Cell Calcium (1998) **24**(2), 105–115

Effects of ryanodine receptor agonist 4-chloro-*m*-cresol on myoplasmic free Ca^{2+} concentration and force of contraction in mouse skeletal muscle

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Summary In single mouse skeletal muscle fibers injected with fluorescent Ca^{2+} indicator Indo–1, 4-chloro-*m*-cresol (chlorocresol, 4-CmC) and its lipophilic analogue 4-chloro-3-ethylphenol (4-CEP) increased resting myoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in a dose-dependent manner. In this regard, 4-CEP was more potent than 4-CmC and both were more potent than caffeine. High concentrations of 4-CmC (1 mM) or 4-CEP (500 μM) caused large and irreversible increase in resting $[Ca^{2+}]_i$ leading to contracture. 4-CmC potentiated the $[Ca^{2+}]_i$ increase and force of contraction induced by tetanic stimulation. Unlike caffeine, 4-CmC did not affect the activity of sarcoplasmic reticulum Ca^{2+} pump or the myofibrillar Ca^{2+} sensitivity. A low concentration of 4-CEP (20 μM) had no effect on resting $[Ca^{2+}]_i$ on its own, but it enhanced the resting $[Ca^{2+}]_i$ increase induced by caffeine and also potentiated the $[Ca^{2+}]_i$ increase and contraction induced by tetanic stimulation. However, a relatively high concentration of 4-CEP (200 μM) inhibited tetanic stimulation-induced $[Ca^{2+}]_i$ increase and contraction. Dantrolene, a muscle relaxant, inhibited 4-CmC-induced $[Ca^{2+}]_i$ increase under resting conditions. However, when 4-CEP was applied in the presence of dantrolene, there was an exaggerated increase in $[Ca^{2+}]_i$. We conclude that 4-CmC and 4-CEP are potent agonists that can increase $[Ca^{2+}]_i$ rapidly and reversibly by activating ryanodine receptors in situ in intact skeletal muscle fibers. These compounds, specially 4-CmC, may be useful for mechanistic and functional studies of ryanodine receptors and excitation-contraction coupling in skeletal muscles.

INTRODUCTION

Ryanodine receptors (RY receptors) are Ca^{2+} release channels in the sarcoplasmic reticulum (SR) that play a crucial role in excitation–contraction coupling in striated muscles [1]. Functional RY receptors are tetramers of 560 subunits. cDNAs for three RY receptors have been cloned [2]. RY_1 and RY_2 receptors are the major isoforms expressed in skeletal and cardiac muscles, respectively. The immunophilins FKBP12 and FKBP12.6 associate with RY_1 and RY_2 receptors, respectively, and stabilize the channel [3]. RY_1 receptor is activated in response to depolarization of transverse tubular membrane probably through a mechanical interaction with the dihydropyridine receptor. In heart, depolarization-induced Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channels activates RY_2 receptor by a mechanism called Ca^{2+}-induced Ca^{2+} release [4,5]. Mutations in RY receptors have been associated with several disease conditions.

Received 8 May *1998*
Revised 25 June 1998
Accepted 30 June 1998

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Abbreviations used: RY receptor, ryanodine receptor; 4-CmC, 4-chloro-*m*-cresol; 4-CEP, 4-chloro-3-ethylphenol; $[Ca^{2+}]_i$, myoplasmic free $[Ca^{2+}]$, SR, sarcoplasmic reticulum.

Malignant hyperthermia is a pharmacogenetic disorder characterised by enhanced sensitivity of RY_1 receptor to halogenated anesthetics and muscle relaxants like succinylcholine leading to Ca^{2+} release, sustained muscle contracture and hypermetabolic state [6].

A wide variety of structurally unrelated substances have been demonstrated to release Ca^{2+} from SR [7]. These include ryanodine, caffeine as well as sulfhydryl oxidizing agents and drugs like halothane and doxorubicine. The effects of such substances on RY receptors have been characterised to a variable extent and only a few of them are considered useful as pharmacological or diagnostic tools in studies involving RY receptors. Ryanodine, a plant alkaloid binds to specific high or low affinity sites on the RY receptors and can have either stimulatory or inhibitory effects. Studies in cell free systems show that ryanodine usually activates the channel when used at nanomolar concentrations and inhibits the channel when used at micromolar concentrations [8]. The molecular mechanism by which this occurs is not well understood. Ryanodine is often difficult to use because of its slow and essentially irreversible nature of binding to the receptor. Such difficulty becomes more pronounced when ryanodine is to be used in experiments with intact cells and distinction between its stimulatory and inhibitory action becomes essential for correct interpretation of the results.

Caffeine is the most widely used activator of RY receptors but is also far from being a satisfactory one. It needs to be used at millimolar concentrations that may have many nonspecific actions. Caffeine inhibits SR Ca^{2+} pump, increases myofibrillar Ca^{2+} sensitivity, inhibits several ion channels, and increases cellular cAMP level [9,10]. Furthermore, despite many studies, it is still unknown which site(s) on the RY receptors mediates caffeine effect. Some studies suggest that the caffeine-binding site may lie within the amino terminal region of RY receptors [11] but it may even be on a different protein closely associated with RY receptors [12].

Thus, there is clearly a lack of suitable ligands that could be used for mechanistic studies of RY receptors or for diagnostic purposes. Recently, a new group of RY receptor agonists have been described. These are 4-chloro-*m*-cresol (chlorocresol, 4-CmC) a preservative commonly present in some pharmaceutical preparations and its lipophilic analog 4-chloro-3-ethyl phenol (4-CEP) [13]. These monocyclic hydrocarbons directly activate RY receptors in planar lipid bilayer and release Ca^{2+} from fractionated skeletal muscle terminal cisternae [14–16]. These agents are being used for studying RY receptors in different cells [13,17]. A common use of 4-CmC is in the skeletal muscle cells where the agent is being used in in vitro contracture tests for diagnosing susceptibility to malignant hyperthermia [18]. However, while numerous studies have characterised effects of caffeine on skeletal muscle, many aspects of the effects of 4-CmC and related compounds remain to be studied. There is currently no information on the pattern of $[Ca^{2+}]_i$ changes induced by 4-CmC in intact skeletal muscle fibers and how such changes are affected by other RY receptor agonists and antagonists. In the present study, we have characterised $[Ca^{2+}]_i$ changes induced by these compounds and their effects on contractility of skeletal muscle fibers. Our results show that 4-CmC potently and reversibly activates RY receptors in situ in skeletal muscle fibers without affecting SR Ca^{2+} pumping or myofibrillar Ca^{2+} sensitivity.

MATERIALS AND METHODS

Dissection and mounting

Male mice (NMRI strain) were killed by rapid cervical dislocation. Single fibers were dissected from the flexor brevis muscle of the hind limb [19]. Isolated fibers were mounted in a chamber between an Akers 801 force transducer and an adjustable holder allowing the fiber to be stretched to the length giving maximum force in response to electrical stimulation.

Solutions and drugs

Experiments were performed at room temperature (22–24°C). The fiber was continuously superfused by a standard Tyrode solution containing (in mM): NaCl 121, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 24, glucose 5.5, fetal calf serum 0.2%. The solution was gassed with 95% O_2 and 5% CO_2 which gave a bath pH of 7.4. The rate of superfusion was 1 ml/min and the volume of the muscle chamber was 200 µl. Thus, changes in bathing solutions were accomplished within ~12 s. 4-CEP, 4-CmC and forskolin were dissolved in DMSO and final concentration of DMSO in the solution was < 0.001%. Caffeine and dantrolene were dissolved directly in the Tyrode solution.

$[Ca^{2+}]_i$ measurement

Indo-1 pentapotassium salt was pressure injected into the myoplasm after impalement of the fiber with a micropipette containing 10 mM of the dye dissolved in 150 mM KCl and 10 mM HEPES (pH 7.3). The experimental chamber was mounted on a microscope equipped for fluorescence and photometry. Fluorescence was measured with a system consisting of a xenon lamp, a monochromator and two photomultiplier tubes (PTI,

Photo Med GmbH, Wedel, Germany). The excitation light was set to 360 nm and the emitted light at 405 and 495 nm was measured. The Ca^{2+}-dependent signal was obtained as the ratio of the light emitted at 405 to that at 495 nm. The fluorescence ratio was converted to $[Ca^{2+}]_i$ by an intracellular calibration [20]. The dissociation constant for Indo–1 was 283 nM. In experiments where the effect on resting $[Ca^{2+}]_i$ was studied, fibers were exposed to 4-CEP for 10 min and to 4-CmC until a stable $[Ca^{2+}]_i$ was obtained. $[Ca^{2+}]_i$ was measured as the mean over 10 s before and at the end of drug exposure, respectively. While caffeine quenched the fluorescence of Indo–1, 4-CmC and 4-CEP had no obvious interference with fluorescence signals.

Assessment of contractile function and Ca^{2+} handling during tetanic stimulation

Tetanic contractions were produced by giving a train (total duration 350 ms) of supramaximal current pulses (duration 0.5 ms). Standard stimulation frequency was 100 Hz except in experiments where the force–$[Ca^{2+}]_i$ relationship was studied. In experiments with 4-CmC, tetanic contractions were produced at 1 min interval. In most experiments with 4-CEP, single 100 Hz contractions were produced under control conditions, after 10 min exposure to the substance, and after at least 10 min washout.

Analysis of SR Ca^{2+} pump function

The methods and assumptions involved in the analysis of SR pump function have been described before [21]). The effect of 4-CmC on SR Ca^{2+} uptake was studied by analyzing the late slow phase of $[Ca^{2+}]_i$ decline after tetanic stimulations ($[Ca^{2+}]_i$ tails) [21]. For this purpose, we used averaged $[Ca^{2+}]_i$ traces from three 100 Hz tetani obtained under control conditions and three 100 Hz tetani produced after $[Ca^{2+}]_i$ had stabilised after exposure to 4-CmC. $[Ca^{2+}]_i$ tails were fitted to double exponential functions. Ca^{2+} uptake is proportional to the rate of $[Ca^{2+}]_i$ changes ($d[Ca^{2+}]_i/dt$) and thus a plot of $d[Ca^{2+}]_i/dt$ versus $[Ca^{2+}]_i$ would represent SR pump function curve. $d[Ca^{2+}]_i/dt$ was measured at regular intervals on these curve fits and was plotted against $[Ca^{2+}]_i$. The following equation was fitted to the data points:

$$d[Ca^{2+}]_i/dt = A\,[Ca^{2+}]_i^N - L \qquad \text{Eq. 1}$$

where A reflects the rate of SR Ca^{2+} uptake and L is the SR Ca^{2+} leak. N is a power function and was set to 4, which gave a reasonable fit in all experiments. This was done because small changes of N will have a very large effect on A, which makes changes of A impossible to assess with any accuracy.

The effect of 4-CmC on the myofibrillar Ca^{2+} sensitivity was studied by giving tetani at 20–100 Hz and fitting force–$[Ca^{2+}]_i$ data-points to a Hill equation [21]:

$$P = P_{max}\,[Ca^{2+}]_i^N/(Ca_{50}{}^N + [Ca^{2+}]_i^N) \qquad \text{Eq. 2}$$

where P_{max} is the force at saturating $[Ca^{2+}]_i$, P is the force in percent of P_{max}, Ca_{50} is the $[Ca^{2+}]_i$ giving 50% of P_{max}, and N is a constant that describes the steepness of the function. P_{max} was obtained by producing a 100 Hz tetanus in the presence of 5 mM caffeine and was taken as 100% in each experiment.

Measurement of membrane potential

Membrane potential was measured in fibers of soleus muscle from the same type of mice. Individual soleus fibers were impaled by microelectrode filled with 3 M KCl and the membrane potential was recorded. A single action potential was produced by a brief shock (< 1 ms) from an extracellular blunt microelectrode positioned over the muscle fiber that was impaled by the measuring microelectrode.

Statistics

Values are presented as means ± SEM. Student's paired or unpaired *t*-tests were used as test for significance and significance level was set at 0.05 throughout.

Materials

4-chloro-*m*-cresol and 4-chloro-3-ethylphenol were from Aldrich (Steinheim, Germany). Indo–1 was from Molecular Probes Europe (Leiden, The Netherlands). Other reagents were from Sigma (St Louis, MO, USA).

RESULTS

Effects of 4-CmC and 4-CEP on resting $[Ca^{2+}]_i$

We first examined whether 4-CmC and 4-CEP could act on the resting conformation of RY receptor in skeletal muscle and activate the channel in situ. Figure 1A shows the typical effect of 200 μM 4-CmC on resting $[Ca^{2+}]_i$ in a single muscle fiber. $[Ca^{2+}]_i$ began to rise almost immediately on arrival of 4-CmC to the chamber, increased slowly to a plateau and reversed on wash out of the substance. The increase in $[Ca^{2+}]_i$ caused by 4-CmC was dose-dependent, the lowest concentration yielding a significant rise being 100 μM (Fig. 1B,E). Repeated application of 4-CmC to the same fiber induced repeated increase in $[Ca^{2+}]_i$. 4-CEP also increased $[Ca^{2+}]_i$ in a dose-dependent manner and proved more potent than 4-CmC (Fig. 1C–E). In the experiment shown in Figure 1D, 20 μM

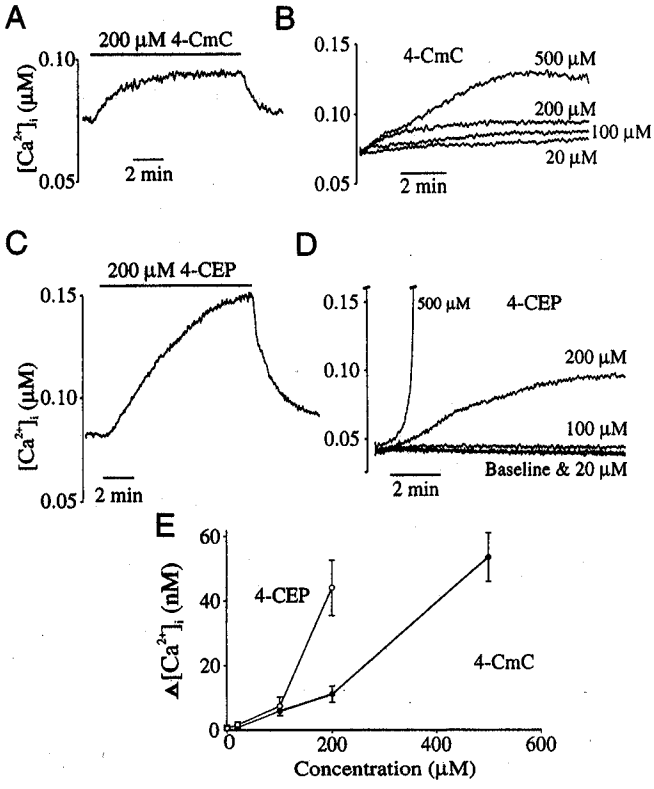

Fig. 1 Effect of 4-CmC and 4-CEP on $[Ca^{2+}]_i$ under resting conditions. (**A**) Original record from one representative fiber showing the time course of changes in $[Ca^{2+}]_i$ during exposure to 4-CmC. Period of exposure to 200 μM 4-CmC is indicated by the horizontal bar above the record. (**B**) The magnitude of $[Ca^{2+}]_i$ increase on exposure to different concentrations of 4-CmC. Superimposed $[Ca^{2+}]_i$ traces obtained from one fiber are shown. Increasing concentrations of 4-CmC were applied to the fiber successively over 10 min periods; applications start at the beginning of each trace. After each application, the substance was washed out and $[Ca^{2+}]_i$ allowed to return to the basal level before a higher concentration of the agent was applied. (**C**) Original record from one fiber showing the time course of changes in $[Ca^{2+}]_i$ during exposure to 200 μM 4-CEP and the subsequent wash-out. (**D**) Superimposed $[Ca^{2+}]_i$ traces obtained from one fiber on exposure to different concentrations of 4-CEP. $[Ca^{2+}]_i$ was allowed to return to base line between the applications (as in B). 500 μM 4-CEP increased $[Ca^{2+}]_i$ to > 5 μM leading to contracture. The increase in $[Ca^{2+}]_i$ by 500 μM 4-CEP was not reversible. (**E**) Comparison of dose-dependent increase in $[Ca^{2+}]_i$ by 4-CmC and 4-CEP. $\Delta[Ca^{2+}]_i$ is the difference between $[Ca^{2+}]_i$ measured over 10 s before and at the end of application of the compounds. Data points represent mean and standard error of 4–10 measurements at different concentrations.

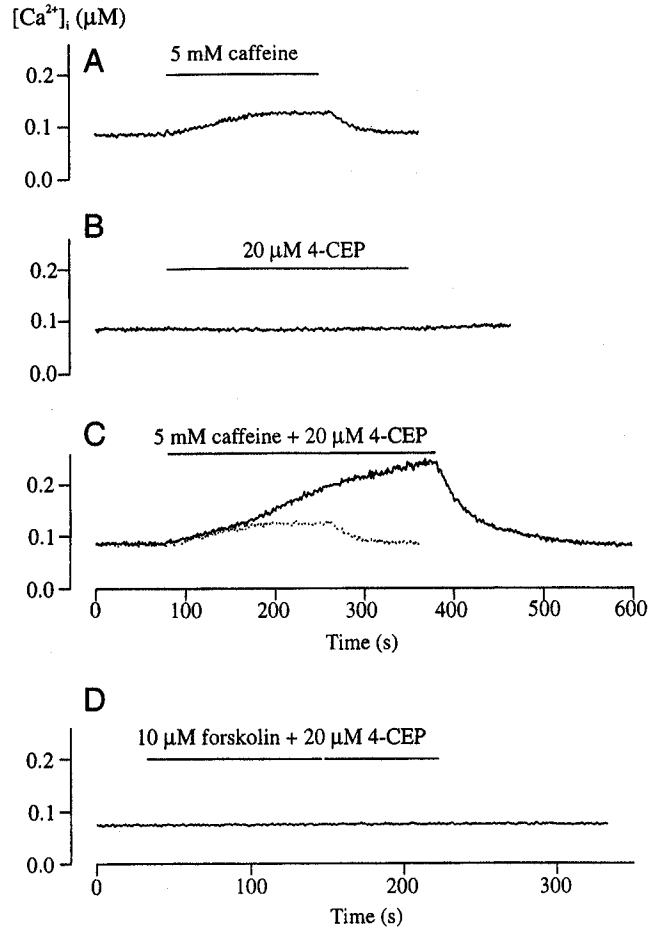

Fig. 2 4-CEP enhances caffeine-induced increase in $[Ca^{2+}]_i$. Figure shows traces obtained from one fiber in the presence of 5 mM caffeine (**A**), 20 μM 4-CEP (**B**), and 5 mM caffeine plus 20 μM 4-CEP (**C**). 5 mM caffeine increased $[Ca^{2+}]_i$ which reached a plateau and reversed on wash out (**A**). 20 μM 4-CEP did not increase $[Ca^{2+}]_i$. (**B**). Superimposed traces obtained on application of 5 mM caffeine alone (dotted line; from A) or 5 mM caffeine plus 20 μM 4-CEP (solid line) are shown in (**C**). (**D**) shows lack of response of another fiber to 10 μM forskolin and 20 μM 4-CEP.

4-CEP did not increase $[Ca^{2+}]_i$, whereas 100 μM of the substance induced a small increase (~6 nM, as measured at the end of 10 min exposure). A larger increase in $[Ca^{2+}]_i$ (~50 nM) was achieved with 200 μM 4-CEP. At 200 and 500 μM concentrations, the maximal increases in $[Ca^{2+}]_i$ were significantly higher with 4-CEP than with 4-CmC (Fig. 1B,D,E). The rates of rise of $[Ca^{2+}]_i$ on application of 200 μM of the substances were, however, faster with 4-CmC ($t_{1/2} = 82 \pm 15$ s, $n = 5$) than with 4-CEP ($t_{1/2} = 198 \pm 28$ s, $n = 10$) ($P = 0.016$) (cf. Fig. 1A and 1C). 500 μM 4-CEP increased $[Ca^{2+}]_i$ to very high level (e.g. > 5 μM) often close to the saturation point of Indo-1, thus making exact measurement of $[Ca^{2+}]_i$ difficult. At this concentration, 4-CEP induced an increase in resting force which started at a $[Ca^{2+}]_i$ of 356 ± 29 nM ($n = 4$) and led invariably to an irreversible contracture. With 4-CmC, 1 mM of the substance was required for such effect. The large $[Ca^{2+}]_i$ increase achieved with 500 μM 4-CEP or 1 mM 4-CmC did not reverse on wash out of the substances.

4-CEP enhanced Ca^{2+} release by caffeine

To investigate whether the increase in $[Ca^{2+}]_i$ in intact muscle fibers induced by 4-CEP exposure involves activation of RY receptor, we examined the effect of this agent on Ca^{2+} release by caffeine, a well-studied agonist of the receptor. Figure 2 shows original records from one fiber first exposed to caffeine (5 mM), then to a subthreshold concentration of 4-CEP (20 μM), and finally to both caffeine and 4-CEP. It can be seen that caffeine induced an increase in $[Ca^{2+}]_i$ which reached a plateau and readily reversed on washout of the substance (Fig. 2A). However, when caffeine was added together with 4-CEP (Fig. 2C), $[Ca^{2+}]_i$ continued to increase instead of stabilizing at a plateau as was observed with caffeine alone. Mean data from these experiments showed that caffeine (5 mM) significantly increased $[Ca^{2+}]_i$ by 21 ± 6 nM from a basal value of 60 nM, whereas 4-CEP had no significant effect (see Fig. 1). When caffeine and 4-CEP were added together in the same fibers, resting $[Ca^{2+}]_i$ increased by 95 ± 27 nM, as measured at the end of 5 min exposure. Thus 20 μM 4-CEP which alone did not increase resting $[Ca^{2+}]_i$ (cf. Fig. 2*B*) markedly potentiated the Ca^{2+}-releasing effect of caffeine. Caffeine increased $[Ca^{2+}]_i$ more rapidly ($t_{1/2} = 28 \pm 4$ s, $n = 4$) than 200 μM 4-CmC or 4-CEP (cf. Fig. 2A & Fig. 1A,C). In all experiments where caffeine and 4-CEP were added together, the initial rate of increase of $[Ca^{2+}]_i$ was similar to that obtained with caffeine alone (see Fig. 2C) suggesting that the potentiating effect of 4-CEP sets in after a delay. The continuous rise of $[Ca^{2+}]_i$ achieved with caffeine and 4-CEP reversed on wash out of the substances.

Caffeine could have potentiated the effect of 4-CEP by increasing cellular cAMP level [22,23]. To test this, we used forskolin that elevates the cAMP level by activating adenylylcyclase. Forskolin (10 μM) did not increase $[Ca^{2+}]_i$ by itself in any of the experiments ($n = 3$). As shown in Figure 2D, combination of 10 μM forskolin and 20 μM 4-CEP also did not increase $[Ca^{2+}]_i$. In one of these experiments, the fiber was exposed to forskolin for 5 min before addition of 4-CEP, but still no increase of $[Ca^{2+}]_i$ was observed.

Effects of dantrolene on Ca^{2+} release by 4-CmC and 4-CEP

Dantrolene, a muscle relaxant, is an inhibitor of RY receptors but has also been demonstrated to have a stimulatory effect on these channels [24]. In our experiments, application of 100 μM dantrolene reduced the resting $[Ca^{2+}]_i$ by 30 ± 4 nM from a mean starting level of 87 nM ($n = 8$). As shown in Figure 3A, 100 μM dantrolene completely blocked the $[Ca^{2+}]_i$-elevating effect of 200 μM 4-CmC; similar results were obtained in three fibers. The effect of dantrolene on 4-CEP-induced $[Ca^{2+}]_i$ increase was complex and is illustrated in Figure 3B. As stated above, when applied to resting muscle, dantrolene reduced basal $[Ca^{2+}]_i$, presumably by reducing the Ca^{2+} leak through the RY receptors and, thus, promoting sequestration of the ion in the SR. Subsequent addition of 200 μM 4-CEP had no obvious effect on $[Ca^{2+}]_i$ for 2–4 min: but, thereafter, 4-CEP induced an abrupt and large increase in $[Ca^{2+}]_i$ in all experiments ($n = 4$) despite the continued presence of dantrolene. The total increase in $[Ca^{2+}]_i$ with 4-CEP and dantrolene applied together was larger than that with the same concentration 4-CEP alone (cf. Fig. 3B & Fig. 1C) and a contracture developed in all experiments when the drugs were added together.

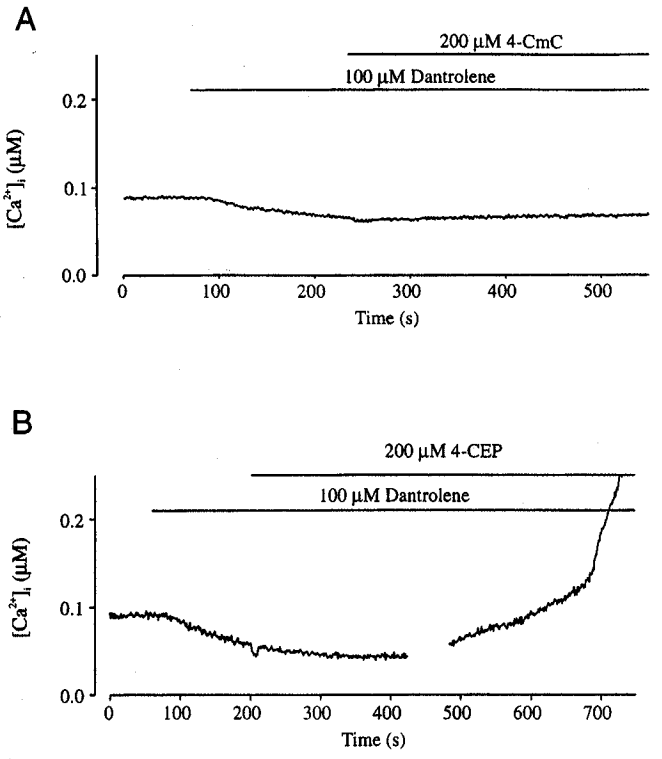

Fig. 3 Effects of dantrolene on $[Ca^{2+}]_i$ changes induced by 4-CmC and 4-CEP. (**A**) Original record from one representative fiber exposed to dantrolene and 4-CmC. Dantrolene (100 μM) reduced basal $[Ca^{2+}]_i$. Addition of 4-CmC (200 μM) in the presence of dantrolene did not affect $[Ca^{2+}]_i$. (**B**) $[Ca^{2+}]_i$ trace obtained from another muscle fiber. Dantrolene (100 μM) and 4-CEP (200 μM) were added at times indicated by the horizontal bars.

Effect of 4-CmC on $[Ca^{2+}]_i$ and force of contraction during tetanic stimulation

To test whether 4-CmC could affect depolarization-induced activation of RY receptor and contraction, we

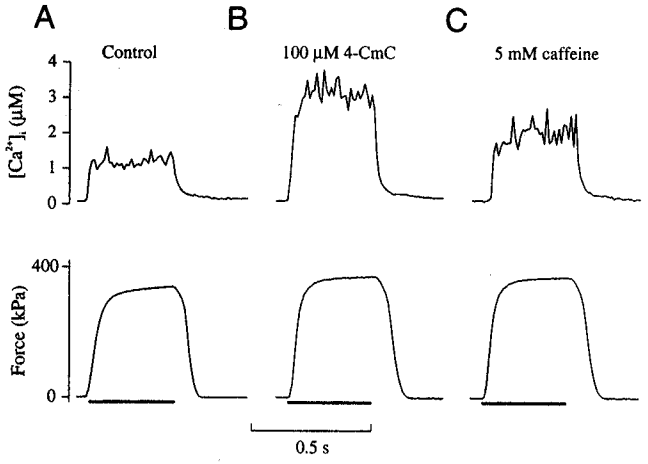

Fig. 4 Effects of 4-CmC on $[Ca^{2+}]_i$ and force of contraction induced by tetanic stimulation. The muscle fiber was stimulated by giving a train of supramaximal current pulses at 100 Hz. The upper panel shows changes in $[Ca^{2+}]_i$ and the lower panel force of contraction measured simultaneously. Responses obtained under control conditions (**A**), after application of 100 μM 4-CmC (**B**), and in the presence of 5 mM caffeine after wash-out of 4-CmC (**C**) are shown. Horizontal lines below force records indicate period of stimulation.

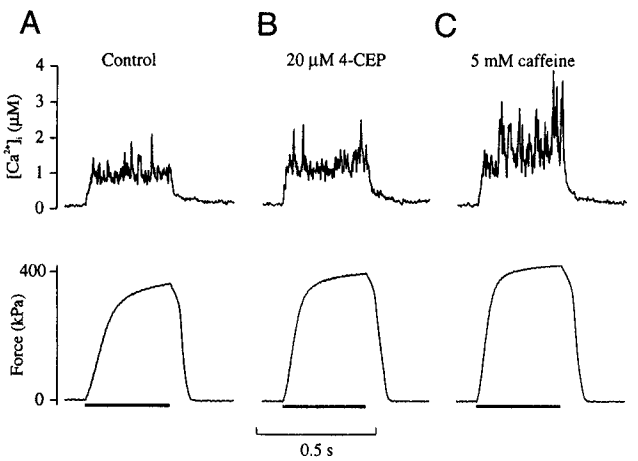

Fig. 5 Effects of 4-CEP on $[Ca^{2+}]_i$ and force of contraction induced by tetanic stimulation. Conditions of experiments were as described in the caption to Figure 4. $[Ca^{2+}]_i$ changes (upper panel) and force of contraction (lower panel) induced by tetanic stimulation under control conditions (**A**), in the presence of 20 μM 4-CEP (**B**) and in the presence of 5 mM caffeine (**C**) are shown.

produced contraction by 350 ms tetanic stimulation in the presence of 20–200 μM 4-CmC. In these experiments, the mean amplitudes of $[Ca^{2+}]_i$ and force of contraction during the last 100 ms of stimulation in the treated groups were compared with that in the controls. 20 μM 4-CmC had minimal stimulatory effect on tetanic $[Ca^{2+}]_i$ and force of contraction (data not shown). As illustrated in Figure 4B, 100 μM 4-CmC increased both tetanic $[Ca^{2+}]_i$ (upper panel) and force of contraction (lower panel). In this fiber, the tetanic $[Ca^{2+}]_i$ obtained in the presence of 100 μM 4-CmC was higher than that obtained in a subsequent tetanus in the presence of 5 mM caffeine after wash out of 4-CmC. The full effect of 4-CmC on tetanic $[Ca^{2+}]_i$ and force of contraction developed after a 2 min exposure to the substance and, thereafter, remained stable throughout the period of exposure to 4-CmC. $[Ca^{2+}]_i$ during tetanic stimulation in the presence of 100 μM 4-CmC was 1406 ± 368 nM higher than the mean control value of 1437 nM, i.e. an increase of 98 ± 18% ($P = 0.012$). This increase was very similar to that obtained with 5 mM caffeine in the same fibers, which amounted to 1510 ± 569 nM or an increase of 94 ± 27% ($P = 0.045$). The increase in the force of contraction on tetanic stimulation obtained with 100 μM 4-CmC (10 ± 4%, $P = 0.034$) was similar to that obtained with 5 mM caffeine (13 ± 3%, $P = 0.0047$; Fig. 4, lower panel). It may also be noted that the rate of force rise in tetani is markedly higher in the presence of 4-CmC or caffeine as compared to control conditions. The effects of both 4-CmC and caffeine on tetanic contractions were reversible on wash out. We tested effects of 200 μM 4-CmC on tetanic $[Ca^{2+}]_i$ and contraction in three fibers. This concentration of 4-CmC gave an increase of tetanic $[Ca^{2+}]_i$ and force comparable to that obtained with 100 μM of the substance. However, in two of these experiment the tetanic $[Ca^{2+}]_i$ and force started to decline and became irregular after a 4 min exposure to the substance (data not shown).

Effects of 4-CEP on $[Ca^{2+}]_i$ and force of contraction during tetanic stimulation

We repeated the experiments described in the preceding paragraph but now in the presence of 20–200 μM 4-CEP. We produced single tetanic contraction after a 10 min exposure to the substance. As shown in Figure 5B, $[Ca^{2+}]_i$ (upper panel), peak force and rate of force rise (lower panel) during 350 ms tetanic stimulation were increased by 20 μM 4-CEP. In six fibers tested, 20 μM 4-CEP significantly increased tetanic $[Ca^{2+}]_i$ by 561 ± 200 nM from a mean control value of 1075 nM, i.e. an increase of 47 ± 8% ($P = 0.037$). At the same time, tetanic force increased by 9 ± 4% ($P = 0.049$). In three of these experiments, we compared the potentiating effect of 4-CEP to that of 5 mM caffeine. As shown in Figure 5C, compared to the effects of 20 μM 4-CEP, 5 mM caffeine had much larger effect on tetanic $[Ca^{2+}]_i$. Caffeine (5 mM) increased tetanic $[Ca^{2+}]_i$ by 110 ± 16% as compared to 53 ± 16% with 20 μM 4-CEP in the same fibers. In these three fibers, caffeine increased force by 17 ± 6%. These

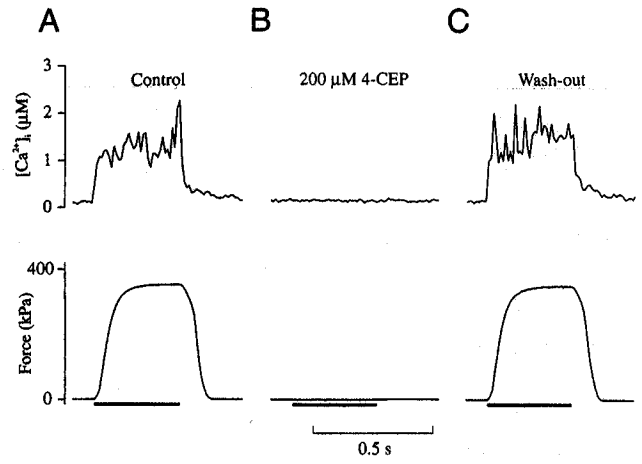

Fig. 6 High concentration of 4-CEP inhibits muscle contraction. A muscle fiber was stimulated at 100 Hz for 350 ms under control conditions (**A**), after a 10 min exposure to 200 μM 4-CEP (**B**) and after a 10 min wash-out (**C**). Upper panel shows $[Ca^{2+}]_i$ changes and lower panel the forces of contractions. The resting $[Ca^{2+}]_i$ was elevated during exposure to 4-CEP but this is not visible with the range used on the y-axis in (B). Tetanic stimulation completely failed to elicit a contraction or elevate $[Ca^{2+}]_i$ in the presence of 200 μM 4-CEP.

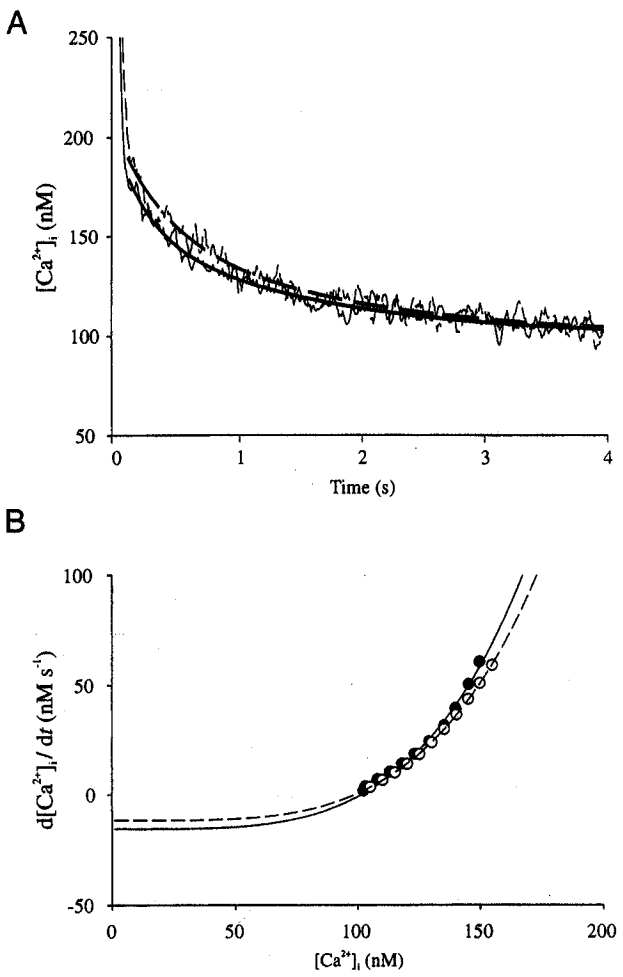

Fig. 7 Effect of 4-CmC on SR Ca^{2+} pumping. (**A**) The noisy records show the late slow phase of decline of $[Ca^{2+}]_i$ after 350 ms 100 Hz tetani in a fiber under control conditions (solid line) and in the presence of 4-CmC (dashed line). Zero time corresponds to the last stimulus. Note the initial (truncated) rapid phase of $[Ca^{2+}]_i$ decline followed by the slow phase. Each record represents the mean of three contractions. The thick dashed lines represent double exponential fits to the slow phase of $[Ca^{2+}]_i$ decline. (**B**) Data points of $d[Ca^{2+}]_i/dt$ against $[Ca^{2+}]_i$ obtained at selected time points from the curve fits in (A): filled circles, control; open circles, 100 μM 4-CmC. Curves shown are fits of Equation 1 to the data points in the control (solid line) and in the presence of 100 μM 4-CmC (dashed line).

effects of caffeine were observed within 30 s of exposure, whereas at least 4 min were required for the full effect of 4-CEP to develop. In another series of experiments, we produced tetanic contraction in three fibers after a 10 min exposure to 100 μM 4-CEP. The increase in tetanic $[Ca^{2+}]_i$ at this concentration was 43 ± 22%, thus very similar to the increase obtained with 20 μM 4-CEP (data not shown).

In contrast to the potentiating effect of 20 and 100 μM 4-CEP on $[Ca^{2+}]_i$ and force of contraction, 200 μM 4-CEP completely inhibited contraction on tetanic stimulation. In experiments illustrated in Figure 6, a muscle fiber was first exposed to 200 μM of 4-CEP for 10 min which increased resting $[Ca^{2+}]_i$. When tetanic stimulation was applied to the fiber at this stage, there was no further increase in $[Ca^{2+}]_i$ and no contraction. Such lack of response to tetanic stimulation was observed in seven out of eight fibers exposed to 200 μM 4-CEP. The fibers recovered from this paralysis upon wash-out (Fig. 6C). After a 20 min wash out, the tetanic $[Ca^{2+}]_i$ and force were 95 ± 6% and 82 ± 6%, respectively, of the controls.

Effect of 4-CmC on SR Ca^{2+} uptake and myofibrillar Ca^{2+} sensitivity

In a previous report, we demonstrated that caffeine affects $[Ca^{2+}]_i$ and muscle contraction by slowing activity of the SR Ca^{2+} pump and by increasing myofibrillar Ca^{2+} sensitivity [9]. To test whether 4-CmC affects the SR Ca^{2+} pump , we analysed the rate of decline of $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ tail) after tetanic contractions. Figure 7A shows original traces of the decline of $[Ca^{2+}]_i$ after 350 ms tetanic stimulations in controls and in the presence of 100 μM 4-CmC (noisy lines each of which is the average of three tetanic contractions). It can be seen that in the presence of the 4-CmC, the magnitude of $[Ca^{2+}]_i$ is larger at the beginning of the tail, but the rate of decline is faster, so that the two traces become indistinguishable towards the

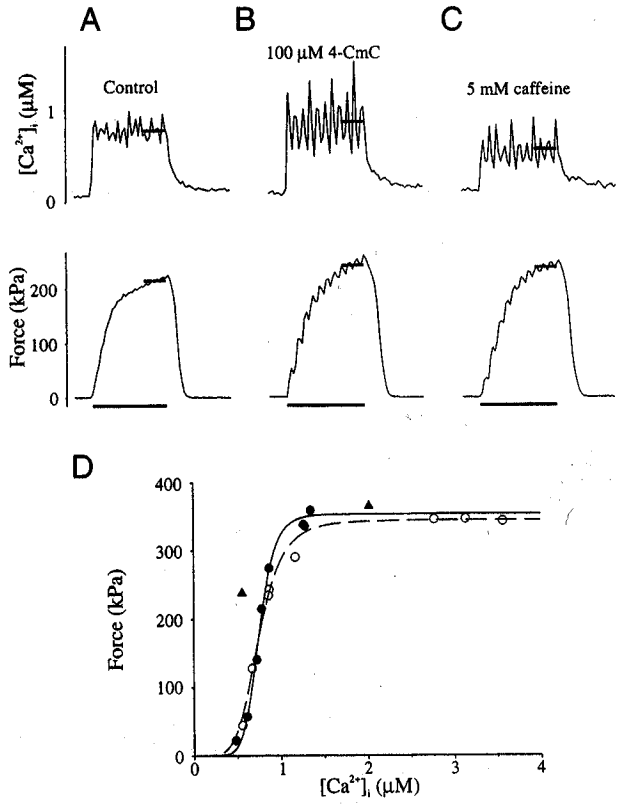

Fig. 8 4-CmC does not affect myofibrillar Ca^{2+} sensitivity. The traces show relationship between $[Ca^{2+}]_i$ (upper panel) and force (lower panel) obtained during tetanic stimulation under different conditions. The muscle fiber was stimulated at 60 Hz under control conditions (**A**), or at 30 Hz in the presence of 100 μM 4-CmC (**B**) or 5 mM caffeine (**C**). Horizontal lines below the lower panel show period of stimulation. $[Ca^{2+}]_i$ and force were averaged over the final 100 ms (shown by horizontal lines in the upper parts of $[Ca^{2+}]_i$ or force traces). Observe that while force is similar in the three situations, $[Ca^{2+}]_i$ is markedly lower with caffeine. (**D**) Plots of the mean force and $[Ca^{2+}]_i$ in the final 100 ms of a series of tetanic stimulation at 20 to 100 Hz under control conditions (filled circles), in the presence of 100 μM 4-CmC (open circles), or in the presence of 5 mM caffeine (filled triangles). The measurements were made from the same fiber as in (A–C). The curves are least squares fits of Equation 2 to the experimental data points in the controls (solid line) and in the presence of 4-CmC (dashed line). A 100 Hz tetanus in the presence of 5 mM caffeine (the filled triangle with higher force) was used to obtain the maximum force at saturating $[Ca^{2+}]_i$ (P_{max}) under control conditions.

end. The thick lines represent two exponential curve fits to the records. These curve fits are used in Figure 7B, where the rate of $[Ca^{2+}]_i$ decline ($d[Ca^{2+}]_i/dt$) is plotted against $[Ca^{2+}]_i$. The data points obtained this way were similar in the presence or absence of 4-CmC. To quantify possible changes, Equation 1 was fitted to the data points with N set to 4 (see Materials and methods). Mean values for A (SR Ca^{2+} pumping) and L (SR Ca^{2+} leak) showed no significant difference. A in controls was $1.83 \pm 0.34 \times 10^{-7}$ $nM^{-3}.s^{-1}$ and in the presence of 4-CmC $1.58 \pm 0.18 \times 10^{-7}$ $nM^{-3}.s^{-1}$ ($P = 0.24$, $n = 3$). L in controls was 12.4 ± 3.8 $nM.s^{-1}$ and with 4-CmC 12.7 ± 1.4 nM s^{-1} ($P = 0.93$, $n = 3$).These analyses suggested no major effect of 4-CmC on SR Ca^{2+} pumping or Ca^{2+} leak.

We tested whether 4-CmC alters myofibrillar Ca^{2+} sensitivity by analysing the force–$[Ca^{2+}]_i$ relationship in the absence or presence of the substance. We varied $[Ca^{2+}]_i$ and force by giving tetanic stimulation at 20–100 Hz. The tension at saturating $[Ca^{2+}]_i$ was obtained by producing 100 Hz tetani in the presence of 5 mM caffeine. A typical experiment is shown in Figure 8, where the upper panel shows $[Ca^{2+}]_i$ traces obtained in the presence or absence of 100 μM 4-CmC and the middle panel shows corresponding forces of contraction (Fig. 8A,B). The traces were chosen such that the forces of contractions were similar. It can be seen that a 60 Hz tetanic contraction in controls and a 30 Hz tetanus in the presence of 4-CmC gave similar $[Ca^{2+}]_i$ (Fig. 8A,B, upper panel) and also similar forces of contractions (Fig. 8A,B, lower panel) suggesting lack of any major effect of 4-CmC on myofibrillar Ca^{2+} sensitivity. For comparison, we have included a 30 Hz tetanus produced in the presence of 5 mM caffeine (Fig. 8C). In this trace, while force is similar to that in the other two tetani, $[Ca^{2+}]_i$ is substantially lower suggesting that myofibrillar sensitivity to Ca^{2+} is increased on application of caffeine. A plot of force versus $[Ca^{2+}]_i$ obtained in the absence or presence of 100 μM 4-CmC showed no difference, whereas the data point from the 30 Hz tetanus in the presence of caffeine lies clearly to the left. Fitting Equation 2 (*see* Materials and methods) to the data points gave similar curves. The values for Ca_{50} (1057 ± 194 versus 1161 ± 57 nM), P_{max} (100% versus 94 ±4%) and N (5.9 ± 1.2 versus 5.1 ± 0.7) in the control groups compared to the 4-CmC group were not significantly different ($n = 4$).

4-CEP inhibited action potential

As described above, 200 μM 4-CEP inhibited muscle contraction on tetanic stimulation. This could be due to inability of RY receptors to respond to membrane depolarization. Alternatively, 4-CEP might affect the resting membrane potential or generation of action potentials. To distinguish between these possibilities, we recorded the membrane potential in fibers of intact soleus muscle. Under control conditions, the resting membrane potential was −67.6 ± 1.9 mV ($n = 10$) and all fibers gave a clear action potential in response to a current pulse. When the muscles were treated for 10 min with 200 μM 4-CEP the membrane was significantly depolarized to −51 ± 2 mM ($n = 20$) and action potential could be produced in only five of the 20 fibers examined (data not shown).

DISCUSSION

Previous studies using fragmented SR demonstrated that the phenolic compound 4-CmC releases Ca^{2+} specifically by activating the RY receptor and that the substance is more potent than caffeine. Here we use a more physiological system and confirm that 4-CmC, and its lipophilic analogue 4-CEP, increase resting $[Ca^{2+}]_i$ in intact skeletal muscle fiber in a dose-dependent manner. The pattern of increase in $[Ca^{2+}]_i$ by 4-CmC resembled closely that by caffeine in being relatively rapid in onset, effective on both the resting and the activated state of RY receptor and completely reversible. These phenolic compounds were clearly more potent than caffeine: the increase in resting $[Ca^{2+}]_i$ by 500 μM 4-CmC being roughly comparable to that by 5 mM caffeine. In this regard, 4-CEP, the more lipophilic compound, was more potent. High concentrations of the compounds caused muscle contracture in the absence of stimulation, an effect that resembles that of high concentrations of caffeine. However, 4-CmC and 4-CEP induced muscle contracture at concentrations ~1/50th to 1/25th that of caffeine [25]. Furthermore, when used at low concentrations, these phenolic compounds did not appear to have any major toxic effect on muscle fibers during acute applications for as long as 10–20 min.

The increase in resting $[Ca^{2+}]_i$ caused by 4-CmC and 4-CEP was due to release of the ion from the SR through RY receptors. This is evidenced by the fact that $[Ca^{2+}]_i$ increase by 4-CmC was inhibited by dantrolene an inhibitor of RY receptors. Dantrolene slightly delayed the $[Ca^{2+}]_i$ increase by 4-CEP but, in this case, the initial inhibition of Ca^{2+} release was soon overcome during continued application of 4-CEP. Our interpretation of this finding is that, in the presence of dantrolene, there is an increased accumulation of Ca^{2+} in the SR and the increased SR Ca^{2+} content eventually overcomes the inhibitory effect of dantrolene. Such a phenomenon has been described for other RY receptor inhibitors [26]. Further evidence that $[Ca^{2+}]_i$ increase by these compounds were due to Ca^{2+} release through RY receptor was obtained from experiments where a subthreshold concentration of 4-CEP enhanced $[Ca^{2+}]_i$ increase by 5 mM caffeine, an observation consistent with previous reports [27]. Such an effect does not appear to be due to cAMP-elevating action of caffeine since forskolin did not have similar effect. The potentiating effect of 4-CEP on caffeine-induced Ca^{2+} release may be due to the fact that the xanthine drug and 4-CEP (or 4-CmC) act on different sites on the RY receptor. The binding site(s) of caffeine on the RY receptors is not known but is likely to be on the cytoplasmic side of the receptor [11,12]. On the other hand, 4-CmC appears to bind on the luminal side of RY receptor [14]. Other mechanisms by which caffeine could potentiate the effect of 4-CEP include inhibition of the activity of SR Ca^{2+} pumps and sensitization of RY receptor to Ca^{2+}-induced Ca^{2+} release [9].

4-CmC and 4-CEP potentiated the $[Ca^{2+}]_i$ increase and force of contraction induced by tetanic stimulation. However, such potentiation was observed within a relatively narrow concentration range and high concentrations of these agents tended to be inhibitory. The underlying mechanism of potentiation of tetanic $[Ca^{2+}]_i$ by these substances could be sensitization of the RY receptor-mediated release process or an inhibition of SR Ca^{2+} pumps. To identify if the latter mechanism contributed to potentiation of tetanic $[Ca^{2+}]_i$ by 4-CmC, we analysed the slow phase of $[Ca^{2+}]_i$ decline following tetani in the presence or absence of 4-CmC. These analyses demonstrated that 4-CmC does not have an inhibitory effect on SR Ca^{2+} pump, a conclusion consistent with previous observations made in cell-free systems [13]. This is in contrast to the effect of caffeine which can reduce the activity of SR Ca^{2+} pumps by as much as 60% [9].

The potentiating effect of 4-CmC on force of contraction during tetanic stimulation could be due to increased $[Ca^{2+}]_i$ in the presence of 4-CmC and/or an increase in myofibrillar Ca^{2+} sensitivity. By analysing the force-$[Ca^{2+}]_i$ relationship obtained in the presence or absence of 4-CmC, we demonstrate that the substance does not increase myofibrillar Ca^{2+} sensitivity. Thus, 4-CmC-mediated increased force of contraction during tetanic stimulation is due to increased $[Ca^{2+}]_i$. This is in contrast to the effect of caffeine which significantly increases both tetanic $[Ca^{2+}]_i$ and myofibrillar Ca^{2+}-sensitivity [9].

High concentrations of 4-CEP (e.g. 200 μM) inhibited the $[Ca^{2+}]_i$ increase and contraction on tetanic stimulation. This appears to be due to an inhibitory effect of the compound on the excitability of the sarcolemma since, at this concentration, the compound inhibited generation of action potentials. The underlying mechanism of this inhibition is not clear, but may be related to the observed depolarization of resting fibers. It is possible that this lipophilic substance may inhibit K^+ permeability and, thereby, elevate basal membrane potential leading to difficulty in the generation of action potentials. A similar inhibition might also explain the reduced tetanic $[Ca^{2+}]_i$ and force production observed in some experiments with 200 μM 4-CmC. A depressive effect of 4-CEP on contraction has also been described in smooth muscle cells [28].

Thus, we demonstrate that 4-CmC and 4-CEP activate RY receptor in intact skeletal muscle fiber and, thereby, increase $[Ca^{2+}]_i$ and potentiate muscle contraction on electrical stimulation. Although 4-CEP was more potent

than 4-CmC in increasing resting $[Ca^{2+}]_i$, it appears less suitable for usage in physiological studies. This is because the onset of 4-CEP action is slower and it readily causes inhibition of contraction.

Our study shows that 4-CmC has several advantages over the pharmacological tools commonly used for studying RY receptor namely caffeine and ryanodine. This phenolic compound is more potent than caffeine, does not affect SR Ca^{2+} pumping or leak, and does not alter myofibrillar Ca^{2+} sensitivity. In comparison to the effect of ryanodine, 4-CmC is only activatory and the effect sets in more rapidly. The effect is also fully reversible provided the dose is not high enough to induce contracture. This substance and its derivatives, thus have potential to become useful tools for mechanistic or functional studies of RY receptors in skeletal muscle and probably also in other cell types.

ACKNOWLEDGEMENTS

Financial support was obtained from the Swedish Medical Research Council (Project no 10842), The Swedish National Center for Sports Research, The Swedish Society of Medicine, National Board of Health and Welfare and Funds of the Karolinska Institute. FHA was supported by Baylor College of Medicine-Karolinska Institute Research Exchange Program. HW and MSI have positions at the Swedish Medical Research Council.

REFERENCES

1. Franzini-Armstrong C., Protasi F. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol Rev* 1997; **77**: 699–729.
2. Sorrentino V. The ryanodine receptor family of intracellular calcium release channels. *Adv Pharmacol* 1995; **33**: 67–90.
3. Brillantes A.B., Ondrias K., Scott A. et al. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* 1994; **77**: 513–523.
4. Cheng H., Lederer M.R., Xiao R.P. et al. Excitation-contraction coupling in heart: new insights from Ca^{2+} sparks. *Cell Calcium* 1996; **20**: 129–140.
5. Islam M.S. PhD Thesis. Karolinska Institute, Stockholm, Sweden: 1997.
6. Fujii J., Otsu K., Zorzato F. et al. Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science* 1991; **253**: 448–451.
7. Palade P. Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum. II. Releases involving a Ca^{2+}-induced Ca^{2+} release channel. *J Biol Chem* 1987; **262**: 6142–6148.
8. Lai F.A., Misra M., Xu L., Smith H.A., Meissner G. The ryanodine receptor-Ca^{2+} release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. *J Biol Chem* 1989; **264**: 16776–16785.
9. Allen D.G., Westerblad H. The effects of caffeine on intracellular calcium, force and the rate of relaxation of mouse skeletal muscle. *J Physiol* 1995; **487**: 331–442.
10. Islam M.S., Larsson O., Nilsson T., Berggren P.O. Effects of caffeine on cytoplasmic free Ca^{2+} concentration in pancreatic β-cells are mediated by interaction with ATP-sensitive K^+ channels and L-type voltage-gated Ca^{2+} channels but not the ryanodine receptor. *Biochem J* 1995; **306**: 679–686.
11. Bhat M.B., Zhao J., Zang W. et al. Caffeine-induced release of intracellular Ca^{2+} from Chinese hamster ovary cells expressing skeletal muscle ryanodine receptor. Effects on full-length and carboxyl-terminal portion of Ca^{2+} release channels. *J Gen Physiol* 1997; **110**: 749–762.
12. Yoshikawa K., Furukawa K., Yamamoto M., Momose K., Ohizumi Y. [^{3}H]-9-Methyl-7-bromoeudistomin D, a caffeine-like powerful Ca^{2+} releaser, binds to caffeine-binding sites distinct from the ryanodine receptors in brain microsomes. *FEBS Lett* 1995; **373**: 250–254.
13. Zorzato F., Scutari E., Tegazzin V., Clementi E., Treves S. Chlorocresol: an activator of ryanodine receptor-mediated Ca^{2+} release. *Mol Pharmacol* 1993; **44**: 1192–1201.
14. Herrmann-Frank A., Richter M., Sarkozi S., Mohr U., Lehmann-Horn F. 4-Chloro-*m*-cresol, a potent and specific activator of the skeletal muscle ryanodine receptor. *Biochim Biophys Acta* 1996; **1289**: 31–40.
15. Larini F., Menegazzi P., Baricordi O., Zorzato F., Treves S. A ryanodine receptor-like Ca^{2+} channel is expressed in nonexcitable cells. *Mol Pharmacol* 1995; **47**: 21–28.
16. Ording H., Glahn K., Gardi T., Fagerlund T., Bendixen D. 4-Chloro-*m*-cresol test – a possible supplementary test for diagnosis of malignant hyperthermia susceptibility. *Acta Anaesthesiol Scand* 1997; **41**: 967–972.
17. Islam M.S., Leibiger I., Leibiger B. et al. *In situ* activation of type 2 ryanodine receptor in pancreatic β-cells requires cAMP-dependent phosphorylation. *Proc Natl Acad Sci USA* 1998; **95**: 6145–6150.
18. Herrmann-Frank A., Richter M., Lehmann-Horn F. 4-Chloro-*m*-cresol: a specific tool to distinguish between malignant hyperthermia-susceptible and normal muscle. *Biochem Pharmacol* 1996; **52**: 149–155.
19. Lännergren J., Westerblad H. The temperature dependence of isometric contractions of single, intact fibres dissected from a mouse foot muscle. *J Physiol* 1987; **390**: 285–293.
20. Andrade F.H., Reid M.B., Allen D.G., Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from mouse. *J Physiol* 1998; **509**: 565–575.
21. Westerblad H., Allen D.G. The influence of intracellular pH on contraction, relaxation and $[Ca^{2+}]_i$ in intact single fibres from mouse muscle. *J Physiol* 1993; **466**: 611–628.
22. Carins S.P., Westerblad H., Allen D.G. Changes of tension and $[Ca^{2+}]_i$ during β-adrenoceptor activation of single, intact fibres from mouse skeletal muscle. *Pflügers Arch* 1993; **425**: 150–155.
23. Mayrleitner M., Chandler R., Schindler H., Fleischer S. Phosphorylation with protein kinases modulates calcium loading of terminal cisternae of sarcoplasmic reticulum from skeletal muscle. *Cell Calcium* 1995; **18**: 197–206.
24. Nelson T.E., Lin M., Zapata-Sudo G., Sudo R.T. Dantrolene sodium can increase or attenuate activity of skeletal muscle ryanodine receptor calcium release channel. Clinical implications. *Anesthesiology* 1996; **84**: 1368–1379.

25. Lännergren J., Westerblad H. Force decline due to fatigue and intracellular acidification in isolated fibres from mouse skeletal muscle. *J Physiol* 1991; **434**: 307–322.
26. Overend C.L., O'Neill S.C., Eisner D.A. The effect of tetracaine on stimulated contractions, sarcoplasmic reticulum Ca^{2+} content and membrane current in isolated rat ventricular myocytes. *J Physiol* 1998; **507**: 759–769.
27. Tegazzin V., Scutari E., Treves S., Zorzato F. Chlorocresol, an additive to commercial succinylcholine, induces contracture of human malignant hyperthermia-susceptible muscles via activation of the ryanodine receptor Ca^{2+} channel. *Anesthesiology* 1996; **84**: 1380–1385.
28. Low A.M., Sormaz L., Kwan C.Y., Daniel E.E. Actions of 4-chloro-3-ethyl phenol on internal Ca^{2+} stores in vascular smooth muscle and endothelial cells. *Br J Pharmacol* 1997; **122**: 504–510.