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4-chloro-orto-cresol activates ryanodine receptor more selectively and potently than 4-chloro-meta-cresol

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ABSTRACT

In this study we performed the comprehensive pharmacological analysis of two stereoisomers of 4-chloro-meta-cresol (4CMC), a popular ryanodine receptor (RyR) agonist used in muscle research. Experiments investigating the Ca^{2+}-releasing action of the isomers demonstrated that the most potent isomer was 4-chloro-orto-cresol (4COC) (EC_{50} = 55 ± 14 μM), although 3-chloro-para-cresol (3CPC) was more effective, as it was able to induce higher magnitude of Ca^{2+} flux from isolated terminal cisterna vesicles. Nevertheless, 3CPC stimulated the hydrolytic activity of the sarcoplasmic reticulum ATP-ase (SERCA) with an EC_{50} of 91 ± 17 μM, while 4COC affected SERCA only in the millimolar range (IC_{50} = 1370 ± 88 μM). IC_{50} of 4CMC for SERCA pump was 167 ± 8 μM, indicating that 4CMC is not a specific RyR agonist either, as it activated RyR in a similar concentration (EC_{50} = 121 ± 20 μM).

Our data suggest that the use of 4COC might be more beneficial than 4CMC in experiments, when Ca^{2+} release should be triggered through RyRs without influencing SERCA activity.

1. Introduction

Excitation-contraction coupling (ECC) in skeletal muscle involves a series of molecular events between action potential and contraction. The crucial step of ECC is the conversion of the action potential of the sarcolemma into Ca^{2+} release from the sarcoplasmic reticulum (SR). As a consequence, myoplasmatic [Ca^{2+}] increases, which activates the myofilaments to generate contractile force. The signal transduction between the surface and intracellular membranes is mediated by the communication between the voltage sensor (formed by the dihydropyridine receptor (DHPR)) and the SR Ca^{2+} release channel (ryanodine receptor, RyR)) [1–3].

The most widely used RyR agonist is caffeine, which provided invaluable information about the coupling process and the function of RyR in other signaling pathways as well as in the diagnosis of Malignant Hyperthermia susceptibility (MHS) [4–7]. MHS is an inherited muscle disorder, linked to certain point mutations of the skeletal muscle type RyR (RyR1) and characterized by the hypersensitivity of the Ca^{2+} release machinery to therapeutic doses of succinyl choline and volatile anesthetics. When exposed to these triggering drugs, MHS patients develop generalized muscle spasm, muscle work-related hyperthermia, lactoacidosis, hyperkalemia and concomitant arrhythmias, leading to death unless the RyR blocker dantrolene is applied [8]. As the mutant RyRs are more susceptible to activation by caffeine than healthy RyRs, caffeine may be used in diagnostic, *in vitro* contracture tests of muscle biopsies from MH susceptible suspects before general anesthesia [9].

Although, caffeine is a very popular research tool, it is not an ideal one, because it is very lipophilic, difficult to wash out; and evokes Ca^{2+} release only in millimolar concentrations, so it makes caffeine quite inconvenient to work with [10]. In addition, is has many side-effects as

Abbreviations: 4CMC4, chloro-meta-cresol; 4COC4, chloro-orto-cresol; 3CPC3, chloro-para-cresol; Ry, Rryanodinereceptor; SERC, Asarcoplasmicreticulum Ca^{2+}ATP-ase; ECC, excitation-contractioncoupling; SR, sarcoplasmicreticulum; MHS, malignanthyperthermia susceptibility; TC, terminalcisternae; HSR, VheavySR vesicles; LSRV, longitudinalSR vesicles; RR, rutheniumred; P_o, openprobability

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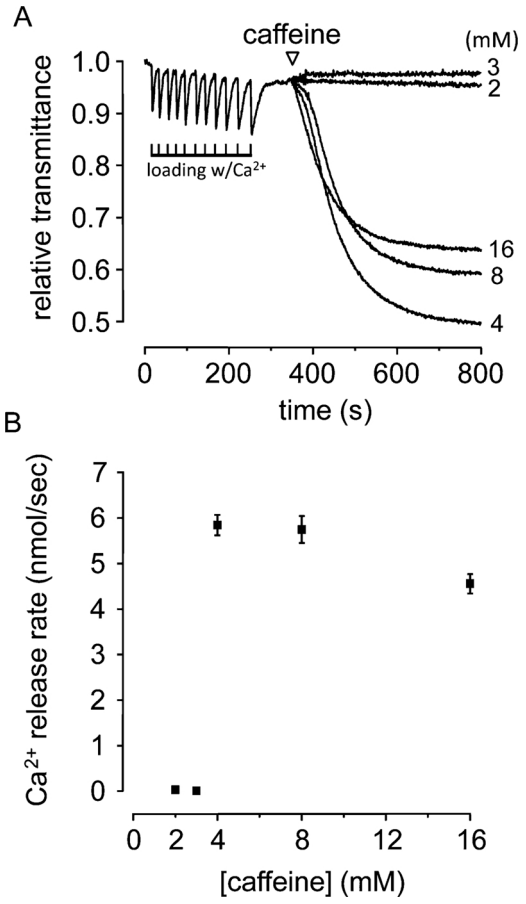

Fig. 1. The effect of caffeine on the Ca^{2+} flux from HSRV.
Representative records of transmittance of a buffer containing Ca^{2+}-loaded HSRV and Ca^{2+} indicator before and during the treatment with 2, 3, 4, 8 and 16 mM caffeine. Ca^{2+}-loading of vesicles were made actively by the SERCA pump and induced by 11 subsequent injection of Ca^{2+} (A). Ca^{2+} injections are shown by ticks, caffeine addition is labelled by arrowhead. Average slope of Ca^{2+} release curves in the presence of 2, 3, 4, 8 and 16 mM caffeine are shown in panel B (n = 3–5).

it enhances the Ca^{2+} sensitivity of myofibrils, increases cAMP level and inhibits the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), IP_3 receptors and other ion channels, therefore may influence Ca^{2+}-handling indirectly too [11–15]. The nonspecific actions on integrated membrane proteins are believed to develop because caffeine significantly accumulates in lipid membranes at the effective concentrations and changes the fluidity of the bilayer membrane.

Also, we encounter technical difficulties of caffeine when applying in our Ca^{2+} release assay, using heavy SR vesicles (HSRV), derived from TC of skeletal muscle. Example of such experiments are demonstrated in Fig. 1A, where HSRV were suspended in a buffer containing the metallochromic indicator antipyrylazo III. The extravesicular Ca^{2+}-level is reported by the transmittance of the medium, so that decrease in the dye transmittance signal represents an increase in extravesicular [Ca^{2+}]. In the beginning of experiments, 11 aliquots of small portions of Ca^{2+} was injected into the solution and after each addition, Ca^{2+} was allowed to be loaded into HSRV lumen by the sarco-endoplasmic reticulum ATP-ase (SERCA), as shown by slow recovery of transmittance after Ca^{2+} injections. Thereafter, different concentrations of caffeine (from 10x stock solutions) was injected into the medium. 4 mM or higher concentrations triggered a fast decline in transmittance - being indicative of the release of previously loaded Ca^{2+} - while 3 mM or lower concentrations failed to trigger Ca^{2+}-release. It should be noted that we had difficulty standardizing the experimental procedure as the quantitative reproducibility of experiments was hindered by the fact that the effect of caffeine depended on the degree of HSRV Ca^{2+}-load. Unfortunately, under these loading conditions we failed to observe a gradual caffeine response (rather an all-or-nothing action), as shown by the average Ca^{2+}-release rates plotted as the function of caffeine concentrations in Fig. 1B. More surprisingly, the amplitude of transmittance-change (which is proportional to the amount of Ca^{2+} released) was much lower for 16 then 4 mM, which may be attributed to an unknown, non-specific effect of caffeine (Fig. 1A).

The technical disadvantages of caffeine could be overcome by using more potent and specific RyR activators such as chlorocresol. The Ca^{2+}-releasing action of chlorocresol was discovered in the 90's, when the question, whether the preservatives, such as chlorocresol used in commercially available anesthetic products contribute to the severity of MH seizures was addressed by Zorzato et al. [16]. Chlorocresols were shown to release Ca^{2+} from SR terminal cisterna (TC) vesicles, increase intracellular [Ca^{2+}] in muscle fibers and cause muscle contractures. Since the earliest studies, 4-chloro-m-cresol (4CMC) became a widely used research chemical in studying Ca^{2+} signaling, as it was suggested to be the most potent chlorocresol stereoisomer [16–18]. More recently, experiments on COS-7 cells, which lack functional RyR provided indirect evidence that 4CMC might inhibit SERCA [19]. 4CMC was also suggested to be used in the diagnosis of MH susceptibility instead of caffeine [20]. While there is an obvious need for more suitable (more potent and RyR-selective) RyR ligands, the detailed pharmacological analysis of chlorocresol stereoisomers on either RyR or SERCA activity have not been performed yet. Therefore, in our attempt to find a better RyR agonist among chlorocresol stereoisomers, we determined their pharmacodynamic properties using skeletal muscle SR vesicles in Ca^{2+} release and ATP-ase activity assays. The chemical structure of chlorocresols tested in this study (4-chloro-meta-cresol (4CMC), 3-chloro-para-cresol (3CPC) and 4-chloro-orto-cresol (4COC)) is displayed in Fig. 2.

2. Material and methods

2.1. Materials

Phospholipids were from Avanti Polar Lipids. All other chemicals were purchased from Sigma-Aldrich. Chlorocresol stock solutions were

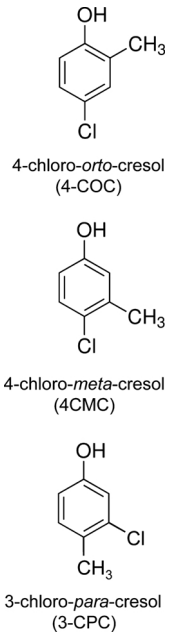

Fig. 2. Structure of chlorocresol stereoisomers.

made using DMSO.

2.2. Microsome isolation

Sarcoplasmic reticulum terminal cisternae (Heavy SR vesicles, HSRV)- and longitudinal SR vesicles (LSRV) were isolated from rabbit fast-twitch skeletal muscle by differential centrifugation as described previously [21]. All steps were performed at 4 °C. The solutions were supplemented with protease inhibitors (200 μM pefabloc SC, 0.1 μM aprotinin, 1 μM leupeptin, 0.2 μM pepstatin A, 500 μM benzamidine). The muscle was homogenized in 450 mL buffer (containing: 100 mM NaCl, 20 mM EGTA, 20 mM Na-HEPES; at pH = 7.5). Thereafter, cell debris was pelleted at 3500 × g, for 35 min using a tabletop centrifuge equipped with a swingout rotor. The supernatant was further centrifuged at 40,000 × g, for 30 min in a Ti45 rotor. The resulting pellet containing crude microsomes were resuspended in 600 mM KCl, 10 mM K-Pipes, 250 mM sucrose, 1 mM EGTA, 0.9 mM $CaCl_2$ containing solution at pH = 7.0 and left in cold room for 1 h. This microsome suspension was centrifuged at 109000xg, for 30 min and the pellet was resuspended and loaded onto a 20–45 % linear sucrose gradient (containing: 105 mM NaCl, 10 mM Pipes, 0.1 mM EGTA, 0.09 mM $CaCl_2$ at pH = 7.0). After overnight centrifugation at 90,000 × *g* in a SW27 rotor, two visible rings, corresponding to LSRV and HSRV were collected from the 30–32 % and the 36–38 % regions of the sucrose gradient, respectively. The microsomes were washed in a 10x volume of buffer (475 mM sucrose, 1 mM NaCl, 10 mM Pipes, pH = 7.0) and collected again by centrifugation at 124,000 × *g* for 60 min in the Ti45 rotor. The pellet was resuspended in a buffer (300 mM sucrose, 10 mM K-PIPES pH = 7.0) at a final protein concentration of > 20 mg/mL. Microsomes were aliquoted and rapidly frozen in liquid nitrogen and stored at −70 °C until further use.

2.3. Calcium flux measurements

HSRV (0.5 mg protein/experiment) was suspended in 1.9 mL buffer (92.5 mM KCl, 18.5 mM MOPS, 1 $MgCl_2$ mM, 1 mM ATP, 250 μM antipyrylazo III pH = 7.0, 37 °C) in a glass cuvette [23]. The extravesicular [Ca^{2+}] of the solution was followed by measuring the transmittance of the metallochromic dye antipyrylazo III at 710 nm using a spectrofluorimeter (Spex Fluoromax). HSRV was loaded with Ca^{2+} by utilizing SERCA activity. Ca^{2+} uptake of HSRV was initiated by the addition of appropriate doses of $CaCl_2$. After Ca^{2+} uptake was complete, Ca^{2+} release was triggered by different doses of chlorocresols. The rate of Ca^{2+} release was obtained by measuring the slope of the initial segment of the curve. Light intensities were normalized to the average of the first 5 data points. In some experiments vesicles were pretreated with ruthenium red (RR) in order to verify the role of RyR during the process.

2.4. RyR reconstitution and single-channel current recording

Measurements of channel activity were carried out using HSRV fused into planar lipid bilayers [21]. Bilayers were formed across a 200 μm wide hole drilled into the wall of a delrin cup (Warner Instruments Inc., Hamden, CT, U.S.A.), containing a recording solution (50 mM $Cs-CH_3O_3S$, 100 μM K_2H_2EGTA, 150 μM $CaCl_2$, 20 mM HEPES, pH 7.2). The lipid solution contained phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (Avanti Polar Lipids) in the ratio of 5:4:1 and dissolved in *n*-decane in the final lipid concentration of 20 mg/mL. Fusion of HSRV with the bilayer was induced by increasing the [Cs^+] by 450 mM in the *cis* chamber (corresponding to the cytoplasmic side of the channel). The other chamber was referred as *trans* and was kept on ground potential. The current was driven by the Cs^+ concentration gradient while the membrane potential was held at 0 mV. After successful incorporation of RyR, free [Ca^{2+}] in the *cis* chamber was decreased from 50 μM, to 100 nM by addition of EGTA. Thereafter, chlorocresol was applied to the cytoplasmic side of RyR. Currents were processed using an Axopatch 200 amplifier, filtered at 1 kHz through an eight-pole lowpass Bessel filter, digitized at 3 kHz and recorded using pCLAMP 6.03 software (Axon Instruments, Foster City, CA, U.S.A.). Open probabilities were calculated with Clampfit 10 software.

2.5. ATP-ase activity measurements

ATPase activity of LSRV was determined by using a coupled enzyme assay in a medium containing 100 mM KCl, 20 mM Tris−HCl, 5 mM $MgCl_2$, 5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.001 mM A23187 ionophore, 0.2 mM NADH, 7.5 U/mL pyruvate kinase, and 18 U/mL lactate dehydrogenase (pH = 7.5) at 37 °C. The assay was performed at the free [Ca^{2+}] of 1 μM to allow maximal SERCA activity. Ionized Ca^{2+} concentration of the solution was determined using a computer program by Fabiato [22]. A23187 was applied to prevent accumulation of Ca^{2+} inside LSRV. 5.5 μg/mL of protein was used in each experiment. Total hydrolytic activity was measured as the decrease of optical density of the NADH absorbance peak at 340 nm. Data were expressed in micromoles of inorganic phosphate per milligrams of protein per min (abbreviated as I.U.) [21]. Chlorocresols were added to the medium 1 min before recording.

2.6. Measurement of contractility

Extensor digitorum longus muscles were dissected from rats and their ends were attached between the bottom of a chamber and the arm of a force transducer in a vertical position. Muscle tone was measured under isometric condition after the preload of the muscle was adjusted to 10 g. The muscle was bathed in Tyrode's solution, which was continuously oxygenated and kept at 37 °C during the experiments. Different concentrations of 4COC were established using appropriate doses of a 500 mM 4COC stock solution.

3. Results and discussion

3.1. Studies on microsomes

The effect of chlorocresol stereoisomers 4CMC, 3CPC and 4COC were studied on the rate and magnitude of Ca^{2+} efflux from HSRV and on the SERCA pump activity of LSRV.

3.1.1. Ca^{2+}-release experiments

HSRV were suspended in a buffer containing the Ca^{2+} indicator APIII and loaded with equal amounts of Ca^{2+} in each experiment using the Ca^{2+} pump. Thereafter, different doses of chlorocresols were injected into the cuvette, which was followed by a rapid decrease of transmittance of the imaging medium, indicating that chlorocresols induced Ca^{2+} release from HSRV by stimulating RyRs (Fig. 3A, D, G). The specificity of the reaction was verified by experiments, in which chlorocresols (500 μM) were applied in the presence of the RyR inhibitor ruthenium red (RR, 5 μM) [23]. In these cases, no Ca^{2+} release was detected. The Ca^{2+} efflux rate was determined at different concentrations of chlorocresols by a linear fit of the initial phase of the intensity change. The reciprocal values of slopes were plotted against the corresponding concentration values. Data were fitted using the Hill equation, which revealed EC_{50} values of 175 ± 39 μM for 4CMC, 182 ± 37 μM for 3CPC and 113 ± 36 μM for 4COC, respectively (Fig. 3B, E, H). The relative amount of released Ca^{2+} was also measured at different chlorocresol concentrations and appeared to be dose dependent. Half-effective concentrations were 121 ± 20, 71 ± 7 and 55 ± 14 μM for 4CMC, 3CPC and 4COC, respectively (Fig. 3C, F, I). Interestingly, the total amount of Ca^{2+} released by the highest concentration (500 μM) was different for each isomer. As measured by the relative steady state transmittance, 3CPC released twice as much Ca^{2+}

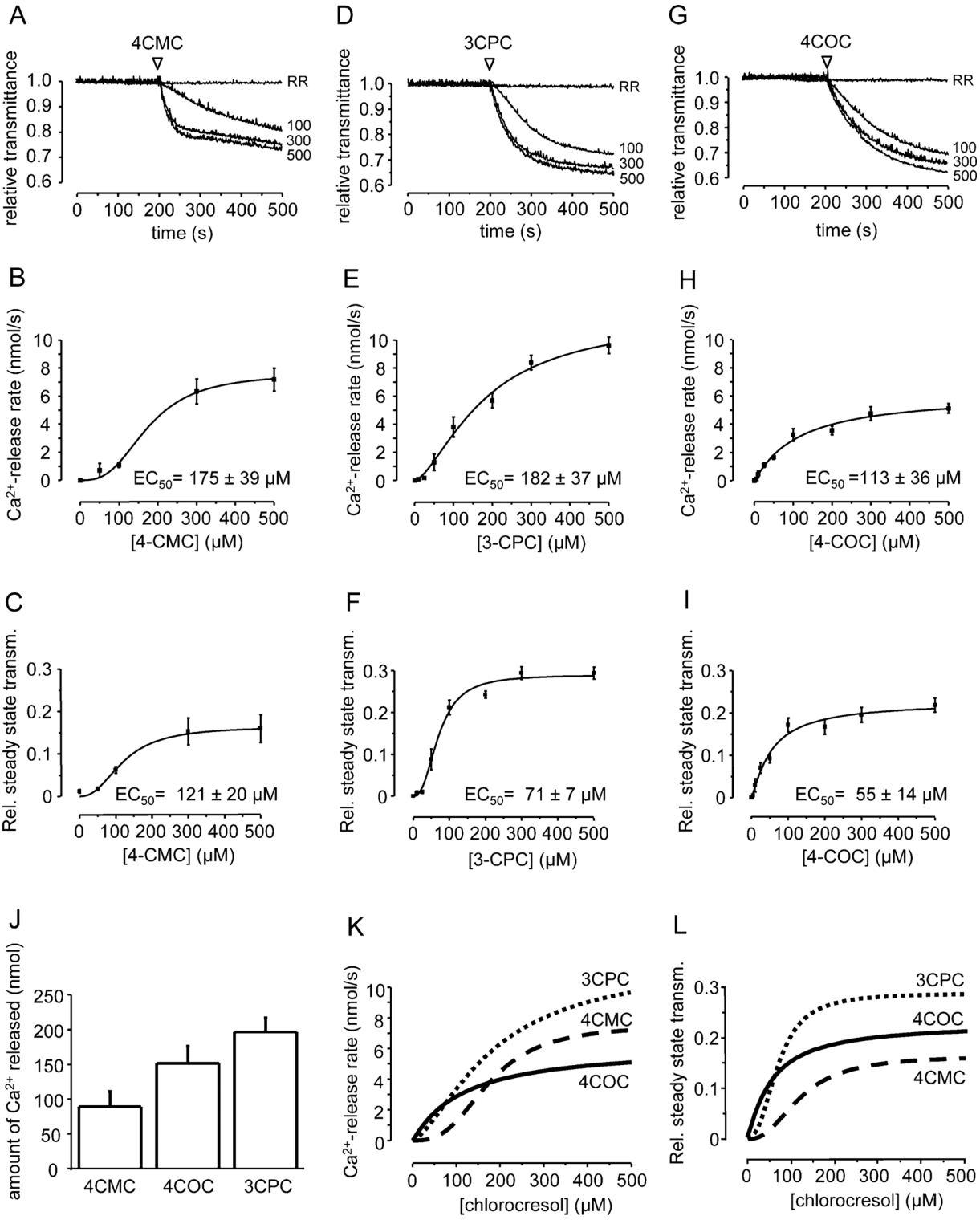

Fig. 3. The effect of chlorocresols on the Ca^{2+} flux from HSRV.
Representative records of transmittance of a buffer containing Ca^{2+}-loaded HSRV and Ca^{2+} indicator before and during the treatment with 100, 300 or 500 μM 4CMC, 3CPC or 4COC are shown in A, D and G, respectively. Injection of chlorocresol stock solutions are labelled by empty arrowheads. Some experiments were performed in the presence of ruthenium red (RR). 4CMC, 3CPC or 4COC-evoked Ca^{2+} flux rates and the relative magnitude of Ca^{2+} release (relative steady-state transmission) were analyzed, plotted as a function of chlorocresol concentrations and presented in B, E, H, and C, F, I, respectively ($n \geq 4$). EC_{50} values are shown in each graph. The total amount of Ca^{2+} released by 500 μM 4CMC, 3CPC or 4COC is shown in J. Hill-fits of the concentration dependence of Ca^{2+} release rates and relative steady-state transmissions of different chlorocresols are illustrated in one graph to serve better comparison (K and L, respectively).

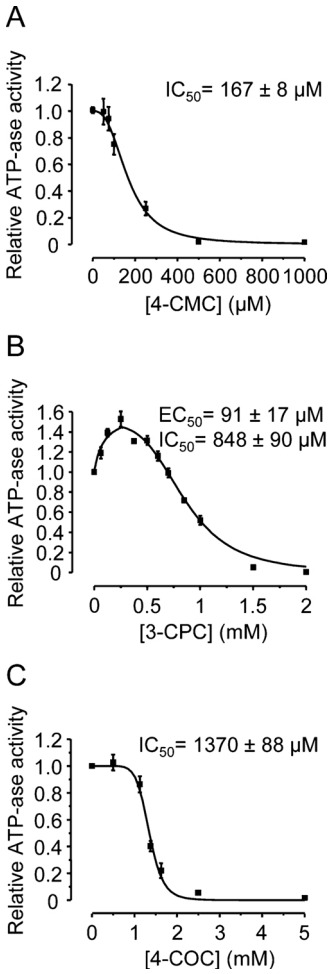

Fig. 4. The effect of chlorocresols on the hydrolytic activity of SERCA.
Relative ATP-ase activities are plotted as the function of 4CMC, 3CPC or 4COC concentrations, fitted with Hill-function and shown in A, B and C, respectively ($n \geq 4$). IC_{50} and EC_{50} values of Hill-fits are included to the graphs.

as 4CMC, while the average of this value was also higher for 4COC, but this was not significant statistically (Fig. 3J). Phenol and toluol were also tested, however they failed to trigger Ca^{2+} efflux from HSRV when used in concentrations up to 2 mM, indicating that chloride is an essential component of these RyR agonists (data not shown).

3.1.2. ATP-ase activity measurements

The effect of the isomers on the SR Ca^{2+}-pump was studied by measuring the hydrolytic activity of LSRV. The specific ATP-ase activity was determined in the presence of 10 μM thapsigargin, a specific inhibitor of SERCA, demonstrating that > 90 % of our sample's ATP-ase activity was attributable to SERCA function (not shown). Calculated pump activities at different chlorocresol concentrations were normalized to the control activity and plotted against cresol concentration. Fig. 4A and B demonstrates that 4CMC and 4COC inhibited ATP-ase activity. Hill-fit of these datasets resulted in IC_{50} of 167 ± 8 μM for 4CMC and 1370 ± 88 μM for 4COC. Interestingly, the effect of 3CPC was biphasic, as it stimulated the pump with an EC_{50} of 91 ± 17 μM and inhibited it with an IC_{50} of 848 ± 90 μM (Fig. 4C). Phenol and toluol did not alter ATP-ase activity significantly (data not shown).

In summary (see Fig. 3K and L), the most potent RyR agonist among chlorocresol stereoisomers was 4COC, as its EC_{50} was the smallest regarding both Ca^{2+} flux rate and Ca^{2+} release magnitude. 4CPC and 4CMC was approximately equally potent. 3CPC triggered the fastest rate of Ca^{2+} release and also, it was able to release the highest amount

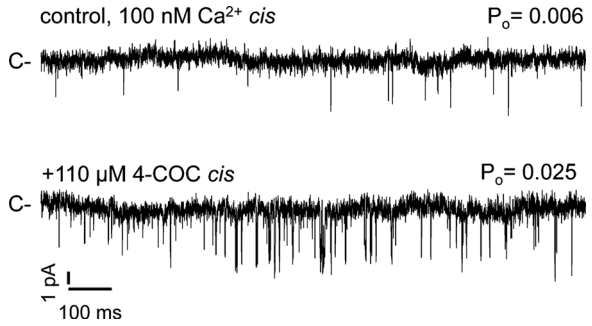

Fig. 5. The effect of 4COC on single RyR channels.
Representative single channel current traces recorded under control conditions (100 nM Ca^{2+} in the cytoplasmic, "*cis*" face of RyR, top trace) and in the presence of 110 μM 4COC (bottom trace), showing that 4COC enhanced the open probability (P_o) of the channel (n = 3). The closed state level of the current is labelled by "C". Downward spikes represent channel openings.

of Ca^{2+} therefore, it was the most effective chemical. However, 4COC elicited the slowest (but still robust) rate of flux, at EC_{50} or lower concentrations it was able to release the largest amount of Ca^{2+} (Fig. 3J) These pharmacological properties make 4COC the most suitable chlorocresol in RyR studies. In addition, 4COC is the most selective chlorocresol, as its half-inhibiting concentration for SERCA was 25 times higher than its half-activating concentration for RyR (55 vs 1370 μM), while similar values for 4CMC are not significantly different (121 vs 167 μM) and 3CPC significantly stimulated SERCA in concentrations < 250 μM, therefore it is not selective at either low or higher concentrations.

Considering these pharmacological features, we suggest the application of 4COC instead of 4CMC, because it is a more selective and more effective Ca^{2+}-releasing agent than 4CMC, which could be advantageous under certain experimental conditions.

3.2. Ion current measurements on single RyR channels

In order to gain more information about the molecular mechanism of the action of 4COC, it was that we further tested on single RyR currents reconstituted in lipid bilayers as described in Methods. A representative current trace is shown in Fig. 5, where downward deflections indicate channel openings. Under control conditions, RyR currents were recorded in 100 nM Ca^{2+} on the cytoplasmic side of the channel, which enables low open probability, as indicated by scarce spikes of channel openings. When the channel was treated with 110 μM 4COC, the open probability (P_o) substantially increased, due to the higher number of open events.

3.3. Muscle contracture test

In our last series of experiments, we tested whether 4COC triggered mechanical activity of skeletal muscle. To this end, the tone of Extensor digitorum longus from rats was measured using a force transducer. The muscles were treated with different doses of 4COC by adding several aliquots of 4COC solution subsequently (Fig. 6). First, 1.6 mM 4COC concentration has been established, which was ineffective. This treatment was followed by additional steps of 4COC injections to raise the concentration by 0.2 mM increments until 2 mM, which slightly enhanced the tone of the muscle. When 4COC concentration reached 2.2 mM, suddenly a robust increase of the tone was observed (Fig. 6A). When in different experiments 4COC was raised to 2.8, (instead of 2.2) in the last step of treatment, the amplitude and the slope of contracture was apparently much higher (Fig. 6B), indicating that the effect of 4COC was concentration dependent. Vehicle (DMSO) was also added to EDLs in increasing doses, but it failed to cause contracture even when it reached twice as high concentration as it was present during 2.8 mM

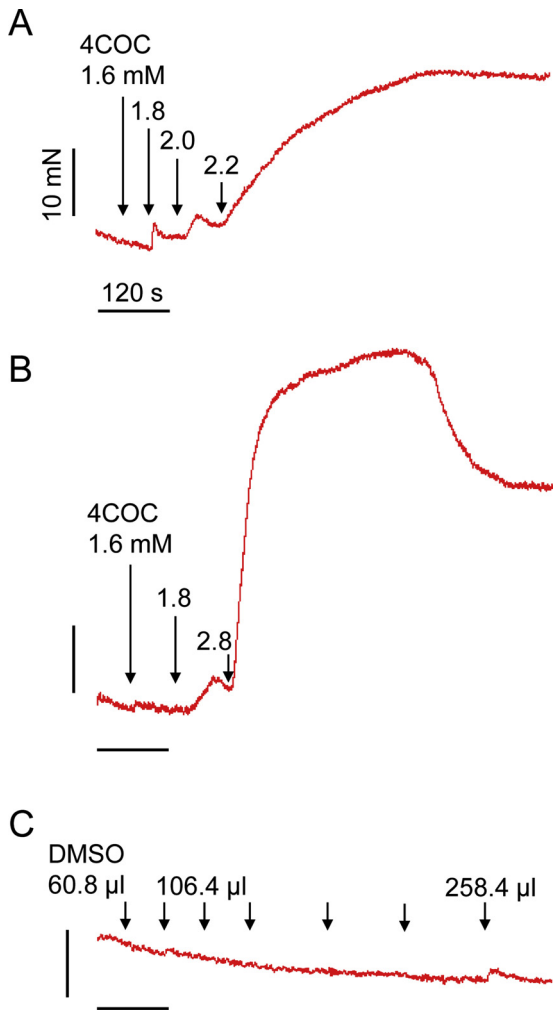

Fig. 6. Muscle contracture evoked by 4COC.
Representative records of muscle tone during 4COC treatment. Applications of indicated doses of 4COC are marked by arrows in A and B. Cumulative doses of vehicle were tested in C, as indicated (n = 3).

4COC treatment (Fig. 6C). These results suggest that 4COC causes skeletal muscle contracture by specifically activating the Ca^{2+}-release apparatus.

3.4. Conclusions

Alltogether, our comprehensive pharmacological analysis suggests that the action of 4COC is qualitatively not different from that of 4CMC, but it is a more selective agonist of the skeletal muscle isoform of the RyR (RyR1), that is, 4COC may cause less interference due to changes of SERCA activity.

Credit author statement

Mariann Skaliczki, Zsuzsanna É Magyar, Tünde Kovács, Miklós Bárdi, Szabolcs Novák, Gyula Diszházi, Judit Péli-Szabó- Performed experiments, Reviewed and Edited manuscript

Balázs Lukács, Sándor Sárközi, Ildikó Márton – Analysed and interpreted data, Reviewed and Edited manuscript, data presentation

István Jóna, Péter Nánási, János Almássy -Designed experiments, Supervised experiments, evaluated data and wrote manuscript, Reviewed and Edited manuscript

Declaration of Competing Interest

The authors declare that they have no known competing interest.

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