Commonly used ryanodine receptor activator, 4-chloro-*m*-cresol (4CmC), is also an inhibitor of SERCA Ca^{2+} pumps

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Abstract:

4-Chloro-*m*-cresol (4CmC) is an extensively used activator of ryanodine receptors (RyRs). Studies have shown that 4CmC, at a concentration of 1 mM, is sufficient to cause Ca^{2+} release through RyRs. Here, we show that mM concentrations of 4CmC also inhibit the sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase (SERCA), (IC_{50} 2–3mM) and cause Ca^{2+} release. 4CmC also causes increased intracellular $[\text{Ca}^{2+}]$ levels in COS-7 cells, which lack functional RyRs. Thus, any increase in $[\text{Ca}^{2+}]$ levels associated with use of 4CmC ($\geq 1\text{mM}$) could lead to non-specific Ca^{2+} changes due to SERCA inhibition rather than RyR activation.

Key words: 4-chloro-*m*-cresol, ryanodine receptor, SERCA, Ca^{2+} pumps

Abbreviations: $[\text{Ca}^{2+}]_i$ – intracellular Ca^{2+} concentrations, 4CmC – 4-chloro-*m*-cresol, RyR – ryanodine receptor, SERCA – sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase, SR – sarcoplasmic reticulum

Introduction

4-Chloro-*m*-cresol (4CmC) is a disinfectant and preservative that is a potent activator of ryanodine receptor (RyR) Ca^{2+} channels, inducing Ca^{2+} release [22]. 4CmC is a widely used pharmacological agent for investigating the function of RyR Ca^{2+} channels in tissues, cells and membranes [10, 11, 22]. To date, over 100 publications have utilized 4CmC to study the physiological or molecular function of RyRs. Pharmacological studies have shown that concentrations of

about 1 mM are required to induce maximal Ca^{2+} release in both skeletal muscle sarcoplasmic reticulum (SR) membranes and in pheochromocytoma (PC12) cells [22]. Indeed, 4CmC has proven to be a better activator of RyRs than other commonly used agonists such as caffeine, which requires a concentration in the range of 5–10 mM to induce maximal Ca^{2+} release [18].

More recent studies have shown that the effects of 4CmC on RyRs are isoform specific, with RyR1 and RyR2 being more sensitive to the effects of 4CmC than RyR3 [12] (The EC_{50} for RyR3 is 1.5 mM, compared to 0.2–0.4 mM for RyR1 and RyR2 isoforms [7, 22]). Site-directed mutagenesis studies have also shown that 4CmC affects RyR1 by binding close to the Gln⁴⁰²⁰ and Lys⁴⁰²¹ residues, which are believed to form part of the M2 transmembrane helix that is partially exposed to the surface of the cell [5].

4CmC has also been used as a potential diagnostic tool to identify malignant Hyperthermia-susceptible (MHS) muscles. Studies by Herrmann-Frank et al., [9] have shown that 4CmC increases the affinity for [³H]-ryanodine binding to MHS SR vesicles, compared to normal muscle SR vesicles.

Many papers using 4CmC to investigate the role of RyRs in cells and tissues tend to use it at concentrations between 0.2 to 1 mM, but some studies have employed concentrations of several mM (up to 10 mM) [2, 6–8]. Here, we report that 4CmC, at concentrations at or above 1 mM, can cause significant inhibition of sarcoplasmic endoplasmic reticulum Ca²⁺-ATPase (SERCA) Ca²⁺ pumps. As Ca²⁺ pumps co-localize to membranes containing RyRs, any increases in intracellular [Ca²⁺] levels (as well as Ca²⁺-dependent processes) associated with 4CmC could be either non-specific or exaggerated, due to its effects on SERCA.

Materials and Methods

Chemicals

4-Chloro-*m*-cresol (4-Chloro-3-methylphenol, 4CmC, purity 99%) was purchased from Sigma-Aldrich Co. Ltd., UK.

Membrane purification

Sarcoplasmic reticulum (SR) was prepared from rabbit skeletal muscle as described in Michelangeli and Munkonge [15]. Porcine brain microsomes were prepared as described in [13].

Ca²⁺ uptake and release assays

SR (5 µg) was added to a stirred cuvette containing 2 ml of 40 mM Tris/phosphate, 100 mM KCl at pH 7.2 and 37°C, in the presence of 250 nM Fluo-3 (free acid), 10 µg/ml creatine kinase, and 10 mM phosphocreatine. 4CmC (0 to 3 mM) was then added 5 min prior to the initiation of Ca²⁺ uptake by the addition of 1.5 mM Mg-ATP. Initial rates of Ca²⁺ uptake were typically determined by measuring the [Ca²⁺] change over the first two minutes after ATP addition. Fluorescence changes were monitored in a Perkin-Elmer LS50B spectrofluorimeter, using excitation and emis-

sion wavelengths of 506 nm and 526 nm, respectively. Ca²⁺ concentrations were determined using the following equation:

$$[\text{Ca}^{2+}]_i = K_d \times [(F - F_{\min}) / (F_{\max} - F)]$$

where K_d is the dissociation constant for Ca²⁺ binding to fluo-3 (900 nM at 37°C, pH 7.2, in 40 mM Tris and 100 mM KCl), F is the fluorescence intensity of the sample, and $F_{\min}$ and $F_{\max}$ are the fluorescence intensities in the presence of 1.25 mM EGTA and 2 mM CaCl₂, respectively (Mezna and Michelangeli [13]). Percentage Ca²⁺ release (%) was determined by comparing the amount of Ca²⁺ released by 4CmC with that observed with 12.5 µg/ml A23187.

Ca²⁺-ATPase activities

The effect of 4-chloro-*m*-cresol on SERCA activity in rabbit skeletal muscle SR was investigated at pH 7.2 and 25°C, using a coupled enzyme assay as previously described in [14, 15]. Ten µg/ml of SERCA was added to a buffer containing 40 mM HEPES/KOH (pH 7.2), 1 mM EGTA, 5 mM MgSO₄, 2 mM ATP, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 8.0 units pyruvate kinase, and 20 units lactate dehydrogenase. SR Ca²⁺-ATPase was incubated for 10 min in 2.5 ml of assay buffer. ATPase activity was initiated by the addition of 90 µl of 25 mM CaCl₂, to give a free Ca²⁺ concentration of 6.5 µM (pCa 5.2).

The Ca²⁺-dependent ATPase activity assay in porcine brain microsomes were performed using the phosphate liberation assay [17]. Microsomal membranes (50 µg) were resuspended in 1 ml of buffer containing 45 mM Hepes/KOH (pH 7.2), 6 mM MgCl₂, 2 mM NaN₃, 0.25 M sucrose, 12.5 µg/ml A23187 ionophore, and EGTA with CaCl₂ added to give a free [Ca²⁺] of 1 µM. Assays were pre-incubated at 37°C for 10 min in the presence of a range of concentrations of 4CmC prior to activation with ATP (final conc. 6 mM). The reaction was stopped after 30 min and the amount of inorganic phosphate in the supernatant was then determined as described in [17].

Intracellular [Ca²⁺] measurements in COS-7 cells

COS-7 cells were cultured onto glass coverslips coated with 2% gelatin. Once they reached ≥ 60% confluence they were loaded with Fluo-3AM as described in [17].

The Fluo-3-loaded cells were then placed in Ca^{2+} free Hank's buffer (also containing 1 mM EGTA) and changes in Fluo-3 fluorescence of individual cells (upon exposure to 1 mM 4CmC or 5 mM caffeine) were recorded using a Nikon TS100F epi-fluorescence microscope. Changes in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were presented as the ratio of fluorescence intensities with respect to the initial fluorescence intensity prior to drug addition (F/F_0), as previously described in [17].

Inhibition data were fitted to a non-competitive inhibition equation using the FigP (Biosoft UK) program. Student's *t*-test was used to calculate statistical significance.

Fig. 1. Effects of 4CmC on Ca^{2+} uptake and release in skeletal muscle SR. **(A)** Shows traces of Ca^{2+} uptake initiated by the addition of 1.5 mM Mg-ATP (indicated by the arrow) in SR vesicles measured at pH 7.2 and 37°C. The trace in the absence of 4CmC is shown by the solid line and the trace in the presence of 2 mM 4CmC is shown by the dotted line. These traces are representative of 2 or 3 experiments. **(B)** Shows the dose-dependent inhibition of 4CmC on the initial rates of ATP-induced Ca^{2+} uptake in SR vesicles. Statistical significance (*p*) was determined by Student's *t*-test where * presents a *p* value of ≤ 0.05, and ** a *p* value of ≤ 0.01. (Inset) Shows a representative trace of Ca^{2+} release induced by 2 mM of 4CmC (arrow), when added to SR vesicles preloaded with Ca^{2+} . The degree of Ca^{2+} release is represented as the percent (%) of that released by 12.5 $\mu\text{g}/\text{ml}$ Ca^{2+} ionophore (A23187)

Results

Figure 1A shows typical traces of ATP-dependent Ca^{2+} uptake into rabbit skeletal muscle SR in the absence and presence of 4CmC (2 mM). The membranes were pre-incubated with 4CmC for 5 min prior to the addition of 1.5 mM ATP, to initiate Ca^{2+} uptake. The traces clearly show that the addition of 4CmC reduces the rate of Ca^{2+} uptake compared to the control trace (in the absence of 4CmC). Figure 1B, shows how the initial rate of ATP-dependent Ca^{2+} uptake (measured over the first 2 min after ATP addition) is decreased when SR membranes are pre-incubated

Fig. 2. Inhibition of SERCA Ca^{2+} pump activity by 4CmC. **(A)** Shows the inhibition of skeletal muscle SR Ca^{2+} ATPase (SERCA1) activity by 4CmC when measured at pH 7.2 and 25 °C, using the coupled enzyme assay. Each data point represents the mean ± SD of 3 measurements. **(B)** Shows the inhibition of porcine microsomal Ca^{2+} ATPase (SERCA2B) activity by 4CmC when measured at pH 7.2 and 37 °C. Each data point represents the mean ± SD of between 3 to 5 measurements. Statistical significance (*p*) was determined by Student's *t*-test where * presents a value of ≤ 0.05, and ** a value of ≤ 0.01

with up to 3 mM of 4CmC. The effect of 4CmC is dose-dependent with an IC₅₀ value for Ca²⁺ uptake inhibition of 2.0 ± 0.3 mM. This inhibition was found to be statistically significant compared to controls that were not treated with 4CmC (i.e., probability value $p \leq 0.05$ at 1 mM, and $p \leq 0.01$ at 2 mM). Figure 1 (inset) also shows that 2 mM of 4CmC can also cause Ca²⁺ release from SR vesicles pre-loaded with Ca²⁺, from which approximately 10% of the accumulated Ca²⁺ was released. This degree of Ca²⁺ release from the SR is comparable to the release observed after the application of caffeine and other RyR activators under these experimental conditions [17, 18]. Taken together, the results presented in Figure 1 suggest that 4CmC (at concentrations at or above 1 mM) activates a Ca²⁺ efflux pathway in SR vesicles by opening RyR Ca²⁺ channels, or by inhibiting Ca²⁺ influx pathways in these vesicles, by inhibiting SERCA Ca²⁺ pumps.

Figure 2A shows the effect of 4CmC on skeletal muscle SR Ca²⁺ ATPase activity, which contains SERCA isoform type 1 [21]. The SR vesicles were pre-incubated with 4CmC for 5 min prior to measuring activity. Ca²⁺ ATPase activity was inhibited by 4CmC in a dose-dependent manner over a concentration range up to 5 mM. The data fit well to the equation assuming non-competitive inhibition (goodness-of-fit: χ^2 was 0.98), and the apparent inhibition constant (K_i) was determined to be 2.8 ± 0.3 mM. Statistical analysis showed that there was a statistically significant difference in activities (compared to control) at 4CmC concentrations of 1 mM ($p \leq 0.01$).

In order to assess whether 4CmC inhibition of SERCA Ca²⁺ pumps is isoform-specific, its effects on brain membranes, which are abundant with SERCA isoform 2B [1, 21], were tested. Figure 2B shows the effect of 4CmC on Ca²⁺-dependent ATPase activity from porcine brain microsomes. 4CmC inhibits this Ca²⁺ ATPase with an IC₅₀ of 3.6 ± 0.5 mM. The data again fitted well to a non-competitive inhibition equation (goodness-of-fit χ^2 was 0.98). Statistical analysis also showed that the probability of inhibition by 4CmC was significant at concentrations at or above 1 mM ($p \leq 0.05$).

COS-7 cells have previously been shown to exhibit little [³H]ryanodine binding, lack immuno-reactivity with RyR antibodies, and lack caffeine-induced [Ca²⁺]_i increases [19, 20]. Therefore, these cells were ideal to study the non-RyR effects of 4CmC on intracellular [Ca²⁺]_i levels. Figure 3 shows the effects of 4CmC (1 mM) and caffeine (5 mM) on intracellular

Fig. 3. Effects of 4CmC and caffeine on [Ca²⁺]_i levels in COS-7 cells. The graph shows the trace of relative fluorescence intensities (F/F₀) of fluo-3-loaded COS-7 cells *versus* time, when exposed to either 1 mM 4CmC or 5 mM caffeine (arrow indicates points of addition). Changes in F/F₀ directly relate to changes in [Ca²⁺]_i. Each trace is the mean of 6–8 individual cells within the FOV of the microscope and are typical of 3 independent experiments

[Ca²⁺]_i levels in COS-7 cells. The traces represent the mean relative fluorescence change averaged for 6–8 cells within the field-of-view (FOV) of the microscope, and are typical of the effects observed in 3 replicate experiments. As can be seen, 1 mM of 4CmC induced a robust transient [Ca²⁺]_i increase, while 5 mM caffeine (which we found to have a maximal effect upon the RyR [18]) did not illicit a response. A small decrease in fluorescence due to caffeine quenching of Fluo-3 was observed, and this phenomenon has been reported in other studies [3].

Discussion

This study shows that at or above 1 mM concentrations of 4CmC significant inhibition of SERCA Ca²⁺-ATPases occurs. Furthermore, 4CmC inhibits SERCA 1 better than SERCA 2, which could also explain the findings of Choisy et al. [4], who showed that the potency of 4CmC to cause the contraction of fast-twitch muscles (which contain SERCA 1) is higher than its potency to induce contraction of slow-twitch muscles (with contain SERCA 2).

As SERCA are involved in Ca²⁺ uptake into organelles that also contain RyRs [16], it is clear that when using 4CmC to activate RyRs and induce Ca²⁺ release, the contribution of store leakage through Ca²⁺

pump inhibition must be considered. Since the EC_{50} for activation of RyR1 and RyR2 by 4CmC is 0.2–0.4 mM [7, 22], we recommend that when using 4CmC to study RyR channels in tissues, cells or membranes, concentrations should be kept as low as possible (i.e., ideally less than 0.5 mM), to avoid any non-specific effects caused by Ca^{2+} pump inhibition. Finally, as the EC_{50} for activation of RyR3 by 4CmC is 1.5 mM [12] (similar to the values determined here for Ca^{2+} ATPase inhibition), we recommend that 4CmC be avoided when investigating the properties of this RyR isoform.

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References:

- Babb-Aissa F, Raeymaekers L, Wuytack F, Dode L, Castels R: Distribution and isoforms diversity of the organellar Ca^{2+} pumps in the brain. *Mol Chem Neuropathol*, 1998, 33, 199–208.
- Bina S, Cowan G, Karaian J, Muldoon S, Mongan P: Effects of caffeine, halothane and 4-chloro-m-cresol on skeletal muscle lactate and pyruvate in malignant hyperthermia-susceptible and normal swine as assessed by microdialysis. *Anesthesiology*, 2006, 104, 90–100.
- Brown GR, Sayers LG, Kirk CJ, Michell RH, Michelangeli F: The opening of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel in rat cerebellum is inhibited by caffeine. *Biochem J*, 1992, 282, 309–312.
- Choisy S, Huchet-Cadiou C, Léoty C: Differential effects of 4-chloro-m-cresol and caffeine on skinned fibres from rat fast and slow skeletal muscles. *J. Pharmacol Exp Ther*, 2000, 294, 884–893.
- Fessenden JD, Feng W, Pessah IN, Allen, PD: Amino acid residues Gln⁴⁰²⁰ and Lys⁴⁰²¹ of the ryanodine receptor type 1 are required for activation by 4-chloro-m-cresol. *J Biol Chem*, 2006, 281, 21022–21031.
- Freymond D, Guignet R, Lhote P, Passaquin AC, Rüegg UT: Calcium homeostasis and glucose uptake of murine myotubes exposed to insulin, caffeine and 4-chloro-m-cresol. *Acta Physiol Scand*, 2002, 176, 282–292.
- George CH, Higgs GV, Lai FA: Ryanodine receptor mutations associated with stress-induced ventricular tachycardia mediate increased calcium release in stimulated cardiomyocytes. *Circ Res*, 2003, 93, 531–540.
- Hauser CJ, Kannan KB, Deitch EA, Itagaki K: Non-specific effects of 4-chloro-m-cresol may cause calcium flux and respiratory burst in human neutrophils. *Biochem Biophys Res Commun*, 2005, 336, 1087–1095.
- 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.
- Kabbara AA, Allen DG: Measurement of sarcoplasmic reticulum Ca^{2+} content in intact amphibian skeletal muscle fibres with 4-chloro-m-cresol. *Cell Calcium*, 1999, 25, 227–235.
- Luo D, Sun H, Xiao RP, Han Q: Caffeine induced Ca^{2+} release and capacitative Ca^{2+} entry in human embryonic kidney (HEK293) cells. *Eur J Pharmacol*, 2005, 509, 109–115.
- Matyash M, Matyash V, Nolte C, Sorrentino V, Kettenman H: Requirement of functional ryanodine receptor type 3 for astrocyte migration. *FASEB J*, 2002, 16, 84–86.
- Mezna M, Michelangeli F: The effects of inositol 1,4,5-trisphosphate (InsP₃) analogues on the transient kinetics of Ca^{2+} release from cerebellar microsomes. *J Biol Chem*, 1996, 271, 31818–31823.
- Michelangeli F, Colyer J, Eas, JM, Lee AG: Effect of pH on the activity of the Ca^{2+} -Mg²⁺-activated ATPase of sarcoplasmic reticulum. *Biochem J*, 1990, 267, 423–429.
- Michelangeli F, Munkonge FM: Methods of reconstitution of the purified sarcoplasmic reticulum (Ca^{2+} -Mg²⁺)ATPase using bile salt detergents to form membranes of lipid to protein ratios or sealed vesicles. *Anal Biochem*, 1991, 194, 231–236.
- Michelangeli F, Ogunbayo OA, Wootton LL: A plethora of interacting organellar Ca^{2+} stores. *Curr Opin Cell Biol*, 2005, 17, 135–140.
- Ogunbayo OA, Lai PF, Connolly TJ, Michelangeli F: Terabromobisphenol A (TBBPA) induces cell death in TM4 Sertoli cells by modulating Ca^{2+} transport proteins and causing dysregulation of Ca^{2+} homeostasis. *Toxicol In-Vitro*, 2008, 22, 943–952.
- Tovey SC, Longland CL, Mezna M, Michelangeli F: 2-Hydroxycarbazole induces Ca^{2+} release from sarcoplasmic reticulum by activating the ryanodine receptor. *Eur J Pharmacol*, 2005, 354, 245–251.
- Treves S, Larini F, Menegazzi P, Steinberg TH, Koval M, Vilsen B, Andersen JP, Zorzato F: Alteration of intracellular Ca^{2+} transients in COS-7 cells transfected with the cDNA encoding skeletal-muscle ryanodine receptor carrying a mutation associated with malignant hyperthermia. *Biochem J*, 1994, 381, 661–665.
- Treves S, Pouliquin P, Moccagatta L, Zorzato F: Functional properties of EGFP-tagged skeletal muscle calcium-release channel (ryanodine receptor) expressed in COS-7 cells: sensitivity to caffeine and 4-chloro-m-cresol. *Cell Calcium*, 2002, 31, 1–12.
- Wu KD, Lee WS, Wey J, Bungard D, Lytton J: Localization and quantification of endoplasmic reticulum Ca^{2+} -ATPase isoforms transcripts. *Am J Physiol*, 1995, 269, C775–C784.
- 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.

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