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

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ARTICLE INFO

Keywords:
Skeletal muscle
Ryanodine receptor
SERCA
4-chloro-meta-cresol
Chloro-ortho-cresol
3-chloro-para-cresol

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 Ca²⁺-releasing action of the isomers demonstrated that the most potent isomer was 4-chloro-ortho-cresol (4COC) (EC₅₀ = 55 ± 14 μM), although 3-chloro-para-cresol (3CPC) was more effective, as it was able to induce higher magnitude of Ca²⁺ flux from isolated terminal cisterna vesicles. Nevertheless, 3CPC stimulated the hydrolytic activity of the sarcoplasmic reticulum ATP-ase (SERCA) with an EC₅₀ of 91 ± 17 μM, while 4COC affected SERCA only in the millimolar range (IC₅₀ = 1370 ± 88 μM). IC₅₀ 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 (EC₅₀ = 121 ± 20 μM).

Our data suggest that the use of 4COC might be more beneficial than 4CMC in experiments, when Ca²⁺ 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 Ca²⁺ release from the sarcoplasmic reticulum (SR). As a consequence, myoplasmatic [Ca²⁺] 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 Ca²⁺ 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 Ca²⁺ release machinery to therapeutic doses of succinylcholine 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 Ca²⁺ 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-ortho-cresol; 3CPC3, chloro-para-cresol; Ry, Ryanodinereceptor; SERC, Asarcoplasmicreticulum Ca²⁺ ATP-ase; ECC, excitation-contractioncoupling; SR, sarcoplasmicreticulum; MHS, malignanthyperthermia susceptibility; TC, terminalcisternae; HSR, VheavySR vesicles; LSRV, longitudinalSR vesicles; RR, rutheniumred; P₀, openprobability

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https://doi.org/10.1016/j.ceca.2020.102213
Received 17 January 2020; Received in revised form 25 April 2020; Accepted 25 April 2020
Available online 01 May 2020

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it enhances the Ca\(^{2+}\) sensitivity of myofibrils, increases cAMP level and inhibits the sarcoplasmic 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 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).

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 than 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 integrative 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-paras-cresol (3CPC) and 4-chloro-or-ortho-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 prepared from 10x stock solutions in DMSO. As the experiments were performed on Ca\(^{2+}\)-loaded vesicles, 4CMC was added to the solutions in a final concentration of 4 mM. The medium was continuously gassed with 5% CO\(_2\) in air to maintain pH. The experiments were performed at room temperature (22 ± 1°C).
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 CaCl2 containing solution at pH = 7.0 and left in cold room for 1 h. This microsome suspension was centrifuged at 109,000 × g, 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 CaCl2 at pH = 7.0). After overnight centrifugation at 90,000 × g, 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 mgMgCl2, 1 mM ATP, 250 μM antipyrylazo III pH = 7.0, 37 °C) in a glass cuvette [23]. The extra- vesicular [Ca2+] of the solution was followed by measuring the transmittance of the metallochromic dye antipyrylazo III at 710 nm using a spectrophotometer (Spectrafluorometer, Spex Fluoromax). HSRV was loaded with Ca2+ by utilizing SERCA activity. Ca2+ uptake of HSRV was initiated by the addition of appropriate doses of CaCl2. After Ca2+ uptake was complete, Ca2+ release was triggered by different doses of chlorocresols. The rate of Ca2+ 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 CsCl–CH3O3S, 100 mM K2H2EGTA, 150 mM CaCl2, 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 [Ca2+] 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. ATPase 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 MgCl2, 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 [Ca2+] of 1 μM to allow maximal SERCA activity. Ionized Ca2+ concentration of the solution was determined using a computer program by Fabiato [22]. A23187 was applied to prevent accumulation of Ca2+ 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 Ca2+ efflux from HSRV and on the SERCA pump activity of LSRV.

3.1.1. Ca2+ release experiments

HSRV were suspended in a buffer containing the Ca2+ indicator APIII and loaded with equal amounts of Ca2+ in each experiment using the Ca2+ 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 Ca2+ release from HSRV by stimulating RyRs (Fig. 3A, D, G). The specificitiy 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 Ca2+ release was detected. The Ca2+ 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 EC50 values of 175 ± 39 μM for 4COC, 182 ± 37 μM for 3CPC and 113 ± 36 μM for 4CMC, respectively (Fig. 3B, E, H). The relative amount of released Ca2+ 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 4COC, 3CPC and 4CMC, respectively (Fig. 3C, F, I). Interestingly, the total amount of Ca2+ 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 Ca2+...
Fig. 3. The effect of chlorocresols on the Ca²⁺ flux from HSRV. Representative records of transmittance of a buffer containing Ca²⁺-loaded HSRV and Ca²⁺ 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 Ca²⁺ flux rates and the relative magnitude of Ca²⁺ 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 ≥ 4). EC₅₀ values are shown in each graph. The total amount of Ca²⁺ released by 500 μM 4CMC, 3CPC or 4COC is shown in J. Hill-fits of the concentration dependence of Ca²⁺ release rates and relative steady-state transmissions of different chlorocresols are illustrated in one graph to serve better comparison (K and L, respectively).
3.1.2. ATP-ase activity measurements

ESSENTIAL COMPONENT OF THESE RYR AGONISTS (DATA NOT SHOWN). USED IN CONCENTRATIONS UP TO 2 mM, INDICATING THAT CHLORIDE IS AN ESSENTIAL COMPONENT OF THESE RYR AGONISTS (DATA NOT SHOWN). USED IN CONCENTRATIONS UP TO 2 mM, INDICATING THAT CHLORIDE IS AN ESSENTIAL COMPONENT OF THESE RYR AGONISTS (DATA NOT SHOWN). USED IN CONCENTRATIONS UP TO 2 mM, INDICATING THAT CHLORIDE IS AN ESSENTIAL COMPONENT OF THESE RYR AGONISTS (DATA NOT SHOWN).

As 4CMC, while the average of this value was also higher for 4COC, but this was not significant statistically (Fig. 3I). Phenol and toluidine were also tested, however they failed to trigger Ca2+ 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 Ca2+-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 IC50 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 EC50 of 91 ± 17 μM and inhibited it with an IC50 of 848 ± 90 μM (Fig. 4C). Phenol and toluidine did not alter ATP-ase activity significantly (data not shown).

In summary (Fig. 3K and L), the most potent RyR agonist among chlorocresol stereoisomers was 4COC, as its IC50 was the smallest regarding both Ca2+ flux rate and Ca2+ release magnitude. 4CPC and 4CMC was approximately equally potent. 3CPC triggered the fastest rate of Ca2+ release and also, it was able to release the highest amount of Ca2+ therefore, it was the most effective chemical. However, 4COC elicited the slowest (but still robust) rate of flux, at EC50 or lower concentrations it was able to release the largest amount of Ca2+ (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 Ca2+-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 Ca2+ 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 (Pₒ) 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). These pharmacological features 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 Ca2+-releasing agent than 4CMC, which could be advantageous under certain experimental conditions.
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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 Díszházi, Judit Péli-Szabó- Performed experiments, Reviewed and Edited manuscript
Balázs Lukács, Sándor Sárközi, Ildikó Márton – Analyzed and in-
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.

Acknowledgements

The authors are grateful for Róza Ňri and Õva Ŝagi for their excellent
technical assistance.
JA is supported by the Lajos Szodoray Scholarship of the University of
Debrecen. This work was supported by projects GINOP-2.3.2–15-
2016-00040 and EFOP-3.6.2-16-2017-00006 (to JA and PPN), which
are co-financed by the European Union and the European Regional
Development Fund.

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