Sarcoplasmic Reticulum Ca\textsuperscript{2+} Content Affects 4-CmC and Caffeine Contractures of Rat Skinned Skeletal Muscle Fibers

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Abstract: This study investigated whether the sarcoplasmic reticulum Ca\textsuperscript{2+} content of rat skeletal muscle fibers affected contractile responses obtained by an application of 4-chloro-m-cresol (4-CmC) and caffeine. Contractures were elicited on saponin-skinned fibers under different Ca\textsuperscript{2+} loading conditions. The amplitude of 4-CmC and caffeine contractures of fast-twitch muscle fibers (edl, extensor digitorum longus) differed between the different loading conditions, and this is associated with a greater change in sensitivity to 4-CmC. When the sarcoplasmic reticulum was loaded with a low Ca\textsuperscript{2+} concentration for a short period, the 4-CmC concentration providing half-maximal response was tenfold higher than with a larger sarcoplasmic reticulum Ca\textsuperscript{2+} loading for a longer period, whereas for caffeine this concentration was only twofold higher in the same conditions. These findings indicate that 4-CmC contractile responses of edl muscle fibers are more dependent on luminal Ca\textsuperscript{2+} activity than those of caffeine are. Thus 4-CmC would appear to be of greater interest than caffeine for the study of muscle contractile responses where variations in intracellular Ca\textsuperscript{2+} activity exist. [Japanese Journal of Physiology, 51, 661–669, 2001]

Key words: sarcoplasmic reticulum, Ca\textsuperscript{2+}, 4-chloro-m-cresol, ryanodine receptor.

The sarcoplasmic reticulum of skeletal muscle plays a key role in the regulation of intracellular Ca\textsuperscript{2+} concentration. Ca\textsuperscript{2+} ions are released from this intracellular Ca\textsuperscript{2+} storage by means of the ryanodine receptor and resequestered by the Ca\textsuperscript{2+}-ATPase pump [1–3]. The activity of the ryanodine receptor is controlled primarily by Ca\textsuperscript{2+} and modulated by several endogenous ligands (including Mg\textsuperscript{2+} and ATP). Most of these compounds act on the cytosolic side of the channel [4–6]. Ryanodine receptor–Ca\textsuperscript{2+} channel activity is also regulated by luminal Ca\textsuperscript{2+} [7, 8]. To date, annexin VI [9] and Ca\textsuperscript{2+} are the only molecules proposed to act at the luminal side of the Ca\textsuperscript{2+} channel. Pharmacological substances such as caffeine and ryanodine may activate and/or inhibit the ryanodine receptor in intact and skinned striated muscle fibers and isolated structures containing the ryanodine receptor [6, 10–12]. The effects of caffeine are influenced by cytosolic and luminal Ca\textsuperscript{2+} [10, 13].

Several studies have shown that 4-chloro-m-cresol (4-CmC), a new pharmacological substance, is a potent activator of the ryanodine receptor on muscular and nonmuscular structures [14–18]. 4-CmC has been reported to stimulate Ca\textsuperscript{2+}-activated \textsuperscript{3}H-ryanodine binding on heavy sarcoplasmic reticulum vesicles from rabbit back muscles and to induce transient caffeine-like contractures in intact and malignant hyperthermia-susceptible skeletal muscle fibers at concentrations tenfold lower than for caffeine [15, 16]. These authors proposed that the difference in the sensitivity of skeletal muscles to caffeine and 4-CmC could be related to different binding sites on the ryanodine receptor [16]. Herrmann-Frank et al. [16] suggested that a stronger activation of the isolated ryanodine receptor channel by 4-CmC (when applied to the luminal side of the ryanodine receptor) may be explained by the presence of a 4-CmC binding site.
binding site or sites on this face of the receptor. Recent results showed that micromolar concentrations of 4-CmC induced transient contractures in saponin-skinned fast- and slow-twitch (soleus) rat skeletal muscle fibers through an activation of the ryanodine receptor [20]. Moreover, the release of Ca\(^{2+}\) in edl and soleus muscles by the application of 4-CmC was influenced by the cytosolic Ca\(^{2+}\) concentration.

In this context, the present study attempted to determine whether and to what degree the contractile responses obtained by the application of different 4-CmC and caffeine concentrations were affected by sarcoplasmic reticulum Ca\(^{2+}\) content. Experiments were conducted on saponin-skinned fibers from fast- and slow-twitch rat skeletal muscles in which the sarcoplasmic reticulum and the contractile apparatus were functional. 4-CmC and caffeine contractures were elicited under different Ca\(^{2+}\) loading conditions. The time and the Ca\(^{2+}\) concentration were varied during the loading step to modify sarcoplasmic reticulum Ca\(^{2+}\) content.

The results for edl show that contractile responses induced by 4-CmC were more sensitive to sarcoplasmic reticulum Ca\(^{2+}\) content than those induced by caffeine, and that a decrease in sensitivity to 4-CmC occurred when sarcoplasmic reticulum Ca\(^{2+}\) content was reduced.

METHODS

All procedures were carried out according to a university committee and to the stipulations of the Helsinki Declaration and the Physiological Society of Japan for the care and use of laboratory animals. Adult male Wistar rats weighing 350–380 g were heavily anesthetized with an ether vapor flow until respiratory arrest. The soleus (slow-twitch) and edl (extensor digitorum longus, fast-twitch) muscles were then excised with an ether vapor flow until respiratory arrest. The soleus (slow-twitch) and edl (extensor digitorum longus, fast-twitch) muscles were then excised and placed in an oxygenated buffered physiological solution in a dissecting dish at room temperature. This physiological solution contained (in mM): NaCl (140); KCl (6); CaCl\(_2\) (3); glucose (5); and HEPES (N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid) (5). pH was adjusted to 7.35 with Tris base.

Chemically skinned preparations. Small bundles of two to five fibers (100–250 \(\mu\)m wide and 2–3 mm long) from freshly isolated muscle were manually dissected with fine scissors and forceps under a binocular microscope. Chemical skinning was carried out immediately after dissection. All experiments were conducted on saponin-skinned fibers. Saponinskinning was performed under permanent stirring for 30 min on fibers placed in a relaxing solution containing 50 \(\mu\)g/ml of saponin. This treatment makes the sarcolemma and the T-tubule membranes permeable, leaving the sarcoplasmic reticulum intact [21, 22]. The preservation of the sarcoplasmic reticulum was confirmed by caffeine-induced transient contractures [23]. After the skinning procedure, the fiber was transferred into a relaxing solution without saponin and mounted in an experimental system for the measurement of isometric tension, as described by Huchet and Léoty [24]. This system consists of two stainless-steel tubes fixed to an assembly. One end of the fiber bundle is snared in a loop of fine hair pulled into a tube glued to a fixed rod. The other end of the preparation is similarly snared in a loop of fine hair pulled into a tube glued to a flexible rod (the transducer arm) supporting a metal target opposite the sensor of the displacement measuring system KD 2300 transducer (Kaman, 0.5 S.U., Colorado Springs, CO). The output voltage of the system is proportional to the distance between the sensor and the target. Under a binocular microscope, the fiber is adjusted to slack length, then stretched step by step until the tension (mN/mm\(^2\)) developed at pCa 4.5 (pCa = \(-\log[Ca^{2+}]\)) becomes maximal \(T_{\text{max}}\), i.e., when the fiber reaches 120% of its resting length. Its diameter and length are then measured.

Maximal Ca\(^{2+}\)-activated tension \(T_{\text{max}}\) in mN/mm\(^2\) was recorded at the beginning of the experiments. All experiments were performed at 22°C.

Experimental protocol for uptake and calcium release in saponin-skinned fibers. Saponin-skinned fibers were immersed sequentially in five different solutions, including a sarcoplasmic reticulum Ca\(^{2+}\)-loading solution (solution 3) and a Ca\(^{2+}\)-releasing solution by the application of 4-CmC or caffeine (solution 5).

The ionic composition was the same for relaxing and maximal-activating solutions, i.e., pCa 9.0 and pCa 4.5. However, the concentrations of ethyleneglycol-bis-(\(\beta\)-aminoethyl ether)-N,N,N',N' -tetraacetic acid (EGTA), Mg\(^{2+}\), and Ca\(^{2+}\) were different in the five solutions.

Solution 1 (pCa 9.0) was a high-EGTA (10 mM), high-Mg\(^{2+}\) (1 mM), high-caffeine (25 mM) solution used to deplete the sarcoplasmic reticulum of calcium. Solution 2 (pCa 9.0) was a caffeine-washing solution with the same composition as solution 1, but without caffeine. Solution 3 (pCa 7.0 or 7.5) was obtained by mixing pCa 9.0 and pCa 4.5 and was a high-EGTA (10 mM), high-Mg\(^{2+}\) (1 mM) solution used to load the sarcoplasmic reticulum with calcium. Solution 4 (pCa 7.0) used to prepare the fiber for the next solution, i.e., the Ca\(^{2+}\)-release solution, was a low-EGTA (0.4 mM),

low-Mg$^{2+}$ (0.1 mM) solution to facilitate the opening of the Ca$^{2+}$-release channels. Solution 5, with the same composition as solution 4, was a Ca$^{2+}$-release solution containing 4-CmC (1 μM to 1 mM) or caffeine (0.1 to 10 mM). During a Ca$^{2+}$-load release cycle, the fiber was incubated for 2 min in the first four solutions. It was then maintained in solution 5 to allow a recording of the complete transient tension developed in the presence of 4-CmC or caffeine in the solution.

The purpose of the present work was to test and compare the effect of sarcoplasmic reticulum Ca$^{2+}$ loading on 4-CmC and caffeine contractures of edl and soleus muscles. Two parameters of the loading solution (solution 3) were modified to change sarcoplasmic reticulum Ca$^{2+}$ loading. Two different loading periods (2 min and 15 s) and two Ca$^{2+}$ concentrations (pCa 7.0 and 7.5) of the sarcoplasmic reticulum loading solution were tested. Based on the results of Fryer and Stephenson [25], a maximum loading of the sarcoplasmic reticulum was reached for soleus muscle and near-maximal for edl muscle with 2 min loading in pCa 7.0. Under these conditions, the control loading test was determined to be 2 min loading in pCa 7.0.

To investigate sarcoplasmic reticulum Ca$^{2+}$ loading, the amplitude and the area of 10 mM caffeine contracture were measured. A 10 mM caffeine concentration was chosen because it elicited the release of almost all sarcoplasmic reticulum Ca$^{2+}$ content. Moreover, caffeine produces only slight side effects on the contractile apparatus at this concentration [26]. Furthermore, the total quantity of Ca$^{2+}$ released was estimated by measuring the area under the transient 10 mM caffeine response [27]. The area of the 10 mM caffeine contracture was calculated for different loading periods with different pCa and compared with that obtained in control conditions.

The effects of different loading conditions were tested on 4-CmC (1 μM to 1 mM) and caffeine (0.1 to 10 mM) contractile responses and compared with control conditions (i.e., 2 min loading in pCa 7.0). Amplitude (mN/mm$^2$), time to peak (s), and the half-time of relaxation (s) were measured for 4-CmC and caffeine contractures.

**Composition of saponin-skinned fiber solutions.** The composition of the saponin-skinned fiber solutions was calculated by using the computer program of Godt and Nosek [28] for skinned fibers. The basic solutions (pCa 9.0 [relaxing solution]; 4.5 [maximal-activating solution]; 7.0 used for solutions 4 and 5) were calculated to contain 10 (pCa 9.0 or pCa 4.5) or 0.4 (pCa 7.0) mM EGTA; 30 mM imidazole; 30.6 mM Na$^+$; 1 (pCa 9.0 or pCa 4.5) or 0.1 (pCa 7.0) mM Mg$^{2+}$; 3.16 mM Mg-ATP; 12 mM phosphocreatine, and 0.3 mM dithiothreitol. The pH was adjusted to 7.1 with either HCl or KOH, and an ionic strength of 160 mM was achieved by adding KCl.

In saponin-skinned fiber experiments, the solutions also contained phosphocreatine kinase (17.5 IU/ml) and sodium azide (1 mM). EGTA, phosphocreatine, and caffeine were obtained from the Sigma Chemical Co. (St. Louis, MO). 4-CmC was purchased from Fluka (New Ulm, Germany) and prepared as a stock solution of 0.25 M in dimethyl sulfoxide (DMSO). The maximal concentration of DMSO achieved in final solutions was 0.8%.

**Fitting of curves.** For all experimented fibers, the 4-CmC (or caffeine) concentration giving half-maximal response ($EC_{50}$) was calculated by fitting the 4-CmC (or caffeine) concentration–response curve through a Hill equation. Then for each substance, concentration–response curves representing the mean of 4-CmC (or caffeine) contractures relative amplitudes were plotted by using the mean of $EC_{50}$. Relative amplitude corresponded to the ratio of 4-CmC (or caffeine) contracture amplitude to amplitude of the maximal response achieved by the application of 4-CmC (or caffeine).

**Statistical analysis.** All values are expressed as means±SEM. A Student’s unpaired $t$-test was used to compare the parameters among groups. Statistical significance was reached when $p<0.05$.

**RESULTS**

**Effect of loading time and pCa on sarcoplasmic reticulum Ca$^{2+}$ content of soleus and edl**

After the skinning procedure, edl and soleus were sequentially immersed in five different solutions to load and release Ca$^{2+}$ from the sarcoplasmic reticulum. To modify sarcoplasmic reticulum Ca$^{2+}$ content, the time and the Ca$^{2+}$ concentration were varied during the loading step. Two different Ca$^{2+}$ loading times (15 s and 2 min) and two different Ca$^{2+}$ concentrations (10$^{-7}$ M [pCa 7.0] and 10$^{-7.5}$ M [pCa 7.5]) were tested. To estimate sarcoplasmic reticulum Ca$^{2+}$ content, 10 mM caffeine was then applied, and the amplitude and the area of contracture were measured. In soleus muscle, the amplitude and area of the 10 mM caffeine contractures obtained at 2 min in pCa 7.0 (control conditions) were not significantly different from those obtained after different sarcoplasmic reticulum Ca$^{2+}$ loading conditions. For example, the amplitude of the 10 mM caffeine contracture was 58.0±11.3 mN/mm$^2$ (n=4) at 2 min in pCa 7.0 and 62.2±6.7 mN/mm$^2$ (n=3) at 15 s in pCa 7.5. Conversely, the characteris-
tics of caffeine contracture (10 mM) in edl fibers were modified by loading conditions. Thus the amplitude of the 10 mM caffeine contracture decreased if loading time or Ca\(^{2+}\) concentration of the loading solution was reduced, and it became significantly lower when both these parameters were modified. Furthermore, compared with control conditions, the area of the 10 mM caffeine contracture was reduced by 18.3 ± 3.3\% (n=6, after 15 s in pCa 7.0), 24.3 ± 6.8\% (n=5, after 2 min in pCa 7.5), and 49.6 ± 2.1\% (n=5, after 15 s in pCa 7.5).

Because the Ca\(^{2+}\) loading conditions tested for soleus muscle did not significantly modify the characteristics of 10 mM caffeine contracture, the following experiments were conducted with caffeine and 4-CmC on edl fibers.

**Effects of the loading period or the pCa of the loading solution on 4-CmC and caffeine contractile responses**

Experiments were performed by incubating edl fibers either in a loading solution of pCa 7.0 for different times (15 s or 2 min) or in a loading solution of pCa 7.5 for 2 min. Skinned fibers were then placed in a solution containing different concentrations of 4-CmC (1 \(\mu\)M to 1 mM) or caffeine (0.1 to 10 mM). Figures 1 and 2 show records of a control Ca\(^{2+}\) release cycle (2 min in pCa 7.0) and a shorter cycle (15 s in pCa 7.0) for applications of 10 \(\mu\)M 4-CmC (Fig. 1) or 0.5 mM caffeine (Fig. 2).

For all loading conditions tested, 4-CmC threshold concentration was below 5 \(\mu\)M, and maximal responses (Fig. 3) were obtained for 50 \(\mu\)M (2 min in pCa 7.0, 102.3 ± 17.1 mN/mm\(^2\) [n=3]), 500 \(\mu\)M (15 s in pCa 7.0, 74.5 ± 3.9 mN/mm\(^2\) [n=3]), and 1 mM (2 min in pCa 7.5, 85.5 ± 15.0 mN/mm\(^2\) [n=4]).

As shown by these results, the amplitude of the contractures was modified by loading conditions. These differences were larger for low concentrations of 4-CmC (5 and 10 \(\mu\)M). For example, at 10 \(\mu\)M, the amplitude of the contracture was 34.8 ± 9.9 mN/mm\(^2\) (n=7) and 69.9 ± 21.6 mN/mm\(^2\) (n=3) for 15 s and 2 min, respectively, in pCa 7.0. Moreover, the amplitude of the 5 \(\mu\)M 4-CmC contractile response obtained after 2 min loading in pCa 7.5 was significantly reduced compared with that obtained after 2 min load-

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**Fig. 1.** Effect of sarcoplasmic reticulum Ca\(^{2+}\) loading time on 4-CmC contractures in edl saponin-skinned fibers. Traces correspond to the contractile responses obtained after application of 10 \(\mu\)M 4-CmC on fibers loaded for 15 s or 2 min in pCa 7.0. Experiments were performed at 22°C.

**Fig. 2.** Effect of sarcoplasmic reticulum Ca\(^{2+}\) loading time on caffeine contractures in edl saponin-skinned fibers. Traces correspond to the contractile responses obtained after an application of 0.5 mM caffeine on fibers loaded for 15 s or 2 min in pCa 7.0. Experiments were performed at 22°C.

**Fig. 3.** Maximal contractile response of 4-CmC (□) and caffeine (■) in mN/mm\(^2\), obtained under different loading conditions. All values are compared with the control response, i.e., for 2 min loading at pCa 7.0. *Indicates a significant difference between control and values obtained under different loading conditions (p<0.05). Experiments were performed at 22°C.
ing in pCa 7.0. Indeed, 5 μM 4-CmC induced a contracture with an amplitude of 4.5 ± 2.1 mN/mm² (n = five, 2 min in pCa 7.5) and 60.5 ± 3.8 mN/mm² (n = three, 2 min in pCa 7.0).

For 4-CmC contractures, the relative amplitude of the response was plotted against the different concentrations tested (Fig. 5). The concentration of 4-CmC that produced 50% of the maximal response (EC₅₀) was estimated (Fig. 4B). Compared with the 2 min cycle in pCa 7.0 (EC₅₀ = 6.15 ± 1.12 μM, n = three, control conditions), twice as much 4-CmC was needed to obtain 50% of the maximal response after a shorter loading time (15 s in pCa 7.0: EC₅₀ = 11.80 ± 1.90 μM, n = six) or a higher loading pCa, i.e., a lower Ca²⁺ concentration (2 min in pCa 7.5: EC₅₀ = 15.0 ± 2.90 μM, n = six). These values were significantly different from the control (p < 0.05). These changes are illustrated in Fig. 5 by a shift in the concentration-response curves to larger concentrations of 4-CmC and a decrease in the slope of the curve from 2.3 ± 0.3 (n = three, 2 min in pCa 7.0) to 1.8 ± 0.4 (n = six, 15 s in pCa 7.0) and 1.6 ± 0.3 (n = six, 2 min in pCa 7.5).

The time to peak and the time of half-relaxation of 4-CmC contractures were not significantly different between loading conditions. For example, for 10 μM of 4-CmC, the time to peak of the contracture was 24.7 ± 3.7 s (n = five) and 24.3 ± 2.7 s (n = three) for 15 s and 2 min loading, respectively, in pCa 7.0.

Similar to 4-CmC, the caffeine concentration threshold (0.1 mM) was not changed between the different loading conditions. Moreover, for low concentrations of caffeine (Fig. 2), differences in contractures amplitude existed between two loading conditions (2 min and 15 s in pCa 7.0) but they were less than for 4-CmC. The amplitudes of caffeine contractures were not significantly different between 2 min in pCa 7.0 and 7.5. Contrary to 4-CmC, maximal caffeine response (Fig. 3) was obtained with a similar concentration (10 mM) for all the loading conditions tested. Moreover, the EC₅₀ for caffeine was not significantly different for the three loading conditions tested (Fig. 4A): 0.54 ± 0.09 mM (n = six, 2 min loading in pCa 7.0), 0.74 ± 0.15 mM (n = six, 15 s loading in pCa 7.0), and 0.53 ± 0.10 mM (n = five, 2 min loading in pCa 7.5). Furthermore, the slopes of the curves plotted for these conditions were not significantly modified, as illustrated in Fig. 6.

As for 4-CmC, no significant difference was observed for the time to peak and the time of half-relaxation of the caffeine contractures between the different loading conditions.
These results indicate that different loading times or Ca$^{2+}$ concentrations of the loading solution modified the amplitudes of 4-CmC and caffeine contractures. These changes were more pronounced for 4-CmC than for caffeine. Moreover, the sensitivity of edl muscle to 4-CmC was largely modified compared with that of caffeine. This point was further investigated by testing the effect of a short loading time and a low Ca$^{2+}$ concentration on 4-CmC and caffeine contractures.

**Effects of loading time and the pCa of the loading solution on 4-CmC and caffeine contractures**

4-CmC and caffeine contractures were investigated on edl fibers loaded in a solution of pCa 7.5 for 15 s. The responses were compared with those obtained for fibers loaded in pCa 7.0 for 2 min.

For 15 s loading in pCa 7.5, no contractile response was produced with 5 μM of 4-CmC. Furthermore, contracture amplitudes were significantly lower than for 2 min in pCa 7.0 (p<0.05) for all concentrations of 4-CmC tested. For example, 10 μM of 4-CmC produced a contracture of 21.3±11.4 mN/mm$^2$ (n=8) for 15 s in pCa 7.5 and 69.9±21.6 mN/mm$^2$ (n=3) for 2 min in pCa 7.0. The maximal amplitude of 4-CmC contracture was significantly reduced (p<0.05) compared with that of loading in pCa 7.0 for 2 min (Fig. 3): 57.4±7.2 mN/mm$^2$ (n=3) versus 102.3±17.1 mN/mm$^2$ (n=3). In addition, it was reached for a larger concentration of 4-CmC: 1 mM versus 50 μM.

Similar results were found when caffeine was tested. Interestingly, 0.1 mM of caffeine failed to induce a transient response after 15 s loading in pCa 7.5, whereas for the other loading conditions, a slight response was displayed: 2.5×10$^{-7}$±0% (n=4, 2 min in pCa 7.0), 0.3±0.3% (n=3, 15 s in pCa 7.0), and 1.5±0.8% (n=3, 2 min in pCa 7.5). The amplitude of all caffeine contractures were significantly reduced after 15 s loading in pCa 7.5, compared with 2 min in pCa 7.0 (p<0.05). Contrary to 4-CmC, the caffeine concentration producing the maximal response (10 mM, Fig. 3) was not dependent on loading conditions.

As illustrated in Fig. 4, 4-CmC- and caffeine-EC$_{50}$ were significantly greater for 15 s loading in pCa 7.5 than for 2 min loading in pCa 7.0 (p<0.05). Indeed, 4-CmC-EC$_{50}$ was 6.15±1.12 μM (n=3) for 2 min in pCa 7.0, and 77.44±21.40 μM (n=8) for 15 s in pCa 7.5. Furthermore, as shown in Fig. 5 the shift of the 4-CmC concentration–response curve to larger concentrations was associated with a significantly reduced slope (p<0.05): 0.9±0.2 (n=8) versus 2.3±0.3 (n=3).

The caffeine-EC$_{50}$ was increased only twice as much (Fig. 4A): 0.54±0.09 mM (n=six, 2 min loading in pCa 7.0) and 1.02±0.20 mM (n=five, 15 s loading in pCa 7.5), but the slope of the concentration–response curve was not modified.

**DISCUSSION**

This study compared the contractile responses induced by 4-CmC and caffeine in saponin-skinned fibers of skeletal muscle after different Ca$^{2+}$ loading conditions. In fast-twitch muscle, a short Ca$^{2+}$ loading time induced a decrease in the area of 10 mM caffeine contracture compared with a longer loading period; whereas in slow-twitch fibers, no significant variation was observed. In fast-twitch muscle, contracture amplitude was also reduced when Ca$^{2+}$ uptake was performed in a solution containing a low Ca$^{2+}$ concentration compared with a higher Ca$^{2+}$ concentration. The application of caffeine to saponin-skinned fibers is known to generate contractures with an amplitude and area in relation to the amount of Ca$^{2+}$ released from the sarcoplasmic reticulum [29]. Moreover, the present experiments were conducted by using 10 mM caffeine because this concentration allowed the release of almost all sarcoplasmic reticulum Ca$^{2+}$ content, causing few side effects on the contractile apparatus [26]. Our results also indicate that 4-CmC and caffeine concentration–response curves in fast-twitch muscle were shifted to larger concentrations upon use of low Ca$^{2+}$ concentrations and/or a short loading period. Thus it is likely that the sensitivity of edl muscle to these sub-
stances was reduced when sarcoplasmic reticulum content decreased.

Ca\(^{2+}\) release channel activity is known to be modulated by the luminal Ca\(^{2+}\) of the sarcoplasmic reticulum [30]. In saponin-skinned fibers, caffeine and 4-CmC release Ca\(^{2+}\) by activating the ryanodine receptor [11, 12, 20]. According to the results of the present experiment, it seems likely that sarcoplasmic reticulum Ca\(^{2+}\) content of edl skinned fibers modifies the sensitivity of the ryanodine receptor for 4-CmC and caffeine. Endo [31] has shown that a given level of sarcoplasmic reticulum Ca\(^{2+}\) content is necessary on saponin-skinned fibers before Ca\(^{2+}\) release can be triggered by caffeine. It has also been shown in PC12 cells that Ca\(^{2+}\) released by caffeine is dependent on sarcoplasmic reticulum Ca\(^{2+}\) content [13]. Our results indicate that the lowest loading conditions result in a shift of 4-CmC and caffeine threshold concentrations to larger values. Indeed, for 4-CmC the threshold that was inferior to 5 \(\mu\)M was shifted to a concentration comprised between 5 and 10 \(\mu\)M, and for caffeine it was displaced to a value between 0.1 and 0.5 mM, from 0.1 mM. This result is consistent with the previous work of Dettbarn and Palade [32], showing that an increase in luminal Ca\(^{2+}\) lowered the threshold agonist concentration required to elicit Ca\(^{2+}\) release. Moreover, it has been demonstrated that the caffeine threshold for ryanodine receptor activation is decreased when sarcoplasmic reticulum Ca\(^{2+}\) content is increased [13]. Our results for low concentrations of agonists indicate that the amplitude of responses was increased when sarcoplasmic reticulum Ca\(^{2+}\) content increased. This effect was previously observed by Donoso et al. [33], who showed that the initial rates of Ca\(^{2+}\) release increased with the enhancement of luminal Ca\(^{2+}\).

Our results show that the change of sensitivity in edl fibers to ryanodine receptor agonists associated with the sarcoplasmic reticulum Ca\(^{2+}\) content was more marked for 4-CmC than for caffeine. The apparent affinity (EC\(_{50}\)) of edl fibers for 4-CmC was significantly affected in all loading conditions tested, whereas this effect was observed only for caffeine when a short loading time and a low Ca\(^{2+}\) concentration were tested simultaneously. Thus when sarcoplasmic reticulum Ca\(^{2+}\) content was largely reduced in comparison with control conditions, the apparent affinity of edl fibers was tenfold lower for 4-CmC, but only twofold lower for caffeine. Because the effect of luminal Ca\(^{2+}\) was greater for 4-CmC than for caffeine, it is likely that a stronger interaction exists between the 4-CmC binding site and the site where luminal Ca\(^{2+}\) acts. These results are supported by the proposal of Herrmann-Frank et al. [16] that the greater sensitivity of the ryanodine receptor to Ca\(^{2+}\) in the presence of 4-CmC is due to the close proximity of the 4-CmC binding site (presumably in the luminal part of the ryanodine receptor) to the potential high-affinity Ca\(^{2+}\) site. The major regulatory Ca\(^{2+}\) binding site has been situated in the carboxy-terminal region of the ryanodine receptor and more precisely in a cytoplasmic loop between two transmembrane segments of the ryanodine receptor [34, 35]. Moreover, it has been suggested that luminal Ca\(^{2+}\) affects cytosolic Ca\(^{2+}\) activation and inactivation sites of the ryanodine receptor [36]. Our results for caffeine could be explained by the findings of Bhat et al. [19], indicating that the caffeine binding site is in the NH\(_2\) region of the ryanodine receptor. Thus the few changes observed in caffeine as compared with 4-CmC sensitivity may be indicative of a lower interaction between Ca\(^{2+}\) and caffeine binding sites.

Our previous experiments indicated that cytosolic Ca\(^{2+}\) activity influenced the responses of 4-CmC and caffeine [20] and that this effect was more marked for 4-CmC contractile responses of edl muscle fibers. In that way it could not be excluded that for different loadings of the sarcoplasmic reticulum, Ca\(^{2+}\) released from the sarcoplasmic reticulum was also responsible for the larger modifications in the responses of 4-CmC than in caffeine.

Taken together, these different results argue for a stronger effect of cytosolic and luminal Ca\(^{2+}\) activity of edl muscle fibers on ryanodine receptor sensitivity to 4-CmC, compared with caffeine. Thus 4-CmC would appear to be a better tool than caffeine for sensing the level of disturbance of Ca\(^{2+}\) homeostasis in muscular pathologies, especially in edl muscle. Our results also indicate that Ca\(^{2+}\)-induced modulation of ryanodine receptor activity is a complex mechanism.

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