



4-Chloro-m-cresol: A Specific Tool to Distinguish Between Malignant Hyperthermia-Susceptible and Normal Muscle

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ABSTRACT. Single-channel recordings have indicated that ryanodine receptor (RyR1) mutation Arg615Cys of porcine malignant hyperthermia-susceptible (MHS) muscle is not directly associated with the enhanced caffeine sensitivity of MH(S) muscle [1]. In the present study, the effect of a novel activator of RyR1, 4-chloro-m-cresol (4-CmC), was investigated on high-affinity [³H]ryanodine binding to porcine skeletal sarcoplasmic reticulum. The 4-CmC affinity of [³H]ryanodine binding to MHS vesicles was 2-fold higher compared to that in normal tissue. This enhanced affinity was confirmed when the effect of 4-CmC on [³H]ryanodine binding to the isolated CHAPS-solubilized MHS RyR1 was investigated. 4-CmC is, therefore, suggested to be a potent tool to distinguish between Ca²⁺ release from MHS and normal muscle. *BIOCHEM PHARMACOL* 52;1:149–155, 1996.

KEY WORDS. skeletal muscle; sarcoplasmic reticulum; ryanodine receptor; 4-chloro-m-cresol; caffeine; malignant hyperthermia

Malignant hyperthermia (MH) is a pharmacogenetic skeletal muscle disease that is triggered by inhalation anesthetics and depolarizing muscle relaxants, leading to an abnormally high release of Ca²⁺ from the intracellular Ca²⁺ store, SR[†]. A point mutation (Arg615Cys) in the skeletal muscle RyR1 mediating SR Ca²⁺ release has been linked to porcine stress syndrome in pigs, an equivalent to human RyR1 mutations causing MH [2, 3]. Caffeine, which has been shown to cause SR Ca²⁺ release by direct interaction with RyR1 [4], is used in combination with halothane to determine the susceptibility of patients to MH in the so-called *in vitro* contracture test. MHS muscle both from pigs and humans is characterized by its increased sensitivity to caffeine and halothane [5, 6]. This altered caffeine sensitivity, however, was not confirmed when the caffeine activation of the isolated RyR1 channel from MHS porcine muscle was investigated [1]. Thus, it has been suggested that enhanced caffeine sensitivity might, instead, result from an increased resting myoplasmic Ca²⁺ concentration or an altered Ca²⁺ sensitivity of MHS muscle [1]. Recently, chlorocresols have been shown to activate ryanodine receptor-mediated Ca²⁺

release in skeletal muscle and cerebellum [7]. The most potent compound, 4-CmC, has been found to trigger SR Ca²⁺ release in skeletal muscle by directly activating the isolated RyR1 [8]. In the present study, we investigated the effect of 4-CmC on high-affinity [³H]ryanodine binding to isolated skeletal muscle SR vesicles and to the purified RyR1 from MHS and normal pigs. The activating effect of 4-CmC was compared with the action of caffeine.

Part of this work has been published in abstract form [9].

MATERIALS AND METHODS

Materials

4-CmC was purchased from Fluka (Neu-Ulm, Germany). Ryanodine was from Calbiochem (Bad Soden, Germany) and protease inhibitors were from Boehringer (Mannheim, Germany). [9,21-³H(N)]Ryanodine was obtained from Du Pont NEN (Bad Homburg, Germany). All other chemicals were of analytical grade. Filter membranes for [³H]ryanodine binding were purchased from Schleicher & Schüll (S&S, Dassel, Germany).

Preparation of SR Membranes

A microsomal SR fraction was isolated from the Mm. longissimi dorsi of Pietrain pigs homozygous for the gene causing porcine stress syndrome and of normal German landrace swine, following the method of Mickelson *et al.* [10]. Isolated membranes were resuspended in 0.3 M sucrose, 10 mM K-PIPES, pH 6.8, and either rapidly frozen in

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† Abbreviations: 4-CmC, 4-chloro-m-cresol; MHS, malignant hyperthermia-susceptible; RyR1, skeletal muscle ryanodine receptor; SR, sarcoplasmic reticulum.

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liquid nitrogen and stored at -70°C or immediately used for receptor purification. To prevent proteolysis, the following protease inhibitors were included in various purification steps: 200 μM Pefabloc (4-(2-aminoethyl)benzolsulfonyl-fluorid), 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidine. Protein concentration was determined according to the method of Lowry *et al.* [11], using bovine serum albumin as a standard.

Isolation of the Ca^{2+} Release Channel/Ryanodine Receptor Complex

Purification of the Ca^{2+} release channel/ryanodine receptor complex was carried out as previously described [8]. Briefly, isolated SR membranes (3.5 mg/mL) were solubilized for 2 hr at 4°C in the presence of 1.6% CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate). One part of the solubilized sample was labelled with [^3H]ryanodine. Following centrifugation at $59,000 \times g$, the supernatants containing the solubilized receptor were layered on the top of 10–28% sucrose gradients and centrifuged for 16 hr at $136,000 \times g$ (4°C). Gradient fractions corresponding to the peak of the [^3H]ryanodine-bound receptor were collected, rapidly frozen in liquid nitrogen, and stored at -70°C . Protein concentration of the solubilized receptor was determined according to Kaplan and Pedersen [12]. Throughout receptor purification, protease inhibitors as stated above, as well as 1 μM calpain-inhibitor I (N-acetyl-leu-leu-norleucinal) and 1 μM calpain-inhibitor II (N-acetyl-leu-leu-methioninal) were used.

[^3H]Ryanodine Binding

SR vesicles (at a protein concentration of 400 $\mu\text{g}/\text{mL}$) were incubated with indicated concentrations of [^3H]ryanodine in a medium containing 100 mM KCl, 100–500 μM EGTA, 20 mM Na-PIPES, 200 μM Pefabloc, pH 6.8, for 3 hr at 37°C . Varying concentrations of Ca^{2+} , caffeine, and 4-CmC were added to the incubation medium. Unbound ryanodine was separated from protein-bound ryanodine by filtration of protein aliquots (12.5 μg) through S&S GF51 filters presoaked in 1% polyethylenimine (PEI). Filters were washed 3 times with ice-cold buffer solution as described above. Radioactivity remaining with the filters was measured by liquid scintillation counting. Nonspecific binding was determined by a 1000-fold excess of unlabelled ryanodine.

[^3H]ryanodine binding to the isolated ryanodine receptor was performed as described above, with the following modifications: 20–45 $\mu\text{g}/\text{mL}$ solubilized receptor protein were incubated for 4 hr at 22°C in [^3H]ryanodine-binding buffer solution, as stated above, including 200 μM Pefabloc, 1 μM leupeptin, and 1 mM benzamidine. Filters were soaked in 5% PEI.

Miscellaneous Methods

Free concentrations of Ca^{2+} were calculated, using the computer program and binding constants described by

Fabiato [13]. Dose-response curves were fitted, using non-linear curve-fitting routines based on the Marquardt-Levenberg algorithm. Error bars represent SEM.

RESULTS

[^3H]ryanodine binding to isolated SR vesicles from MHS and normal muscle was determined in the presence of maximally activating Ca^{2+} concentrations as described by Shomer *et al.* [14]. Scatchard analysis revealed a single class of high-affinity binding sites for both tissues (Fig. 1). The

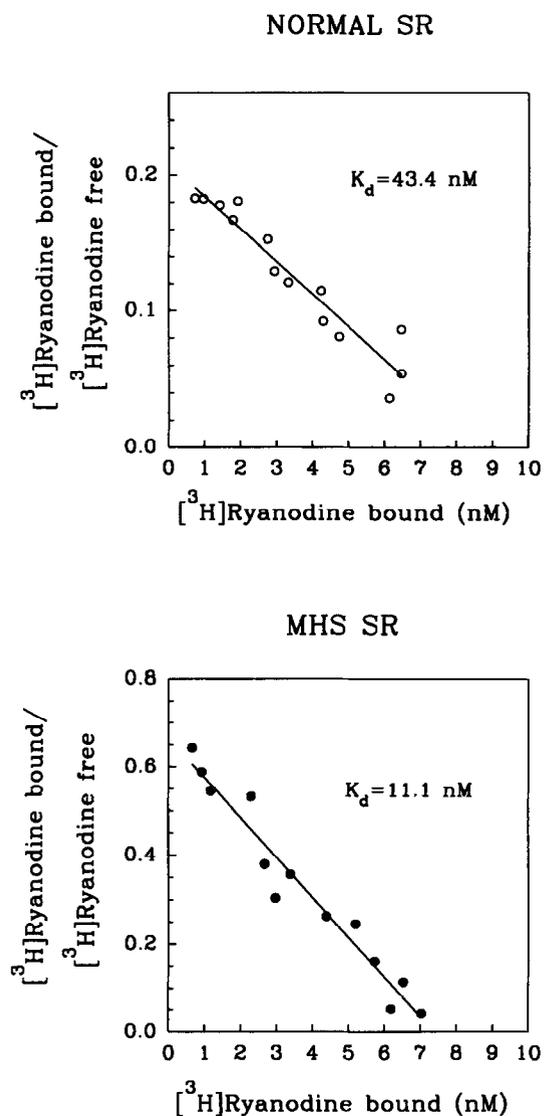


FIG. 1. Scatchard plots of [^3H]ryanodine binding to MHS and normal SR vesicles. Representative Scatchard plots at a Ca^{2+} concentrations of 10 μM Ca^{2+} . Unspecific binding was determined in the presence of 10 μM nonlabeled ryanodine. Resulting K_d values were 43.4 nM for normal SR and 11.1 nM for MHS SR. Corresponding B_{max} values were calculated to be 8.7 pmol/mg protein of normal SR and 7.4 pmol/mg protein of MHS vesicles.

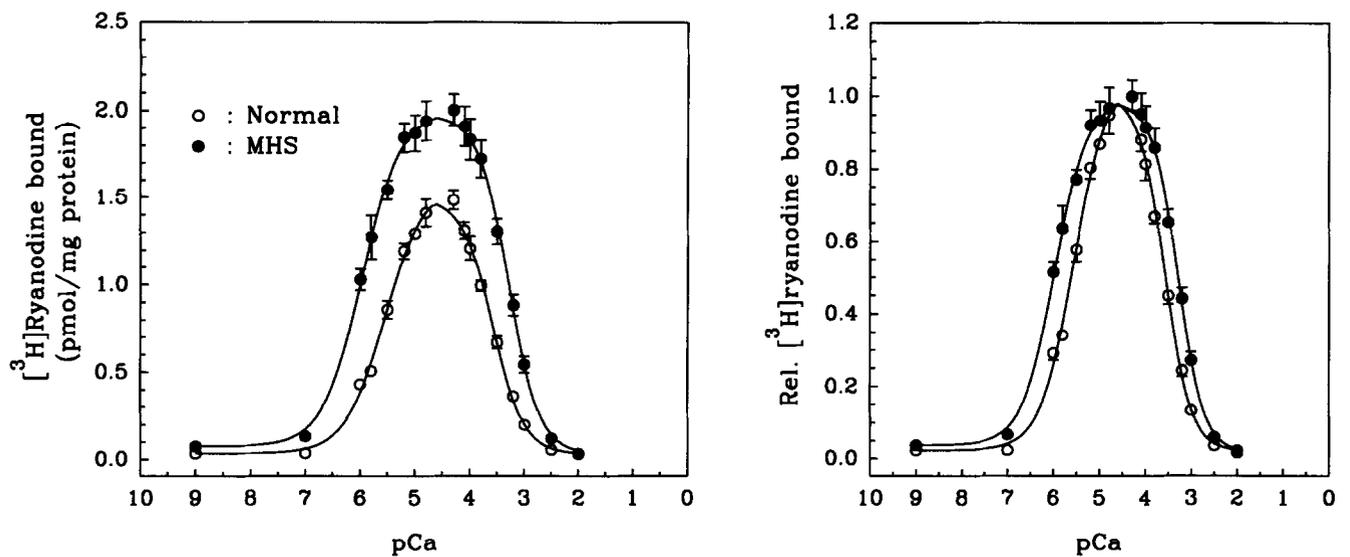


FIG. 2. Ca^{2+} dependence of high-affinity $[^3\text{H}]$ ryanodine binding. Left: Ca^{2+} dependence of $[^3\text{H}]$ ryanodine binding to normal and MHS SR vesicles in the presence of 12 nM $[^3\text{H}]$ ryanodine and indicated concentrations of free Ca^{2+} . Error bars represent standard error ($n = 6$). Right: Corresponding data points from the left panel were normalized to maximal binding values at 10 μM Ca^{2+} .

K_d for MHS vesicles (13.5 ± 2.2 nM, $n = 6$) was approximately 3.5-fold lower than in normal vesicles (47.9 ± 4.5 nM, $n = 8$), and the B_{max} values were not significantly different (MHS: 7.5 ± 1.2 pmol/mg protein ($n = 6$) vs normal: 8.7 ± 1.3 pmol/mg protein ($n = 8$)). To compare the effects of different modulators on $[^3\text{H}]$ ryanodine binding in MHS and normal muscle, the following experiments were carried out at a $[^3\text{H}]$ ryanodine concentration slightly below the K_d of MHS vesicles, 12 nM.

Figure 2 shows the biphasic dependence of high-affinity $[^3\text{H}]$ ryanodine binding on cytoplasmic Ca^{2+} concentration. Ca^{2+} activated $[^3\text{H}]$ ryanodine binding more potently in MHS vesicles. However, maximal activation was found at 10 μM Ca^{2+} for both tissues. To compare the kinetics of Ca^{2+} -activated $[^3\text{H}]$ ryanodine binding, both data sets were

normalized to maximal binding (Fig. 2, right). The threshold of activation was virtually the same for both tissues at 0.1 μM . Differences in binding were observed both for activating and inactivating Ca^{2+} concentrations. The largest differences were found at Ca^{2+} concentrations between 1 ($\text{pCa} = 6$) and 3 μM ($\text{pCa} = 5.5$) and between 159 ($\text{pCa} = 3.8$) and 631 μM ($\text{pCa} = 3.2$) (Table 1).

4-CmC activates $[^3\text{H}]$ ryanodine binding to rabbit SR vesicles in a concentration- and Ca^{2+} -dependent manner [8]. In the experiments presented here, we investigated the activating effect of 4-CmC in the presence of a subthreshold Ca^{2+} concentration (0.1 μM), and compared the activation by 4-CmC with that of caffeine. Caffeine activated $[^3\text{H}]$ ryanodine binding in millimolar concentrations (Fig. 3, top). For maximal activation, concentrations higher than

TABLE 1. $[^3\text{H}]$ ryanodine binding to normal and MHS SR vesicles in the presence of different Ca^{2+} concentrations

pCa	Normal SR (pmol $[^3\text{H}]$ ryanodine bound per mg protein)	MHS SR (pmol $[^3\text{H}]$ ryanodine bound per mg protein)	Normal SR (bound/ B_{max})	MHS SR (bound/ B_{max})
7.0	0.04 ± 0.01	0.14 ± 0.02	0.03 ± 0.002	0.07 ± 0.010
6.0	0.43 ± 0.03	1.03 ± 0.06	0.33 ± 0.002	0.55 ± 0.030
5.8	0.51 ± 0.02	1.28 ± 0.13	0.40 ± 0.012	0.68 ± 0.062
5.5	0.86 ± 0.05	1.55 ± 0.05	0.67 ± 0.033	0.82 ± 0.027
5.2	1.20 ± 0.04	1.85 ± 0.08	0.93 ± 0.030	0.98 ± 0.030
5.0	1.29 ± 0.02	1.88 ± 0.10	1.00 ± 0.010	1.00 ± 0.051
4.0	1.21 ± 0.07	1.84 ± 0.12	0.94 ± 0.045	0.98 ± 0.059
3.8	0.99 ± 0.03	1.72 ± 0.11	0.77 ± 0.020	0.91 ± 0.054
3.5	0.67 ± 0.04	1.31 ± 0.07	0.52 ± 0.023	0.70 ± 0.036
3.2	0.36 ± 0.03	0.88 ± 0.06	0.28 ± 0.016	0.47 ± 0.030
3.0	0.20 ± 0.02	0.54 ± 0.05	0.16 ± 0.012	0.29 ± 0.023

$[^3\text{H}]$ ryanodine binding was performed in low salt (0.1 M KCl), pH 6.8, at 12 nM $[^3\text{H}]$ ryanodine as described in Materials and Methods. Values represent means \pm SE ($n = 6$) of data points shown in Fig. 2.

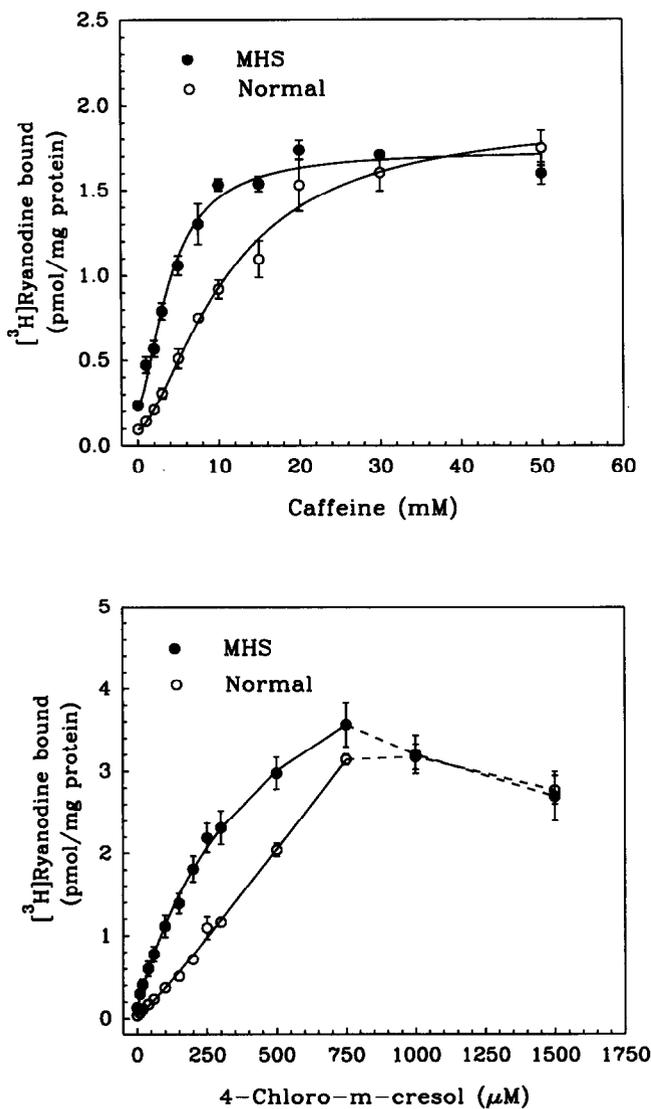


FIG. 3. Activation of [^3H]ryanodine binding by caffeine and 4-chloro-m-cresol (4-CmC). Top: The activating effect of caffeine on [^3H]ryanodine binding to normal (O) and MHS (●) SR vesicles was investigated in the presence of $0.1 \mu\text{M}$ Ca^{2+} and 12 nM [^3H]ryanodine. Data were fitted according to the Hill equation. Resulting EC_{50} values were 10.7 mM ($n = 6$) for normal and 3.4 mM ($n = 6$) for MHS SR. Bottom: The activating effect of 4-CmC was examined under the same conditions as described above. For calculation of the half-maximally activating concentrations, only data points connected by solid lines were taken. Normal SR: $\text{EC}_{50} = 395 \mu\text{M}$ ($n = 6$), MHS SR: $193 \mu\text{M}$ ($n = 6$).

20 mM had to be applied for both tissues. Although caffeine stimulated [^3H]ryanodine binding to both vesicle types to the same extent, affinity to caffeine was 3-fold higher for MHS vesicles. [^3H]ryanodine binding increased half-maximally at 10.7 mM caffeine for normal and at 3.4 mM for MHS vesicles. For comparison, Fig. 3 (bottom) shows the stimulating effect of 4-CmC; it was found to be active in approximately 15 times lower concentrations compared to caffeine. In addition, the extent of maximal stimulation was 2-fold higher. Maximal binding was achieved at 750

μM 4-CmC in both vesicle types, and higher concentrations inhibited binding. The latter effect may be due to a direct interaction of 4-CmC with the lipid membrane. The half-maximally activating concentrations were calculated to be $395 \mu\text{M}$ for normal and $193 \mu\text{M}$ for MHS vesicles.

To investigate whether or not the observed higher affinity of 4-CmC to MHS vesicles is preserved on the receptor level, we tested the effect of 4-CmC on [^3H]ryanodine binding to the isolated RyR1. Binding of ryanodine to the mutant MHS receptor was slightly higher in the absence of caffeine or 4-CmC (Fig. 4). Both caffeine and 4-CmC potentiated ryanodine binding to a 2-fold greater extent in the MHS receptor compared to the normal one. Maximal binding was achieved between 700 and 800 pmol/mg MHS receptor protein in the presence of both compounds (Fig. 4 A, C). To compare the sensitivity of the normal and mutant receptor to caffeine and 4-CmC, data were normalized to maximal binding (Fig. 4 B, D). Contrary to previous electrophysiological data [1], the isolated MHS receptor displayed an enhanced affinity to caffeine ($\text{EC}_{50} = 4.9 \text{ mM}$ ($n = 6$) for the MHS receptor and 7.2 mM ($n = 6$) for the normal receptor). However, maximal activation was only achieved in the presence of high concentrations (i.e., greater than 20 mM). Also, the enhanced 4-CmC affinity of MHS SR vesicles was preserved on the isolated receptor level. Resulting half-maximal activating concentrations were very similar compared to the values obtained for stimulation of ryanodine binding to SR vesicles, and were more than 15-fold lower than those of caffeine ($\text{EC}_{50} = 141 \mu\text{M}$ ($n = 6$), MHS receptor vs $520 \mu\text{M}$ ($n = 6$), normal receptor).

DISCUSSION

The high-molecular-weight ryanodine receptor/ Ca^{2+} release channel complex forms an elaborate tetrameric structure in the sarcoplasmic reticulum. Pharmacological binding studies with the plant alkaloid [^3H]ryanodine revealed one high-affinity binding site per tetramer (reviewed in [15]), likely located at the carboxy terminus of the protein [16]. Ryanodine has also been used as a probe for the functional state of the sarcoplasmic reticulum Ca^{2+} release channel. Ligands that have been shown to activate or inhibit the Ca^{2+} release channel modulate [^3H]ryanodine binding in a similar way [17–19]. In the present study, we utilized high-affinity [^3H]ryanodine binding to isolated SR vesicles and the purified ryanodine receptor to determine the sensitivity of skeletal muscle of pigs, both susceptible and nonsusceptible to malignant hyperthermia, to a novel activator of the SR Ca^{2+} release channel, 4-CmC. 4-CmC activates the purified RyR1 reconstituted into planar lipid bilayers without affecting the conductance of the channel. Studies investigating [^3H]ryanodine binding to rabbit skeletal SR vesicles revealed that 4-CmC enhances the affinity of [^3H]ryanodine binding without changing maximal binding [8].

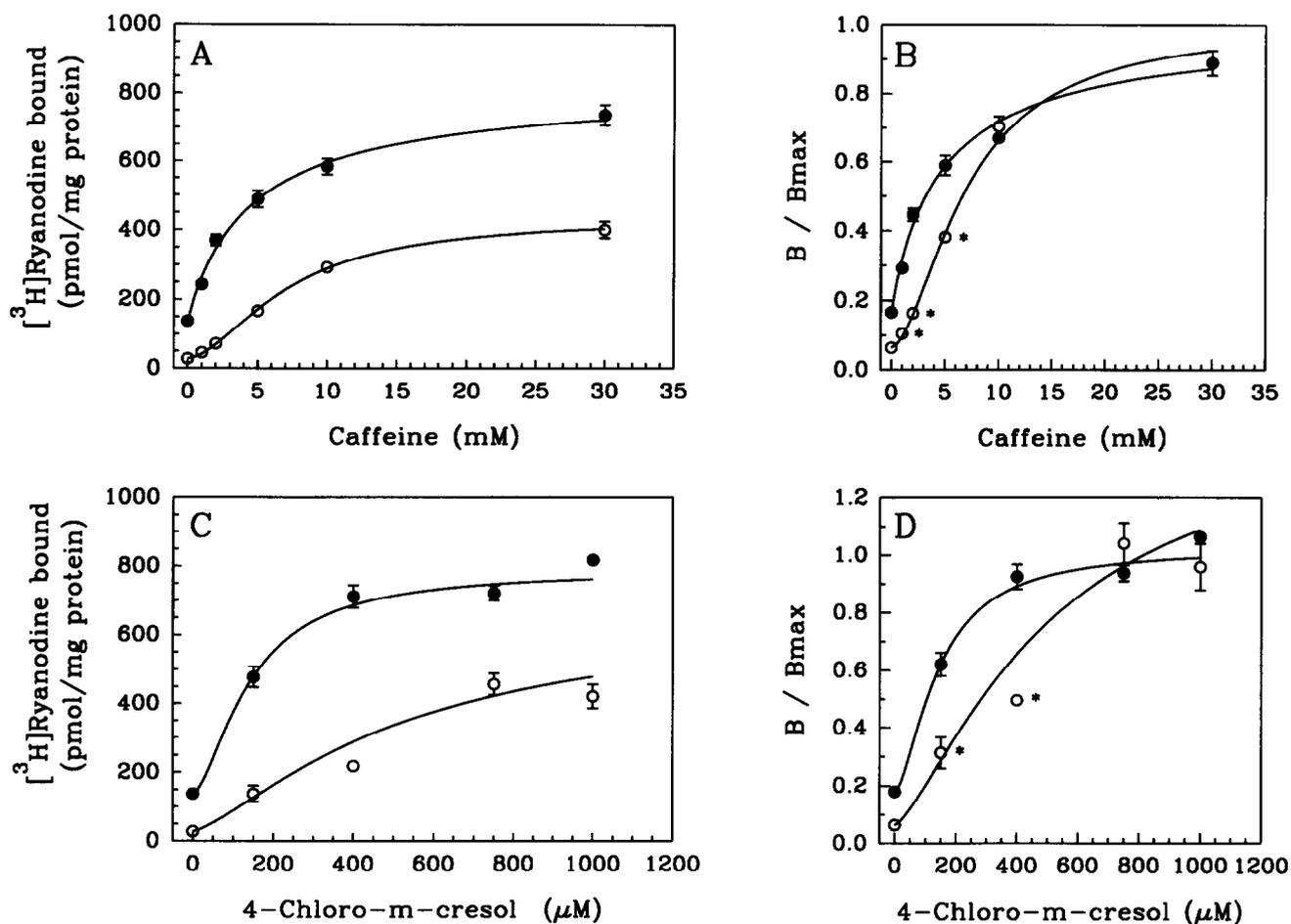


FIG. 4. Activation of $[^3\text{H}]$ ryanodine binding to the isolated ryanodine receptor from normal and MHS SR by caffeine and 4-chloro-m-cresol. A, C: Binding of $[^3\text{H}]$ ryanodine to the purified ryanodine receptors of normal (○) and MHS (●) muscle was performed as described in Materials and Methods at 40 nM Ca^{2+} and 12 nM $[^3\text{H}]$ ryanodine. Data points represent means \pm SE of 6 different experiments. If no error bars are shown, they are encompassed within symbol. B, D: Data points of panels A and C were normalized to maximal binding in the presence of caffeine and 4-CmC, respectively. Normalized graphs were fitted according to the Hill equation. EC_{50} values were for the activating effect of caffeine (A, B) 7.2 mM for the normal vs 4.9 mM for the MHS receptor. For activation by 4-CmC (C, D), half-maximally activating concentrations were 520 μM for the normal and 141 μM for the MHS ryanodine receptor. Data points labeled by * are significantly different at $P < 0.05$ (Student's *t*-test).

$[^3\text{H}]$ ryanodine binding studies were performed in the presence of low salt (0.1 M KCl) at a pH of 6.8. These conditions have been described as optimal for visualizing differences in $[^3\text{H}]$ ryanodine binding to MHS and normal SR vesicles from pig skeletal muscle [14]. MHS vesicles exerted a 3.5-fold higher affinity than normal ones, and maximal binding was not significantly different in these tissues (Fig. 1). Ca^{2+} typically activated binding in concentrations up to 10 μM , and higher concentrations were inhibitory (Fig. 2). Data comparing the Ca^{2+} affinity of MHS and normal vesicles are controversial in the literature. Single-channel recordings revealed that the MHS Ca^{2+} release channel inactivates in the presence of increased Ca^{2+} concentrations, and the Ca^{2+} -dependent activation is not modified [14, 20]. $[^3\text{H}]$ ryanodine binding experiments, however, additionally indicated a higher affinity for the Ca^{2+} activation site in both pig and human MHS muscle [21, 22]. Our own experiments confirm the latter data. Ma-

lor differences were found both for activating and inhibitory Ca^{2+} concentrations (Table 1).

Contracture tests with human or pig skeletal muscle strips have shown that MHS muscle is more sensitive to caffeine [5, 6]. This enhanced sensitivity has also been recorded when SR Ca^{2+} release in various functional assays, using isolated SR vesicles, was investigated [9, 22, 23]. Caffeine-activated $[^3\text{H}]$ ryanodine binding also displayed a higher affinity in SR vesicles isolated from MHS muscle (Fig. 3). However, maximal activation is not fully achieved at concentrations higher than 20 mM. In this concentration range, caffeine has been shown to exert various side effects in intact muscle (e.g., in concentrations higher than 5 mM, caffeine activates steady-state force by increasing the Ca^{2+} sensitivity of the myofilaments) [24]. For comparison, 4-CmC activated $[^3\text{H}]$ ryanodine binding in approximately 15-fold lower concentrations than caffeine, with a significantly higher affinity to MHS vesicles (Fig. 3). In addition,

maximal binding was 2-fold higher in both MHS and normal SR. The absolute differences between corresponding data points of MHS and normal vesicles were also 2-fold higher compared to caffeine-activated [³H]ryanodine binding. These higher differences might facilitate the visualization of disparities between normal and MHS tissue. The above data confirm our previous results concerning the activating effect of 4-CmC on the contracture development of muscle fiber bundles obtained from biopsies of patients susceptible or nonsusceptible to MH [8]. 4-CmC induced caffeine-like contractures in a concentration range between 25 and 300 μM. Sensitivity was calculated to be 3-fold higher for MHS muscle strips [8].

In contrast to caffeine-activated Ca²⁺ release in SR vesicles, the isolated porcine MHS muscle ryanodine receptor, reconstituted into planar lipid bilayers and assessed for single channel recordings, did not display an enhanced affinity for caffeine [1]. Examination of the effects of caffeine and 4-CmC on high-affinity [³H]ryanodine binding to the purified solubilized ryanodine receptor, however, revealed that the MHS receptor exerted a higher affinity for both compounds (Fig. 4). Resulting EC₅₀ values confirmed the data from binding studies to SR vesicles. Although we observed a 2-fold higher potentiation of 4-CmC-activated [³H]ryanodine binding to SR vesicles, maximal binding values were approximately the same both for caffeine- and 4-CmC-induced [³H]ryanodine binding to the isolated receptor. This result indicates the higher specificity of 4-CmC compared to caffeine. Caffeine might additionally bind to other proteins than the ryanodine receptor in native SR vesicles. The enhanced caffeine sensitivity of [³H]ryanodine binding to the isolated MHS receptor contrasts with the data obtained from single-channel recordings [1] but is in agreement with other electrophysiological studies demonstrating a higher sensitivity of SR Ca²⁺ release towards caffeine in cells expressing the mutant RyR1 [25, 26]. Single-channel experiments with the purified ryanodine receptor are currently in progress to compare the affinity of the normal and mutant RyR1 to both caffeine and 4-CmC on the electrophysiological level.

In summary, it has been demonstrated that ryanodine receptor mutation Arg615Cys is causative for the enhanced caffeine and 4-CmC sensitivity of MHS skeletal muscle. 4-CmC has been found to activate [³H]ryanodine binding more specifically and with a several-fold higher affinity than caffeine, suggesting that it is a more potent tool for visualizing differences in SR Ca²⁺ release between MHS and normal muscle.

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