \mu \)M) in slow-twitch skeletal saponin-skinned fibers at pCa 7.5. Caffeine experiments (1 and 2) were conducted on one fiber, and 4-CmC experiments (3 and 4) were conducted on another fiber. Traces 1 and 3 correspond to the contractures obtained respectively with 10 mM caffeine (1) and 1 mM 4-CmC (3), in control conditions, i.e., before ryanodine treatment. Traces 2 and 4 represent respectively the tension developed in the presence of 10 mM caffeine associated with 100 \( \mu \)M ryanodine, and in the presence of 1 mM 4-CmC associated with 100 \( \mu \)M ryanodine, after three running challenges. Tension remaining for trace 2 is due to an effect on contractile apparatus.
of the ryanodine receptor, whereas 4-CmC is more potent in activating the ryanodine receptor when applied on the luminal side (Herrmann-Frank et al., 1996a).

The decrease in caffeine contractures (2.5 and 10 mM) induced by 4-CmC suggests that 4-CmC releases Ca\textsuperscript{2+} from the sarcoplasmic reticulum. Furthermore, caffeine and 4-CmC contractures were totally abolished when ryanodine (100 μM) was associated with each of these substances, which would indicate that 4-CmC and caffeine activate the same Ca\textsuperscript{2+}-release mechanism (i.e., the ryanodine receptor).

In saponin-skinned fibers, edl showed greater sensitivity (a lower threshold and EC\textsubscript{50}) than soleus for 4-CmC, which was probably not due to an inhibitory action on the sarcoplasmic reticulum Ca\textsuperscript{2+} pump. Indeed, Zorzato et al. (1993) concluded that at 1 mM, chlorocresol did not involve inhibition of sarcoplasmic reticulum Ca\textsuperscript{2+} pump on longitudinal sarcoplasmic reticulum vesicles. Moreover, Westerblad et al. (1998) showed that on intact structure, 0.1 mM 4-CmC had no inhibitory effect on the Ca\textsuperscript{2+} ATPase of sarcoplasmic reticulum. Under our conditions of experiments used for saponin-skinned fibers, it is difficult to answer to this question.

Our results also showed that 4-CmC inhibited the caffeine contractures in edl and soleus muscles with a different sensitivity. The difference in 4-CmC sensitivity between fast and slow-twitch skeletal muscles may also have resulted

**Fig. 6.** Effect of 2 mM 4-CmC on Ca\textsuperscript{2+}-activated tension and maximal Ca\textsuperscript{2+}-activated tension (pCa 4.5) of soleus (A) and edl (B). Tension was induced by soaking fibers in a solution of decreasing pCa not containing (a–g, soleus; a–h, edl) or containing (a’–g’, soleus; a’–h’, edl) 2 mM 4-CmC. Values for pCa were a = 7, b = 6.5, c = 6.25, d = 6, e = 5.875, f = 4.5, g = 9 in soleus and a = 7, b = 6.5, c = 6.25, d = 6.125, e = 6.0, f = 5.875, g = 4.5, h = 9 in edl.
equation. Temperature was 22°C.

concentration of 4-CmC tested. Curves were fitted using the modified Hill equation. Maximal tension at pCa 4.5 for each concentration of 4-CmC tested. Curves were fitted using the modified Hill equation. Temperature was 22°C.

Fig. 7. Effects of various concentrations of 4-CmC on myofibrils Ca²⁺ sensitivity of slow- and fast-twitch Triton-skinned fibers from skeletal muscle. Tension-pCa curves were obtained with soleus (A) and edl (B) fibers. Isometric tension-pCa (−log₁₀(Ca²⁺)) relationships were determined in twitch fibers in the absence (n = 12, A; n = 10, B) or presence of 1 (n = 8, A; n = 10, B) or 2 (n = 12, A; n = 9, B) mM 4-CmC. Force is expressed as the percentage of maximal tension at pCa 4.5 for each concentration of 4-CmC tested. Curves were fitted using the modified Hill equation. Temperature was 22°C.

from the presence of various isoforms of ryanodine receptor (RyR1 and/or RyR3) in these two types of muscle (Conti et al., 1996) and/or the difference in ryanodine receptor gating (Shin et al., 1996). Different reports have shown that Ca²⁺ release kinetic is faster in intact fibers (Delbono and Meissner, 1996), in sarcoplasmic reticulum vesicles (Lee et al., 1991), and in skinned fibers (Salviati and Volpe, 1988) of edl than of soleus muscle. Our results indicated that the time to peak of 4-CmC contractures was shorter for edl than for soleus (Table 3).

The increase in cytosolic Ca²⁺ (31–100 nM, i.e., pCa 7.5–7.0) shifted the dose-response curves to lower 4-CmC concentrations in saponin-skinned fibers. These results are similar to those reported by Herrmann-Frank et al. (1996a) for sarcoplasmic reticulum vesicles of skeletal muscle, in which a decrease in cytosolic Ca²⁺ (900–100 nM) shifted the dose-response curve to higher 4-CmC concentrations. Moreover, 4-CmC used to detect pathological muscular structure (malignant hyperthermia), in which the resting myoplasmic Ca²⁺ concentration is increased, is described to make the ryanodine receptor more sensitive to [³H]ryanodine binding (Herrmann-Frank et al., 1996b). In our study, edl exhibited greater sensitivity than soleus to 4-CmC. This effect was more marked for higher than lower cytosolic Ca²⁺ activity, whereas under similar conditions the caffeine sensitivity of skeletal muscles was less affected. This difference between caffeine and 4-CmC could be explained by a 4-CmC binding site, presumably located on the luminal side of the ryanodine receptor and close to the potential high-affinity Ca²⁺ intraregular binding site as suggested by Herrmann-Frank et al. (1996a).

Thus, 4-CmC appears to be a more useful pharmacological tool than caffeine in discriminating between the contractile responses of edl and soleus, especially if the side effects on contractile proteins can be reduced.

Triton X-100-skinned fibers were used to determine the effect of 4-CmC on the myofibrillar responsiveness of mammalian skeletal muscle. These results show that in both edl and soleus muscles, 4-CmC decreased maximal-activated tension in a dose-dependent manner, particularly in edl at concentrations of ≥0.5 mM. It could be proposed that 4-CmC affects the biochemical states of crossbridges during the working cycle. The tension-pCa curves were shifted to lower Ca²⁺ concentrations in soleus and to higher concentrations in edl. As pCa-tension curves are assumed to reflect the Ca²⁺-binding properties of troponin C (TnC), the effect of 4-CmC could be due to its direct action on contractile proteins, and more particularly on TnC. Indeed, striated muscles contain two isoforms derived from a single copy gene: TnC-fast (TnC-f) expressed in fast skeletal muscle and TnC-slow (TnC-s) in slow muscle (Wilkinson, 1980). A major difference between the two TnC isoforms concerns the Ca²⁺ binding loops. Our investigations indicate that the Hill coefficient in edl was significantly decreased by the application of 2 mM 4-CmC but only slightly modified in soleus fibers. Accordingly, one possible explanation for changes in the Ca²⁺ sensitivity of contractile proteins is that Ca²⁺ binding loops were affected by 4-CmC. Further research is required to determine in which way 4-CmC affects myofilaments. Interestingly, the effect of 4-CmC on myofibrillar responsiveness is reminiscent of that of caffeine in skeletal muscles (Wendt and Stephenson, 1983).

In conclusion, these results indicating that micromolar 4-CmC concentrations release Ca²⁺ via activation of the sarcoplasmic reticulum ryanodine receptor in mammalian skeletal muscle strongly support the findings of Herrmann-Frank et al. (1996a,b). The difference in sensitivity to cytosolic Ca²⁺ activity between caffeine and 4-CmC could be of importance to studies of muscular pathology resulting in part from increased intracellular Ca²⁺. Moreover, because edl is more sensitive than soleus to 4-CmC, this substance may be a better tool than caffeine in discriminating between edl and soleus contractile responses.
TABLE 4

Effect of 4-CmC on maximal Ca\textsuperscript{2+}-activated tension (pCa 4.5), pCa\textsubscript{1/2}, and the Hill coefficient in Triton X-100-skinned soleus and edl fibers

The mean ± S.E.M. for pCa\textsubscript{1/2} were obtained by fitting the curves to the Hill equation. The mean ± S.E.M. of the Hill coefficient (n\textsubscript{H}) were obtained from the Hill plot curves; n represents the number of fibers.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.01 mM</th>
<th>0.5 mM</th>
<th>1 mM</th>
<th>2 mM</th>
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<tr>
<td><strong>Soleus</strong></td>
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<tr>
<td>T\textsubscript{max} (mN/mm\textsuperscript{2})</td>
<td>110.2 ± 19.6</td>
<td>102.8 ± 8.1</td>
<td>95.3 ± 15.4\textsuperscript{a}</td>
<td>84.3 ± 11.8\textsuperscript{b}</td>
<td>78.9 ± 14.3\textsuperscript{b}</td>
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<td>pCa\textsubscript{1/2}</td>
<td>6.31 ± 0.03</td>
<td>6.25 ± 0.03</td>
<td>6.37 ± 0.02\textsuperscript{a}</td>
<td>6.41 ± 0.03\textsuperscript{b}</td>
<td>6.49 ± 0.03\textsuperscript{b}</td>
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<tr>
<td>n\textsubscript{H}</td>
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<td>1.73 ± 0.03</td>
<td>1.86 ± 0.05\textsuperscript{a}</td>
<td>1.97 ± 0.08\textsuperscript{b}</td>
<td>1.68 ± 0.04\textsuperscript{b}</td>
</tr>
<tr>
<td>n</td>
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<td>9</td>
<td>8</td>
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<tr>
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<tr>
<td>T\textsubscript{max} (mN/mm\textsuperscript{2})</td>
<td>101.7 ± 12.5</td>
<td>87.8 ± 12.5\textsuperscript{b}</td>
<td>75.9 ± 9.6\textsuperscript{b}</td>
<td>60.2 ± 7.6\textsuperscript{b}</td>
<td>41.3 ± 6.1\textsuperscript{b}</td>
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<td>6.03 ± 0.01\textsuperscript{b}</td>
<td>6.01 ± 0.01\textsuperscript{b}</td>
<td>6.01 ± 0.01\textsuperscript{b}</td>
<td>5.94 ± 0.04\textsuperscript{b}</td>
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<td>n\textsubscript{H}</td>
<td>4.83 ± 0.52</td>
<td>5.80 ± 0.13</td>
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<td>4.22 ± 0.44</td>
<td>2.55 ± 0.11\textsuperscript{b}</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
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<td>9</td>
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</tbody>
</table>

Significant difference of T\textsubscript{max}, pCa\textsubscript{1/2}, and n\textsubscript{H} from the value obtained under control conditions at P < .05.

References


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