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4-chloro-*m*-cresol, a potent and specific activator of the skeletal muscle ryanodine receptor

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Abstract

The aim of the present study was to determine the effects of 4-chloro-*m*-cresol (4-CmC), a preservative often added to drugs intravenously administered, on the skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+} release channel/ryanodine receptor. In heavy SR vesicles obtained from rabbit back muscles, 4-CmC stimulated Ca^{2+}-activated [^{3}H]ryanodine binding with an EC_{50} of about 100 μM. In the same concentration range, 4-CmC directly activated the isolated Ca^{2+} release channel reconstituted into planar lipid bilayers. The sensitivity to 4-CmC was found to be higher when applied to the luminal side of the channel suggesting binding site(s) different from those of nucleotides and caffeine. In skeletal muscle fibre bundles obtained from biopsies of patients susceptible to malignant hyperthermia, a skeletal muscle disease caused by point mutations in the ryanodine receptor, 4-CmC evoked caffeine-like contractures. Contrary to caffeine which induces contractures in millimolar concentrations, the threshold concentration for 4-CmC was 25 μM compared to 75 μM for non-mutated control fibres. Since these data strongly indicate that 4-CmC specifically activates SR Ca^{2+} release also in intact cell systems, this substance might become a powerful tool to investigate ryanodine receptor-mediated Ca^{2+} release in muscle and non-muscle tissue.

Keywords: Ryanodine receptor; Sarcoplasmic reticulum; Calcium channel; Caffeine; Skeletal muscle; Malignant hyperthermia

1. Introduction

In skeletal muscle, contraction is initiated by an action potential at the surface of the muscle fibre which propagates along the transverse tubular system into the cell interior. Via a yet not fully understood process, depolarization of the transverse tubules is sensed to the terminal cisternae of the sarcoplasmic reticulum (SR), leading to a rapid release of Ca^{2+} into the cytoplasmic space (cf. [6,25,31,34]). The release of Ca^{2+} ions from SR is mediated by the high molecular weight ligand-gated Ca^{2+} release channel which serves as the receptor for the plant alkaloid ryanodine (cf. [5,10,16,21,23,36]). Experiments on [^{3}H]ryanodine binding to 'heavy' SR vesicles revealed the presence of both high affinity binding site with an apparent K_d of 5–10 nM and low affinity binding sites in a ratio of 1:3 (cf. [5,10,16]). Meanwhile, a wide spectrum of endogenous and exogenous ligands is known to modify the release channel activity. Among endogenous ligands, Ca^{2+} (μM) and ATP (mM) have been shown to activate the Ca^{2+} release channel while Ca^{2+} in higher concentrations (> 100 μM), Mg^{2+} (mM) and calmodulin (μM) exert inhibitory effects (cf. [5,23,29]). In a similar way, these modulators modify [^{3}H]ryanodine binding [1,4,18,22,24, 30,40]. One of the most widely used exogenous activators of the ryanodine receptor is caffeine. Direct activation of the isolated release channel was first shown by Rousseau et al. [32]. Single channel measurements revealed that caffeine activates SR Ca^{2+} release by increasing the number and duration of open events without changing the conductance of the channel [32]. Caffeine binding sites of the ryanodine receptor, however, have not yet been identified. In spite of the direct activation of SR Ca^{2+} release by caffeine in isolated membrane fractions, caffeine exerts various side effects in muscle cells. In concentrations higher than 5 mM, a common concentration for activating

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SR Ca^{2+} release, caffeine activates steady state force in skinned skeletal muscle fibres by increasing the Ca^{2+} sensitivity of the myofilaments [41]. A possible role of caffeine acting as a phosphodiesterase inhibitor cannot be ruled out although a direct activation of SR Ca^{2+} release in skeletal muscle by cAMP has not been demonstrated. Clinically, the Ca^{2+}-releasing action of caffeine is used to determine the susceptibility of patients to the pharmacogenetic muscle disease, malignant hyperthermia (MH). Physiologically, MH-susceptible muscle is characterized by its higher sensitivity towards Ca^{2+}, caffeine and halothane [[28,38], for review see [20]). Although the enhanced caffeine sensitivity has been confirmed by a numerous number of research groups, this observation was not observed on the single channel level when the isolated mutated ryanodine receptor was reconstituted in artificial lipid bilayers [35].

Recently, chlorocresols, preservatives often added for instance to commercial preparations of succinylcholine, have been shown to activate ryanodine receptor-mediated SR Ca^{2+} release in skeletal muscle and cerebellum [43]. Contrary to caffeine which releases Ca^{2+} from SR in millimolar concentrations, 4-chloro-*m*-cresol (4-CmC), the most potent compound, induced Ca^{2+} release in about 10 times lower concentrations. In the present study, we have investigated the interaction of the skeletal muscle ryanodine receptor with 4-chloro-*m*-cresol on the molecular level using three different approaches: (i) [^{3}H]ryanodine binding with heavy SR (HSR) vesicles from rabbit back muscle, (ii) $^{45}Ca^{2+}$ flux measurements with passively loaded HSR vesicles, and iii) reconstitution of the isolated CHAPS-solubilized Ca^{2+} release channel in planar lipid bilayers. In addition, we report the first observations that 4-chloro-*m*-cresol is capable to elicit contractures in isolated skeletal muscle fibre bundles, with a higher sensitivity in preparation obtained from muscle biopsies of patients susceptible to malignant hyperthermia.

Part of this work has been submitted in abstract form [13,14].

2. Material and methods

2.1. Materials

4-Chloro-*m*-cresol was purchased from Fluka (Neu-Ulm, FRG), ryanodine from Calbiochem (Bad Soden, FRG), protease inhibitors were from Boehringer (Mannheim, FRG), lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA), [9,21-^{3}H(N)]ryanodine and $^{45}CaCl_2$ were from Du Pont NEN (Bad Homburg, FRG). All other chemicals were of analytical grade or higher purity. Filter membranes for [^{3}H]ryanodine binding and $^{45}Ca^{2+}$ flux measurements were purchased from Schleicher & Schüll (S&S, Dassel, FRG).

2.2. Isolation of 'heavy' SR vesicles

'Heavy' SR (HSR) vesicles were isolated from rabbit back muscle, following the method of [17]. HSR fractions were recovered from the 36–38% region of continuous sucrose gradients, pelleted at $124\,000 \times g$ for 60 min and resuspended in 0.3 M sucrose, 10 mM K-Pipes (piperazine-1,4-bis-(2-ethanesulfonic acid)), pH 7.0. Resuspended membranes were either rapidly frozen and stored at −80°C or immediately used for ryanodine receptor purification. To prevent proteolysis, the following protease inhibitors were used in various stages of the isolation: 200 μM Pefabloc (4-(2-aminoethyl)-benzolsulfonylfluoride), 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidine. Protein concentration of HSR vesicles was determined according to the method of [19], using bovine serum albumin as a standard.

2.3. Purification of the Ca^{2+} release channel / ryanodine receptor complex

HSR vesicles (3.5 mg/ml) were solubilized for 2 h at 4°C with 1.6% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in a solution containing 1 M NaCl, 100 μM EGTA, 150 μM $CaCl_2$, 5 mM AMP, 0.5% phosphatidylcholine, 20 mM Na-Pipes, pH 7.2, protease inhibitors as stated above and additionally 1 μM calpain inhibitor I (*N*-acetyl-leu-leu-norleucinal) and 1 μM calpain inhibitor II (*N*-acetyl-leu-leu-methioninal). The extent of ryanodine receptor solubilization as well as the subsequent migration distance of the solubilized receptor in the sucrose gradients was monitored by labelling one part of the solubilized sample with [^{3}H]ryanodine. Following centrifugation at $59\,000 \times g$, the supernatant containing the solubilized receptor was layered on the top of 10–28% sucrose gradients containing 1–0% CHAPS, 0.7 M NaCl, 3.3 mM AMP, 0.5% phosphatidylcholine, 70 μM EGTA, 100 μM $CaCl_2$, 1 mM DTE, 13 mM Na-Pipes, pH 7.2, and all protease inhibitors as used before. Unlabelled and [^{3}H]ryanodine-labelled solubilized SR membranes were centrifuged through parallel sucrose gradients for 16 h at $136\,000 \times g$ (4°C). Gradient fractions corresponding to the peak of the [^{3}H]ryanodine bound receptor were collected, rapidly frozen in liquid nitrogen, and stored at −80°C. Protein concentration of the solubilized receptor was determined according to [15].

2.4. [^{3}H]Ryanodine binding

HSR vesicles (at a protein concentration of 500 μg/ml) were incubated with indicated concentrations of [^{3}H]ryanodine in a medium containing 1 M NaCl, 20 mM Na-Pipes, pH 7.1, and varying concentrations of Ca^{2+} and Mg^{2+} for 3 h at 37°C. The unbound ryanodine was separated from the protein-bound ryanodine by filtration of protein aliquots (12.5 μg) through S&S GF51 filters

followed by washing three times with ice-cold buffer solution as described above. Radioactivity remaining with the filters was measured by liquid scintillation counting. Non-specific binding was determined by a 1000-fold excess of unlabelled ryanodine.

2.5. $^{45}Ca^{2+}$ flux measurements

HSR vesicles (~ 6 mg/ml) were passively loaded for 2 h at room temperature in a medium containing 100 mM NaCl, 20 mM Na-Pipes, pH 7.1, 1 mM $CaCl_2$, and trace amounts of $^{45}CaCl_2$. $^{45}Ca^{2+}$ efflux was initiated by 25-fold dilution of vesicles in a buffer solution containing 100 mM NaCl, 1 mM EGTA, and 1 mM $MgCl_2$, 20 mM Na-Pipes, pH 7.1. After 10 s, vesicles were further diluted in a medium of identical composition containing additionally various amounts of $CaCl_2$ or 4-chloro-*m*-cresol as releasing agents (final dilution 75-fold). Untrapped as well as released $^{45}Ca^{2+}$ was removed by filtration through S&S BA85 filters at indicated time points. $^{45}Ca^{2+}$ efflux was stopped by rinsing filters three times with 1 ml 100 mM NaCl solution, pH 7.1, containing 5 mM $MgCl_2$ and 10 μM ruthenium red as release inhibitors. The remaining $^{45}Ca^{2+}$ inside the vesicles was determined by liquid scintillation counting.

2.6. Planar lipid bilayer measurements

The CHAPS-solubilized ryanodine receptor purified in the absence of [^{3}H]ryanodine was incorporated into planar lipid bilayers of the Mueller-Rudin type. Bilayers were formed across a 250 μm aperture from a mixture of phosphatidylethanolamine, phosphatidylserine and L-α-phosphatidylcholine in the ratio 5:4:1 dissolved in decane (20 mg/ml). Reconstitution was initiated in symmetric buffer solution (250 mM KCl, 100 μM EGTA, 150 μM $CaCl_2$, 20 mM Pipes, pH 7.2), using K^+ as the charge carrier. Small aliquots of the solubilized receptor (< 0.5 μg) were added to one side of the bilayer chamber defined as the cytoplasmic (*cis*) side. Successful incorporation was detected as steplike increase in current. Electrical signals were filtered at 1 kHz through an 8-pole low-pass Bessel filter and digitised at 3 kHz, using Axon Instruments hard- and software. Applied voltages are defined with reference to the *trans* side of the bilayer chamber corresponding to the luminal side of the channel. Open probability values

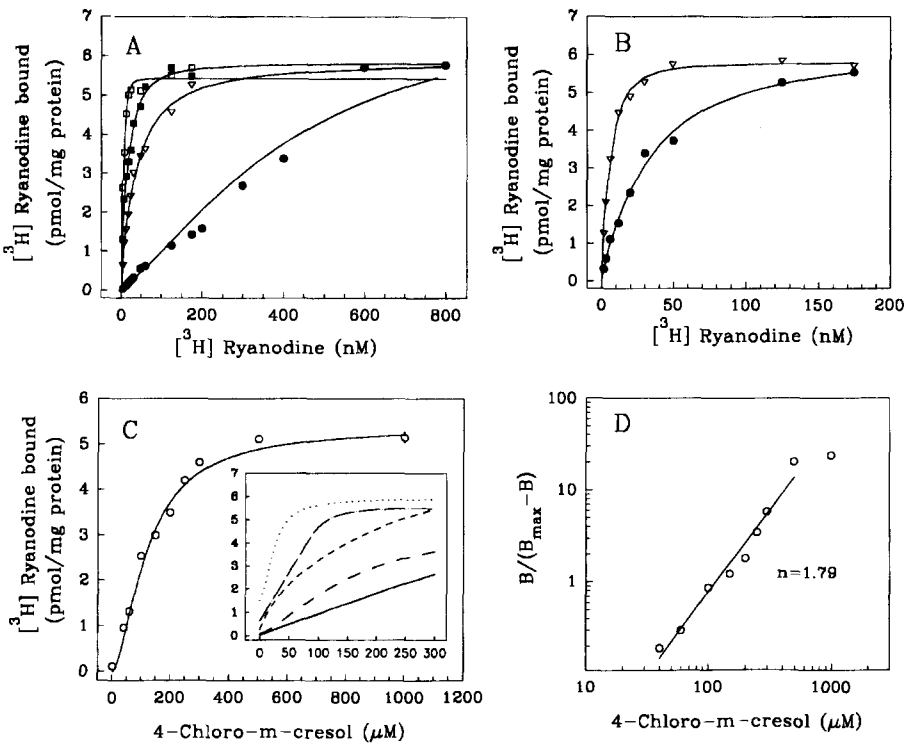

Fig. 1. Potentiation of [^{3}H]ryanodine binding by 4-CmC. A. Specific binding of [^{3}H]ryanodine binding to 'heavy' SR vesicles in the presence of 1 μM Ca^{2+} and various concentrations of 4-CmC: (·) 1 μM Ca^{2+}, (▽) 1 μM Ca^{2+} + 75 μM 4-CmC, (■) 1 μM Ca^{2+} + 150 μM 4-CmC, (□) 1 μM Ca^{2+} + 300 μM 4-CmC. [^{3}H]ryanodine binding was carried out as described in Material and Methods. B: Dependence of [^{3}H]ryanodine binding on 4-CmC at maximally activating Ca^{2+} concentrations. (●) 50 μM μM Ca^{2+}, (▽) 50 μM Ca^{2+} 75 μM 4-CmC. C: Dose-response curve of 4-CmC-stimulated [^{3}H]ryanodine binding. Binding was performed in the presence of 12 nM [^{3}H]ryanodine and 1 μM Ca^{2+}. Inset: Dose-response curves at various [^{3}H]ryanodine concentrations: (——) 3.75 nM, (— —) 7.5 nM, (— — —) 24 nM, (– · –) 60 nM, (· · ·) 175 nM. D: Hill plot of 4-CmC-stimulated [^{3}H]ryanodine binding. The apparant Hill coefficient was determined according to the equation $\log (B/(B_{max} - B)) = \log K + n \cdot \log$ [4-CmC], using data points of the corresponding dose-response curve at 12 nM [^{3}H]ryanodine (panel C).

(P_0) were calculated from representative data segments of 20–60 s duration. The total recording time in each experiment was > 5 min for any experimental condition tested. The experimental temperature was 20°C.

2.7. Contracture measurements

Muscle fibre bundles were dissected from human muscle specimens of the vastus lateralis muscle obtained from 10 individuals who underwent muscle biopsy for the test of susceptibility to malignant hyperthermia. All procedures were in accordance with the Helsinki convention and were approved by the Ethics Committee of the University of Ulm. Dissected muscle bundles of about 30 mm length and 2 mm diameter in average were mounted between the arm of an electro-mechanical transducer (Grass FT03) and a small hook in a temperature-controlled chamber. The in vitro-contracture test was performed and analysed according to the European test protocol (EMHG, 1984). In addition, 4-CmC was added to bundles not used for the diagnostic procedure. Muscle bundles were stimulated with supra maximal pulses of 1 ms duration at a frequency of 0.1 Hz. The bathing solution contained 118 mM NaCl, 3.4 mM KCl, 2.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and indicated concentrations of 4-CmC at pH 7.4 (Krebs-Ringer solution). Solutions were continuously perfused with 95% O_2 and 5% CO_2 and kept at 37°C.

2.8. Miscellaneous methods

Free concentrations of Ca^{2+} and Mg^{2+} were calculated by a computer program using binding constants published by [9]. Hill plots and dose-response curves were fitted using either linear regression analysis or non-linear curve-fitting routines based on the Marquardt-Levenberg algorithm.

3. Results

3.1. Modulation of Ca^{2+}-activated [^{3}H]ryanodine binding by 4-chloro-m-cresol

Since 4-chloro-*m*-cresol (4-CmC) has been shown to activate ryanodine receptor-mediated SR Ca^{2+} release [43],

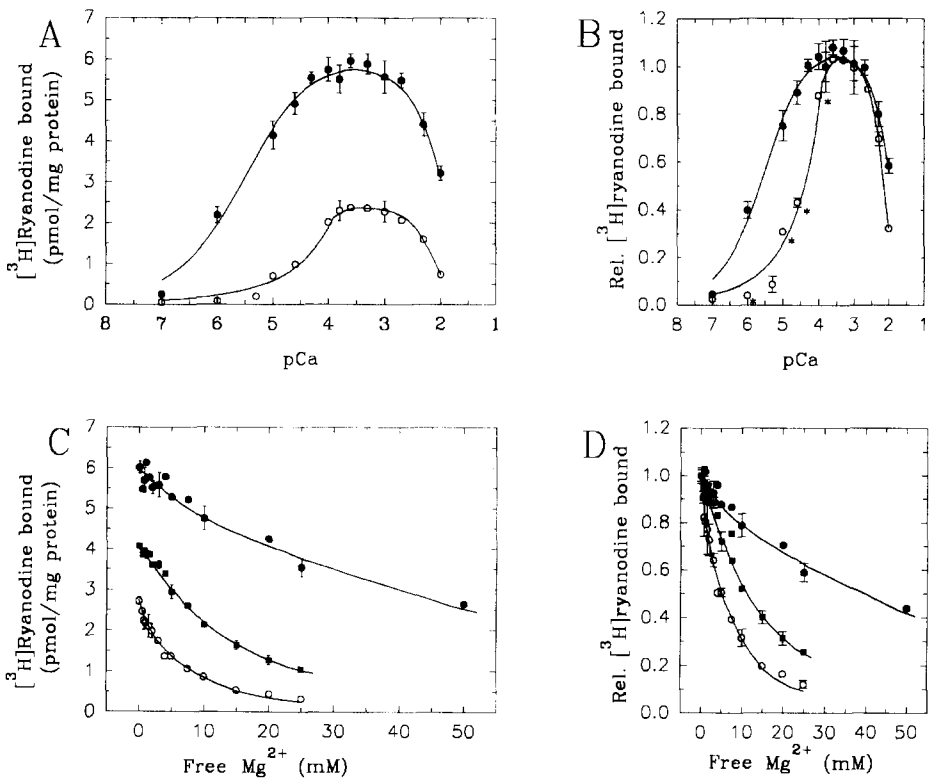

Fig. 2. Modulation of 4-CmC-stimulated [^{3}H]ryanodine binding by Ca^{2+} and Mg^{2+}. Top: Dependency of 4-CmC-stimulated [^{3}H]ryanodine binding on Ca^{2+}. A: Dose-response curves in the absence (○) and presence of 75 μM 4-CmC (•). Error bars indicate S.E.: $n = 6$ (○) and $n = 8$ (•).B: Data from panel A were normalized to maximal [^{3}H]ryanodine binding achieved at 158 μM Ca^{2+}. Data points are derived from the means of panel A. ★ indicates significant differences between corresponding data points (○, •) at $P < 0.05$ (Student's *t*-test). Bottom: Inhibition of [^{3}H]ryanodine binding by Mg^{2+}. C: [^{3}H]ryanodine binding was investigated at the indicated Concentrations of free Mg^{2+} in the presence of 158 μM Ca^{2+} + 250 μM 4-CmC (•), 1 μM Ca^{2+} + 250 μM 4-CmC (•), and 158 μM Ca^{2+} (○). Error bars indicate S.E.: $n = 8$ (•), $n = 4$ (■,○). D: Data from panel C were normalized to maximally bound [^{3}H] ryanodine in the absence of Mg^{2+}. Data points are derived from the means of panel C. The [^{3}H]ryanodine concentration was 6 nM under all conditions tested.

we examined its effect on high-affinity [^{3}H]ryanodine binding. Fig. 1(A,B) shows the kinetic effect of 4-CmC on [^{3}H]ryanodine binding. Both in the presence of subthreshold and nearly maximally activating Ca^{2+} concentrations, 4-CmC increased the affinity of binding to [^{3}H]ryanodine but did not affect maximal binding. At 50 μM Ca^{2+}, the K_d decreased from 11.4 nM (control, $n = 8$) to 4.7 nM ($n = 6$) in the presence of 75 μM 4-CmC. 4-CmC potentiated [^{3}H]ryanodine binding to 'heavy' SR vesicles (HSR) in a concentration-dependent manner (Fig. 1C). At 12 nM [^{3}H]ryanodine, the K_d of high-affinity binding in the absence of 4-CmC (Fig. 1B), the half-maximally stimulating concentration was calculated to be 112 μM. Hill plot analysis yielded a Hill coefficient of 1.8, suggesting the presence of more than one cooperating binding site for 4-CmC (Fig. 1D).

Ca^{2+} and Mg^{2+} are antagonistic regulators of the ryanodine receptor (cf. [5,23,29]). Fig. 2 shows that 4-CmC-stimulated [^{3}H]ryanodine binding is further modified by these ions. 4-CmC potentiated [^{3}H]ryanodine binding when the free Ca^{2+} concentration was higher than 0.1 μM (Fig. 2A). Normalising the data to maximally bound [^{3}H]ryanodine indicates that 4-CmC is most effective at activating Ca^{2+} concentrations between 10 and 50 μM Ca^{2+} (Fig. 2B). Mg^{2+} inhibition of 4-CmC-stimulated [^{3}H]ryanodine binding was investigated in the presence of 1 μM Ca^{2+}, inducing negligible binding of [^{3}H]ryanodine, and in the optimum of Ca^{2+}-activated [^{3}H]ryanodine binding (Fig. 2C,D). In the presence of maximally stimulating Ca^{2+} and 4-CmC concentrations, more than 20 mM Mg^{2+} was necessary to inhibit [^{3}H]ryanodine binding to 50%. Reducing Ca^{2+} to 1 μM, half-maximal inhibition was achieved in the presence of about 10 mM Mg^{2+}. Keeping the high Ca^{2+} concentration but leaving out 4-CmC, reduced the IC_{50} value to about 5 mM (Fig. 2C). Normalising the data showed that the steepness of the dose response curve was stronger increased when 4-CmC was withdrawn from the binding medium compared to Ca^{2+}, indicating that 4-CmC decreases the sensitivity of the ryanodine receptor for Mg^{2+} more strongly than Ca^{2+}.

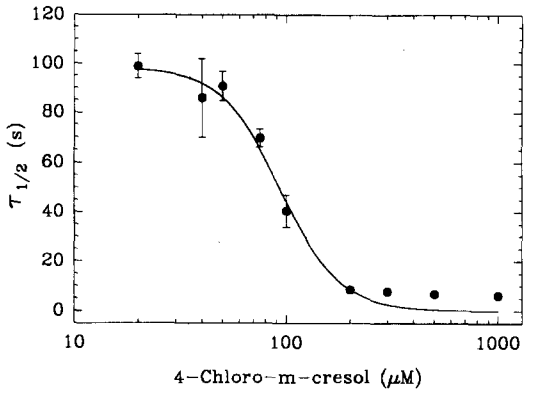

Fig. 3. 4-CmC-induced $^{45}Ca^{2+}$ efflux from passively loaded SR vesicles. 'Heavy' SR vesicles were passively loaded with 1 mM $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles into release media as described in Methods containing 1 mM EGTA, 1 mM $MgCl_2$ and the following concentrations of 4-CmC: 0 mM (○), 20 μM (▽), 40 μM (▼), 50 μM (□), 75 μM (■), 100 μM (△), 200 μM (▲), 300 μM (◇), 500 μM (◆) and 1000 μM (⊙). The straight line indicates leakage of $^{45}Ca^{2+}$ in the presence of the two release inhibitors $MgCl_2$ (5 mM) and ruthenium red (10 μM), the dashed line indicates complete release of $_{45}Ca^{2+}$ stores at 0.1 μM Ca^{2+} + 15 mM caffeine. Inset: Comparison of $_{45}Ca^{2+}$ efflux in the presence of 1 μM free Ca^{2+} (•), and 1 mM EGTA + 100 μM 4-CmC (△).

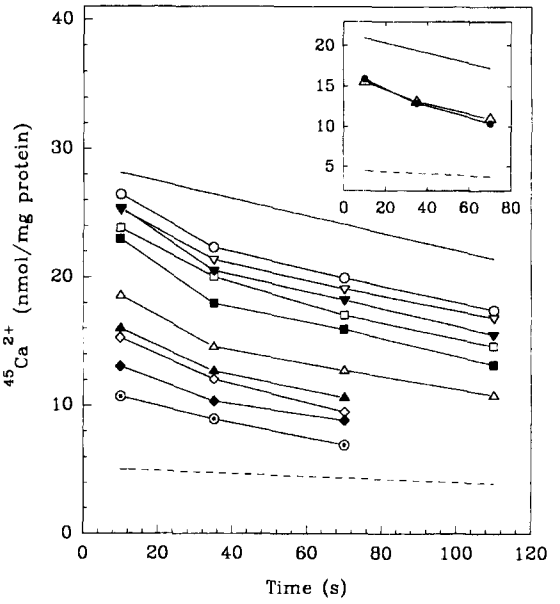

Fig. 4. Dependency of $^{45}Ca^{2+}$ efflux on 4-CmC concentration. Passively $^{45}Ca^{2+}$-loaded SR vesicles were diluted in release media containing the indicated concentrations of 4-CmC. Vesicles released half their $^{45}Ca^{2+}$ stores within the indicated times ($\tau_{1/2}$). Error bars indicate S.E. ($n = 6$).

3.2. Activation of Ca^{2+} release from passively $^{45}Ca^{2+}$-loaded 'heavy' SR vesicles

Comparing the Ca^{2+} dependence of [^{3}H]ryanodine binding with and without 4-CmC indicates that 4-CmC enhances the affinity of Ca^{2+} to the high-affinity Ca^{2+} binding site. However, for potentiation of Ca^{2+}-activated [^{3}H]ryanodine binding, Ca^{2+} concentrations > 0.1 μM are needed. To examine if basal Ca^{2+} is necessary for activating the ryanodine receptor, the effect of 4-CmC on $^{45}Ca^{2+}$ efflux from passively loaded HSR vesicles was investigated in the absence of Ca^{2+}. In a nominally Ca^{2+}-free release medium, 4-CmC activated $^{45}Ca^{2+}$ release in a concentration-dependent way between 50 and 200 μM (Fig. 4). Fig. 3 shows the time-course of $^{45}Ca^{2+}$ efflux from a representative experiment in detail. Without 4-CmC, vesicles released half of their $^{45}Ca^{2+}$ stores in more than 100 s. This time was about 3-times shorter in the presence of 100 μM 4-CmC which is comparable to the releasing effect of 1 μM Ca^{2+} (Fig. 3, inset). In the presence of higher 4-CmC concentrations, the times for releasing half of vesicular $^{45}Ca^{2+}$ ($\tau_{1/2}$) were 10 s, i.e. at the limit or below the first time point which could be measured by the

manual filtration technique. In these cases, the time course was linearly extrapolated between 0 and 10 s to determine $\tau_{1/2}$.

3.3. Direct activation of the isolated Ca^{2+} release channel / ryanodine receptor complex by 4-chloro-m-cresol

In order to prove the direct interaction of 4-CmC with the ryanodine receptor, the purified receptor was incorporated into planar lipid bilayers and the gating behaviour of the incorporated channel was studied. In the presence of submicromolar Ca^{2+}, 4-CmC increased the open probability of the isolated channel in the same concentration range which was effective in [^{3}H]ryanodine binding and $^{45}Ca^{2+}$ flux measurements (Fig. 5A). The *trans* side corresponding to the SR luminal part of the channel was found to be the preferred binding site for 4-CmC. While the threshold concentration of 4-CmC was 50 μM when applied to the *trans* side of the channel, more than 150 μM was needed to significantly activate the channel from the *cis*, i.e. cytoplasmic side. Plotting the open probabilities (P_o) against the applied *trans* 4-CmC concentration, an EC_{50} of 147 μM was obtained (Fig. 5B). Calculation of the Hill coefficient led to a value of 2.7 (Fig. 5C). The increase in P_o is due to prolonged open events in the presence of 4-CmC. When the channel was activated by 50 μM Ca^{2+}, open time histograms could be adequately fitted by a single exponential function (Fig. 6, Table 1). When *cis* [Ca^{2+}] was lowered from 50 to 0.9 μM, the open time constant slightly decreased from 0.43 ms to 0.38 ms ($n = 4$) (Table 1). For fitting open time histograms from single channel recordings obtained in the presence of 0.9 μM Ca^{2+} and an almost maximally activating 4-CmC concentration (250 μM), a double exponential function had to be used. Resulting time constants were $t_1 = 1.38$ ms and $t_2 = 5.26$ ms ($n = 4$) (Table 1).

To investigate if cytoplasmic [Ca^{2+}] is necessary for the activating effect of 4-CmC on the isolated receptor, we investigated the 4-CmC effect at a subthreshold activating Ca^{2+} concentration of 96 nM (Fig. 7A). Compared to the concentration dependence in the presence of higher Ca^{2+}, the dose-response curve was shifted to higher 4-CmC concentrations. Fig. 7B furthermore shows that the 4-CmC-modified channel is still modulated by cytoplasmic Mg^{2+}. When the channel is activated by 0.9 μM Ca^{2+} and 250 μM 4-CmC, about 0.3 mM Mg^{2+} is needed to decrease the initial Po in the absence of Mg^{2+} to 50%.

Nano- to micromolar concentrations of ryanodine trans-

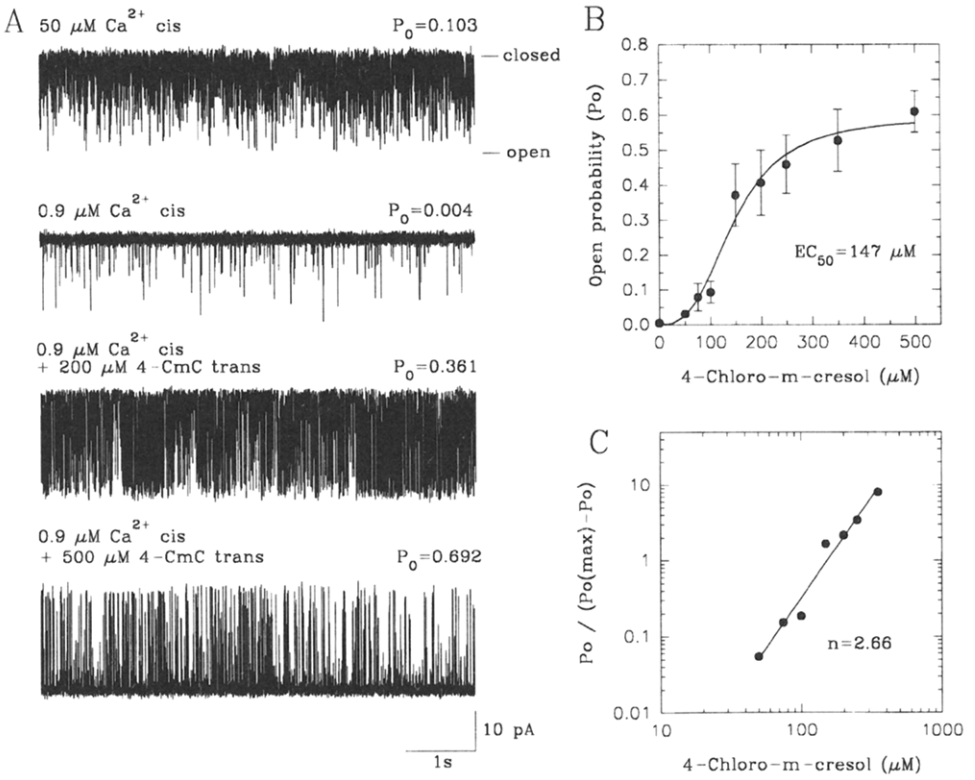

Fig. 5. Activation of the isolated Ca^{2+} release channel by 4-CmC. A: Representative single channel recordings in symmetrical 250 mM KCl, 20 mM Pipes, pH 7.2. The channel was activated by 0.9 μM cytoplasmic (*cis*) Ca^{2+} and indicated concentrations of 4-CmC. Increasing concentrations of 4-CmC were successively added to the luminal (*trans*) side of the channel. Open probabilities (Po) were calculated from data segments of 20 s duration. The holding potential was −45.2 mV. B: Concentration dependence of 4-CmC activated Ca^{2+} release. Data were taken from representative single channel recordings in the presence of 0.9 μM *cis* Ca^{2+} and indicated concentrations of *trans* 4-CmC. Open probabilities (P_o) were calculated from data segments of 20–60 s duration. Error bars indicate S.E. ($n = 9$). C: Corresponding Hill plot of 4-CmC-induced activation of the isolated receptor. The means of the calculated P_o values from panel B were used for calculation.

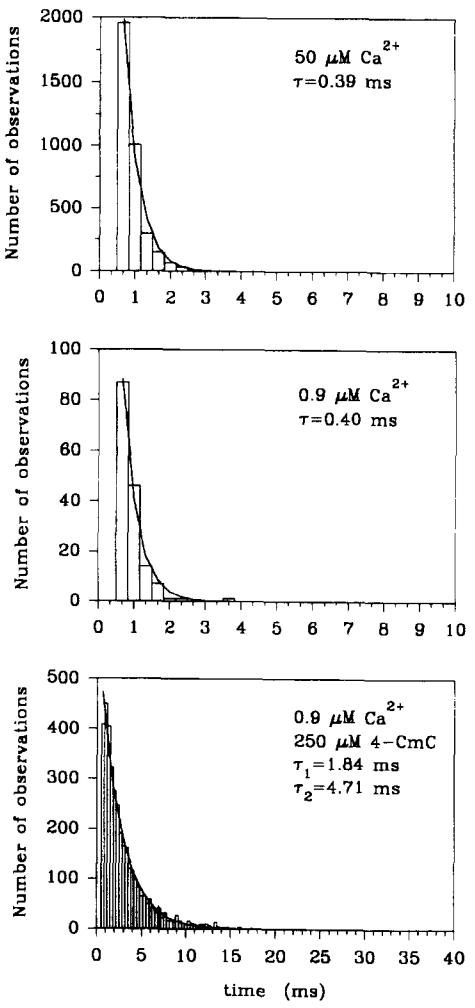

Fig. 6. Open time histograms in the presence and absence of 4-CmC. Representative open time histograms in the presence of indicated *cis* Ca^{2+} and *trans* 4-CmC concentrations. Data are derived from recordings of 20 s duration. Histograms were fitted by a single exponential function in the absence of 4-CmC (top, middle) and by the sum of two exponential functions in the presence of 250 μM 4-CmC with the indicated time constants.

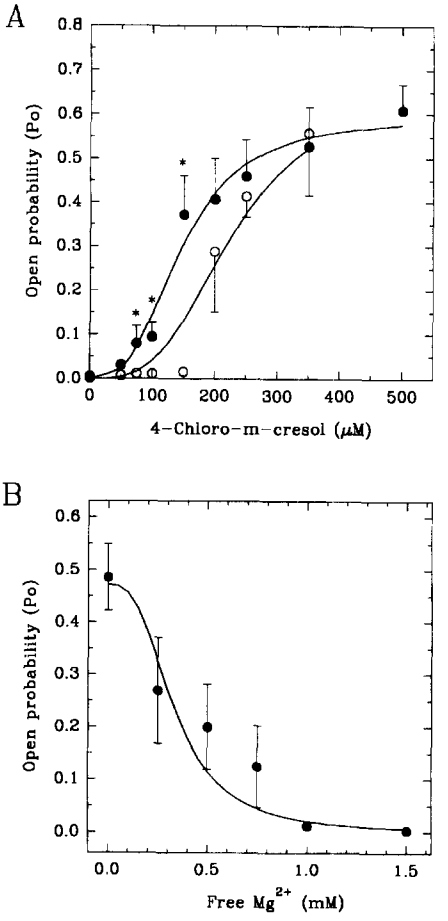

Fig. 7. Modulation of the 4-chloro-m-cresol-activated Ca^{2+} release channel by Ca^{2+} and Mg^{2+}. A: Dependency of open probability on *trans* [4-CmC] in the presence of 0.9 μM (•) and 0.1 μM (○) *cis* Ca^{2+}. Error bars indicate S.E.: $n=9$ for (•) and $n=4$ for (○). At data points labelled with ★, the P_o is significantly higher at $P<0.05$ (Student's *t*-test). B: Inhibition of the 4-CmC-activated Ca^{2+} release channel by *cis* Mg^{2+}. Channels ($n=5$) were initially activated by 0.9 μM *cis* Ca^{2+} and 250 μM 4-CmC.

fer the skeletal muscle ryanodine receptor/Ca^{2+} release channel into a conformation which is characterised by long open intervals and a reduced conductance [18,27,33]. The 4-CmC-modified channel also retained this typical gating property. 1 μM ryanodine transferred the 4-CmC-activated channel into the typically long open state of about 50% of maximal conductance. Further addition of ryanodine (10 μM) closed the channel permanently (data not shown).

Table 1
Open time constants of Ca^{2+} – and 4-CmC-activated Ca^{2+} release channels

	Open time constants	
	T_1	T_2
50 μM Ca^{2+}	0.43 ± 0.06	
0.9 μM Ca^{2+}	0.38 ± 0.01	
0.09 μM Ca^{2+} + 250 μM 4-CmC	1.38 ± 0.32	5.26 ± 2.06

Open time histograms of representative data segments at indicated cytoplasmic Ca^{2+} and luminal 4-CmC concentrations were fitted to a single or to the sum of two exponential functions. Calculated open time constants are expressed as the mean ± S.E. ($n=4$).

3.4. 4-Chloro-m-cresol-induced contractures in fibre bundles of MH muscle

Caffeine in millimolar concentrations induces contractures in isolated skeletal muscle fibres (cf. [8]). According to the European in vitro-contracture test protocol to determine the susceptibility of patients to malignant hyperthermia (MH), caffeine is cumulatively added to the bath in doses between 0.5 and 4 mM. A threshold contracture of ≥ 200 mg at 2 mM caffeine or less is considered to indicate MH susceptibility (MHS) if also the sensitivity to halothane is enhanced. To compare the usefulness of 4-CmC for this protocol, we applied 4-CmC in the same

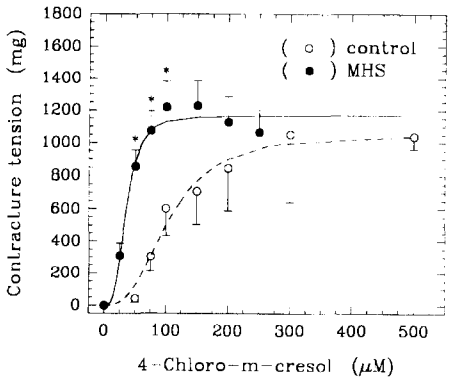

Fig. 8. 4-CmC-induced contractures in isolated skeletal muscle fibre bundles. Small fibre bundles were dissected from human skeletal muscle biopsies obtained from patients susceptible to malignant hyperthermia (•) and non-susceptible indviduals (○). 4-CmC was added in increasing concentrations to the bathing solution. The amplitudes of 4-CmC-induced contractures are plotted against the applied 4-CmC concentration. Data points labelled with ★ are significantly different at $P < 0.05$ (Student's *t*-test). Error bars indicate S.E.: $n = 12$ (•) and $n = 8$ (○).

manner. In control fibres obtained from patients non-susceptible to MH (MHN), 4-CmC in concentrations ≥ 75 μM evoked caffeine-like contractures. After peak activation, force generally decreased to a lower steady state level which was plotted against the 4-CmC concentration in Fig. 8. The half-maximal activating concentration was 109 μM while maximum force was achieved at concentrations higher than 300 μM. By contrast, 25 μM 4-CmC already induced contractures of more than 200 mg in MHS fibre bundles. Fig. 8 shows that the dose-response curve for these fibres is very steep. Maximal force was achieved at 3-fold lower concentrations of 4-CmC as compared to control. The EC_{50} were 35 μM for MHS fibres compared to 104 μM in controls. In both cases, the calculated Hill coefficient was about 3 (MHS: 3.3, control: 2.7) which is identical with the value we obtained for the activation of the isolated release channel.

4. Discussion

Galloway and Denborough [11] first demonstrated that the contracture-inducing effect of commercial preparations of the muscle relaxant agent succinylcholine in vitro is due to admixtures of the preservative 4-chloro-*m*-cresol. Zorzato et al. [43] investigated the Ca^{2+}-releasing effects of various chlorocresol compounds on skeletal muscle SR vesicles and cerebellar microsomes and found the methylated compound 4-chloro-*m*-cresol (4-CmC) to be the most potent one. 4-CmC-induced Ca^{2+} release in SR vesicles was blocked by ruthenium red and 4-CmC did not activate inositoltrisphosphate-mediated Ca^{2+} release in cerebellar microsomes. These observations led to the conclusion that the ryanodine receptor/Ca^{2+} release channel is the preferred binding structure of this agent. In the present study, we have demonstrated that 4-chloro-*m*-cresol directly activates the isolated Ca^{2+} release channel when reconstituted into planar lipid bilayers. Combining the methods of [^{3}H]ryanodine binding, $^{45}Ca^{2+}$ flux measurements, and reconstitution of the isolated ryanodine receptor, we were able to provide a comprehensive pharmacological profile of this compound.

4-CmC increased the K_d of high-affinity [^{3}H]ryanodine binding but did not change the B_{max} values (Fig. 1). Binding was potentiated with an EC_{50} of 112 μM 4-CmC (Fig. 1) which is about 10 times lower than that of caffeine [Herrmann-Frank, unpublished data]. It has been shown that cytoplasmic Ca^{2+} modulates [^{3}H]ryanodine binding in a biphasic manner [4,7,26,35,39]. Ca^{2+} up to 200 μM increases [^{3}H]ryanodine binding while higher concentrations inhibit binding. 4-CmC potentiated [^{3}H]ryanodine binding at Ca^{2+} concentrations higher than 0.1 μM. Normalizing the data to maximally bound [^{3}H]ryanodine (Fig. 2B) revealed that 4-CmC did not change this bell-shaped Ca^{2+} dependence but increased the affinity of the Ca^{2+} activation site while the effects on the Ca^{2+} inhibition site were minor. This observation was confirmed when the Mg^{2+} dependence of [^{3}H]ryanodine was tested in the absence and presence of 4-CmC. In the presence of maximally activating Ca^{2+} and 4-CmC concentrations, more than 20 mM Mg^{2+} was necessary to inhibit binding half maximally (Fig. 2C). Removing 4-CmC from the binding assay decreased the IC_{50} for Mg^{2+} 2-fold more than reducing Ca^{2+} (Fig. 2D). Since Mg^{2+} inhibition of [^{3}H]ryanodine binding is due to a direct competition between Ca^{2+} and Mg^{2+} for the Ca^{2+} activation site [30], these results suggest that 4-CmC renders the ryanodine receptor more sensitive to activating Ca^{2+} concentrations. The effect of 4-CmC appears to be specific. We tested the effects of two further monocyclic carbohydrogens, benzol and toluol, on [^{3}H]ryanodine binding (data not shown). Neither benzol nor the methylated derivative toluol (300 μM) significantly potentiated [^{3}H]ryanodine binding. Also the effect of *m*-cresol was negligibly small (0.3 pmol/mg protein at 300 μM) compared to 4-chloro-*m*-cresol (7.5 pmol/mg protein). Thus the chloride at position 4 in the aromatic ring systems appears to be essential for the activating effect of 4-CmC.

To investigate if 4-CmC directly interacts with the isolated ryanodine receptor, we tested the effect of 4-CmC on the CHAPS-solubilized Ca^{2+} release channel incorporated into planar lipid bilayers. 4-CmC increased the open probability of the isolated channel in a similar concentration range ($EC_{50} = 147$ μM) as it stimulated [^{3}H]ryanodine binding. This activating effect is due to an increase in the number and duration of open events (Fig. 6, Table 1). Testing the effect of 4-CmC on the cytoplasmic and luminal side of the channel, the latter side was found to be the preferred binding domain, i.e. the last 1000 amino acids next to the carboxy terminus of the receptor [37,42]. The corresponding Hill coefficient of 2.7 indicated the

presence of at least 3 binding sites for 4-CmC. The identical value was also obtained when testing the contracture-inducing effect of 4-CmC in isolated muscle fibre bundles while we determined a Hill coefficient of 1.8 for 4-CmC-stimulated [^{3}H]ryanodine binding. A Hill coefficient of 1.8 does not exclude the existence of more than 2 binding sites. Since the high-affinity ryanodine binding site, however, is most likely located between AA 4475 and the carboxyl terminus of the ryanodine receptor [1] which is possibly also a binding domain for 4-CmC, the discrepancy in the Hill coefficients could be alternatively explained by assuming that the binding of ryanodine transfers the channel into a conformation which screens a further 4-CmC binding site.

Testing the Ca^{2+}-dependence of the activating 4-CmC effect on the single channel level confirmed our results from [^{3}H]ryanodine binding. Comparing the effect of 4-CmC at pCa 6 and 7 revealed that Ca^{2+} shifts the dose-response curve to lower 4-CmC concentrations. Our single channel data further revealed that the 4-CmC-activated channel is further sensitive to its endogenous modulator Mg^{2+} and its exogenous ligand ryanodine thus providing a useful tool for further biochemical and physiological studies.

To investigate if the ryanodine receptor is also the preferred binding structure of 4-CmC when applied to intact muscle fibres, we examined the effect of 4-CmC on force development in muscle fibre bundles obtained from biopsies of patients susceptible to malignant hyperthermia (MHS) and patients for whom susceptibility was excluded. 4-CmC induced caffeine-like contractures in both fibre types with a 3-fold higher sensitivity in MHS fibres. The largest differences between the dose-response curve were found at concentrations of 50 and 75 μM 4-CmC. Thus the contracture development in the concentration range between 0 and 100 μM could become a significant parameter to determine the MH susceptibility of patients. We cannot exclude that 4-CmC also increases the sensitivity of the myofilaments as it has been reported for caffeine [41]. Experiments are in progress to elucidate this question.

With the exception of skeletal muscle, *m*-cresol has been shown to potentiate transepithelial sodium transport across frog skin induced by insulin [12]. It has been speculated that *m*-cresol modifies the structure of insulin and that this modification may facilitate the hormone-receptor interaction. Our data strongly indicate a direct interaction of 4-chloro-*m*-cresol with the ryanodine receptor. Most of the known regulators of the Ca^{2+} release channel activate the channel from the cytoplasmic side (cf. [5,23]). The stronger activation of the isolated channel by 4-CmC when applied from the luminal side suggest binding sites for 4-CmC different from those for caffeine and nucleotides. Because of the high electronegativity of chloride in position 4 of the aromatic ring, 4-CmC could interact with the ryanodine receptor by binding to basic amino acids in luminal parts of the receptor. Screening the last C-terminal 1000 amino acids, we found a cluster of 14 arginines between AA 4295 and AA4341 that form a luminal loop between the transmembrane segments M3 and M4 as deduced from the hydrophobicity of the primary sequence [42]. The close neighbourhood of the potential high-affinity Ca^{2+} binding site [2,3] could explain the observed enhancement of Ca^{2+} sensitivity induced by 4-CmC.

In summary, we have successfully demonstrated that 4-CmC is a potent and most likely specific activator of the Ca^{2+} release channel/ryanodine receptor receptor protein. Because of its more than 10-times higher sensitivity compared to caffeine, it could become an interesting tool for both physiological and biochemical studies to characterize ryanodine receptor-mediated Ca^{2+} release, without having the negative side effects described for caffeine. Our data furthermore suggest that this compound might be a powerful pharmacological agent to determine the susceptibility of patients to malignant hyperthermia via the in vitro-contracture test.

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