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Structural Determinants of 4-Chloro-*m*-cresol Required for Activation of Ryanodine Receptor Type 1

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ABSTRACT

4-Chloro-*m*-cresol (4-C*m*C) is a clinically relevant activator of the intracellular Ca^{2+} release channel, the ryanodine receptor isoform 1 (RyR1). In this study, the chemical moieties on the 4-C*m*C molecule required for its activation of RyR1 were determined using structure-activity relationship analysis with a set of commercially available 4-C*m*C analogs. Separate compounds each lacking one of the three functional groups of 4-C*m*C (1-hydroxyl, 3-methyl, or 4-chloro) were poor activators of RyR1. Substitution of different chemical groups for the 1-hydroxyl of 4-C*m*C resulted in compounds that were poor activators of RyR1, suggesting that the hydroxyl group is preferred at this position. Substitution of hydrophobic groups at the 3-position enhanced bioactivity of the compound relative to 4-C*m*C, whereas substitution with hydrophilic groups abolished bioactivity. Likewise, 4-C*m*C analogs with hydrophobic groups substituted into the 4-position enhanced bioactivity, whereas hydrophilic or charged groups diminished bioactivity. 4-C*m*C analogs containing a single hydrophobic group at either the 3- or 4-position as well as 3,5-disubstituted or 3,4,5-trisubstituted phenols were also effective activators of RyR1. These results indicate that the 1-hydroxyl group of 4-C*m*C is required for activation of RyR1 and that hydrophobic groups at the 3,4- and 5-positions are preferred. These findings suggest that the 4-C*m*C binding site on RyR1 most likely consists of a hydrophilic region to interact with the 1-hydroxyl as well as a hydrophobic region(s) to interact with chemical groups at the 3- and/or 4-positions of 4-C*m*C.

Muscle contraction is triggered by Ca^{2+} released from intracellular stores via activation of the ryanodine receptor (RyR), a large homotetrameric cation channel located in the sarcoplasmic reticulum (SR) membrane. RyR activity can be modulated by a diverse array of pharmacological substances that include methylxanthines such as caffeine [an RyR activator (+)], small halogenated hydrocarbons [halothane, enflurane; (+)], aminoglycosides [neomycin; an RyR inhibitor; (−)], plant alkaloids [ryanodine; (±)], polycationic dyes [ruthenium red; (−)], polyamines [poly-L-lysine; (±)], and phenols [4-chloro-*m*-cresol; (+)] (Pessah et al., 1985, 1987; Palade, 1987; Zorzato et al., 1993; el-Hayek et al., 1995; Zucchi and Ronca-Testoni, 1997). The structural diversity of these modulators most likely reflects a range of distinct binding sites for these compounds on the surface of the RyR, although most of these sites have not been identified within the primary sequence of the protein.

One exception, however, is 4-chloro-*m*-cresol (4-C*m*C), a potent activator of both skeletal (RyR1) and cardiac (RyR2) ryanodine receptor isoforms (Zorzato et al., 1993; Choisy et al., 1999). 4-C*m*C, a structural analog of phenol, activates RyR1 with an EC_{50} value of ~50 to 200 μM (Zorzato et al., 1993; Herrmann-Frank et al., 1996b). Sensitivity to activation by 4-C*m*C is increased for RyR1-containing mutations linked to the skeletal muscle disorder malignant hyperthermia, a finding that has led to the clinical use of 4-C*m*C in malignant hyperthermia diagnosis (Herrmann-Frank et al., 1996a; Baur et al., 2000). However, 4-C*m*C is a poor activator of the "brain" RyR isoform RyR3 (Fessenden et al., 2000), because millimolar levels of 4-C*m*C are required to activate this isoform (Matyash et al., 2002). Using RyR1 to RyR3 chimeric proteins, the 4-C*m*C activation site on RyR1 has been localized to residues 4007 to 4180 of the RyR1 primary sequence (Fessenden et al., 2003). In addition, two amino acids within this region ($Gln^{4020}Lys^{4021}$) are critical for 4-C*m*C activation of RyR1 because mutation of these residues to their RyR3 counterparts ($Leu^{3873}Gln^{3874}$) selectively abolishes channel activation by 4-C*m*C (Fessenden et al., 2006).

To obtain additional insights into the structure of the 4-C*m*C binding site on RyR1, a more detailed understanding

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ABBREVIATIONS: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; 4-C*m*C, 4-chloro-*m*-cresol.

of the structural requirements of the 4-C*m*C molecule required for bioactivity is needed. 4-C*m*C is comprised of a single benzene ring substituted with a 1-hydroxyl, 3-methyl, and 4-chloro group. Substitution of an ethyl group at the 3-position enhances RyR1 activation relative to 4-C*m*C (Zorzato et al., 1993; Larini et al., 1995). In addition, 4-C*m*C analogs lacking either the 4-chloro or 3-methyl groups have reduced potency of activation of RyR1 compared with 4-C*m*C (Zorzato et al., 1993). However, little else is known as to the functional significance of these chemical groups and how they contribute to the bioactivity of the 4-C*m*C molecule.

In this study, structural analogs of 4-C*m*C were tested for their ability to activate RyR1 both in vitro using [^{3}H]ryanodine binding analysis and in vivo using intracellular Ca^{2+} release assays in a transgenic muscle cell line. Our results indicate that the 1-hydroxyl group is absolutely required for activation of RyR1 and that a large hydrophobic group at the 3- and/or 4-position significantly improves the ability of the compound to activate RyR1.

Materials and Methods

Materials. The structures and calculated chemical information for all 4-C*m*C analogs used in this study are indicated in Table 1. The EC_{50} values were determined using experiments described under *Results*. The pK_a and log D values were estimated using the ACD/I-Lab Web service (http://www.acdlabs.com/ilab/). Log D (the ratio of the concentration of a compound in octanol to its concentration in water at pH 7.0) is an index of hydrophobicity.

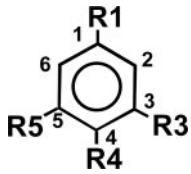
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except compounds **3a**, **6a**, **6c**, and **7c**, which were purchased from Avocado Organics (Heysham, UK). Compound **6b** was synthesized by heating 200 ml of a 100 mM solution of compound **6c** in 100% ethanol with a drop of concentrated sulfuric acid at 100°C for 5 h. The crude ester from the reaction was purified using silica gel column chromatography by elution with hexane/ethyl acetate (3:1). The chromatographically pure material was then analyzed and its chemical structure confirmed by both ^{1}H NMR spectroscopy (400 MHz, $CDCl_3$) and mass spectrometry (fast atom bombardment, positive mode).

SR Membrane Preparation. Junctional sarcoplasmic reticulum membrane preparations used for [^{3}H]ryanodine binding studies were prepared as described previously (Perez et al., 2003). The level of total protein in the preparation was determined using the Bio-Rad protein assay based on the method of Bradford (1976) (Bio-Rad, Hercules, CA). Ten micrograms of purified junctional SR membranes were used per [^{3}H]ryanodine binding assay.

[^{3}H]Ryanodine Binding Assays. Specific binding of [^{3}H]ryanodine (specific activity, 56 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) to SR preparations was used to monitor RyR1 activity as described previously (Pessah et al., 1987). Assays consisted of 5 nM [^{3}H]ryanodine, 10 μg of rabbit skeletal muscle SR vesicles, 250 mM KCl, 20 mM HEPES, pH 7.4 (adjusted with concentrated KOH), and 1 μM $CaCl_2$ (adjusted using 100 μM EGTA) in a final volume of 0.5 ml. Reaction mixtures were allowed to equilibrate with shaking at 37°C for 2 to 3 h followed by rapid filtration through GF/B glass fiber filters (Whatman, Maidstone, UK). Filters were then quickly rinsed three times with 2 ml of wash buffer consisting of 20 mM HEPES, pH 7.4. The amount of [^{3}H]ryanodine bound to each filter was determined by liquid scintillation counting. Specific binding of [^{3}H]ryanodine was determined via subtraction of nonspecific binding measured in the presence of 5 μM unlabeled ryanodine. Nonspecific binding was not altered by increasing [4-C*m*C].

The potency of each compound toward RyR1 activation was deter-

TABLE 1
4-C*m*C analogs used in this study



Compound	R1	R3	R4	R5	EC_{50}	pK_a	Log D
					μM		
1a	OH	CH_3	Cl		129	9.63	2.89
1b	OH	CH_3				10.07	1.94
1c	OH		Cl			9.47	2.40
1d		CH_3	Cl			N.A.	3.27
2a	NH_2	CH_3	Cl			4.08	2.22
2b	Cl	CH_3	Cl			N.A.	3.80
2c	OCH_3	CH_3	Cl			N.A.	3.28
3a	OH	CF_3	Cl		33.1	8.52	4.00
3b	OH	CH_2CH_3	Cl		85.1	9.62	3.42
3c	OH	Cl	Cl		66.1	8.55	3.20
3d	OH	OH	Cl			10.22	1.60
4a	OH	CH_3	Br		110	9.51	2.95
4b	OH	CH_3	$CH(CH_3)_2$		44.7	10.36	3.30
4c	OH	CH_3	CH_3			10.38	2.40
4d	OH	CH_3	NO_2			7.39	N.D.
4e	OH	CH_3	NH_2			10.34	0.17
4f	OH	CH_3	OH			11.96	1.10
5a	OH	CF_3			323.6	8.96	2.90
5b	OH	CF_3		CF_3	30.9	7.96	4.70
6a	OH		$CH(CH_3)_2$		64.6	10.19	2.80
6b	OH		$(CH_2)_4CO_2CH_2CH_3$		75.9	10.14	3.10
6c	OH		$(CH_2)_4CO_2H$			10.12	−0.30
7a	OH	CH_3	Cl	CH_3	63.1	9.76	3.30
7b	OH	CH_3	Br	CH_3	42.7	9.64	3.40
7c	OH	CH_3	I	CH_3	33.9	9.56	3.80

N.A., not applicable; N.D., not determined.

mined via nonlinear regression analysis of sigmoidal concentration dependence curves plotted from individual determinations using Prism, version 4.0, analysis software for Macintosh (GraphPad Software Inc., San Diego, CA). The log EC_{50} values are presented as the mean ± S.D. for the number of individual measurements indicated for each compound. B_{max} represents the level of [^{3}H]ryanodine binding at the highest concentration of compound tested. Differences in B_{max} were assessed using two-way analysis of variance with a Dunnett's post-test. A significant difference was inferred for $p < 0.05$.

Cell Culture. 1B5 myotubes were cultured and differentiated as described previously (Moore et al., 1998; Fessenden et al., 2000). In brief, 1B5 myoblasts propagated in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (v/v) (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin-G, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine at 37°C in 10% CO_2 were seeded onto a 96-well plate and cultured until they attained 50% confluence. Differentiation was initiated by changing the medium to Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated horse serum (v/v) and L-glutamine/antibiotics as described above at 37°C in 18% CO_2. After 7 days, differentiated myotubes were transduced with RyR constructs using equivalent amounts of herpes simplex virus virions (Wang et al., 2000) that contained the cDNAs encoding either rabbit RyR1 or RyR3. After a 2-h incubation at 37°C in antibiotic-free medium, the virus was removed and the myotubes were incubated for 2 days to allow full expression of the RyRs before testing.

Calcium Imaging. Changes in intracellular Ca^{2+} in 1B5 myotubes were measured using the Ca^{2+} indicator dye Fluo-4. 1B5 myotubes were loaded in the presence of 5 μM Fluo-4/acetoxymethyl ester (Invitrogen) for 30 min at 37°C in imaging solution (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 6 mM glucose, 2 mM $CaCl_2$, and 25 mM HEPES, adjusted to pH 7.4 with NaOH). After extensive washing with imaging buffer, changes in intracellular Ca^{2+} were monitored using a 40× objective lens (UPlanApo/340; Olympus; Melville, NY) and a Nikon Diaphot 300 microscope (Nikon). Myotubes were illuminated using a DeltaRam lightsource (Photon Technology International, Monmouth Junction, NJ) set at 495 nm. Emitted fluorescence from the 1B5 myotubes was measured with an XR/Mega-12 intensified charge-coupled device camera (Stanford Photonics Inc., Palo Alto, CA), and resultant images were monitored using QED imaging software (QED Software, Pittsburgh, PA) on a Macintosh G4 computer. Images were acquired at the rate of 12 images per second, and changes in fluorescence of individual myotubes were monitored using regions of interest.

Myotubes transduced with RyR-encoding viruses were challenged with successive 30-s additions of 40 mM caffeine and 0.5 mM 4-C*m*C followed by 30 and 100 μM solutions of the 4-C*m*C analog being tested. All substances were dissolved in imaging solution before use. A 30-s wash was applied between each addition of agonist. These substances were delivered to the myotubes using a Valve Bank 8/II pressure-driven perfusion system (Automate Scientific, Inc., Foster City, CA). Results are presented as a single data trace from a representative RyR-expressing 1B5 myotube.

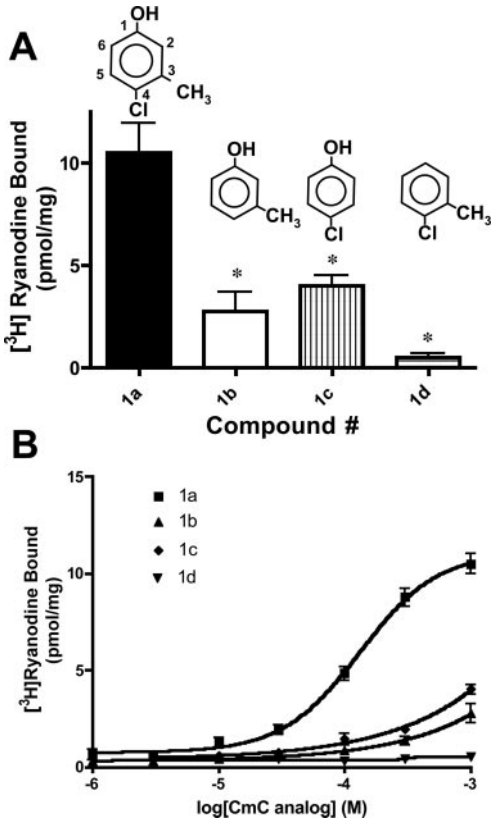


Fig. 1. A, equivalent concentrations (1 mM) of 4-C*m*C, *m*-cresol, 4-chlorophenol, or 2-chlorotoluene were tested for their ability to activate RyR1 in [^{3}H]ryanodine binding measurements as described under *Materials and Methods*. Values represent mean ± S.D. for $n = 8$ (4-C*m*C) or $n = 4$ measurements (all others). Asterisks indicate a significant difference ($p < 0.05$) compared with 4-C*m*C. B, concentration dependence of enhancement of [^{3}H]ryanodine binding was determined for each compound. Values represent mean ± S.D. for the number of determinations indicated in A.

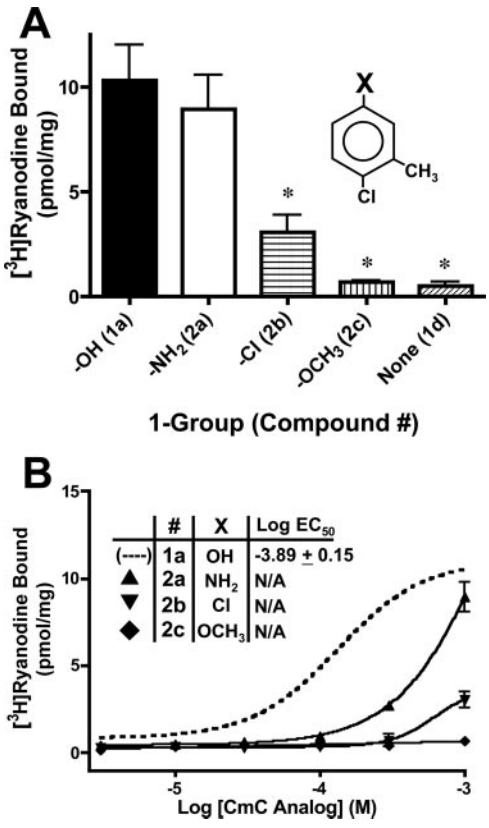


Fig. 2. A, equivalent concentrations (1 mM) of 4-C*m*C as well as analogs containing a 1-amino (**2a**), 1-chloro (**2b**), 1-methoxy (**2c**), or no group (**1d**) in place of the 1-hydroxyl of 4-C*m*C were tested for their ability to activate RyR1 in [^{3}H]ryanodine binding measurements as described under *Materials and Methods*. Values represent mean ± S.D. for $n = 8$ (4-C*m*C) or $n = 4$ measurements (all others). Asterisks indicate a significant difference ($p < 0.05$) compared with 4-C*m*C. B, concentration dependence of enhancement of [^{3}H]ryanodine binding was determined for each compound. Values represent mean ± S.D. for the number of determinations indicated in A. Log EC_{50} values were determined as described under *Materials and Methods*.

Results

4-C*m*C analogs lacking the 1-hydroxyl (compound **1d**; Table 1), 3-methyl (**1c**), or 4-chloro group (**1b**) were tested for their ability to activate RyR1 in [^{3}H]ryanodine binding assays (Fig. 1). The amount of [^{3}H]ryanodine bound after addition of 1 mM *m*-cresol (**1b**), 4-chlorophenol (**1c**), and 2-chlorotoluene (**1d**) was 2.75, 4.01, and 0.53 pmol/mg protein, respectively, compared with 10.3 pmol/mg for 4-C*m*C (**1a**) (Fig. 1A). The concentration dependence of activation by either *m*-cresol or 4-chlorophenol indicated that these compounds were significantly weaker activators of RyR1 ($EC_{50} \gg 1$ mM) than 4-C*m*C, whereas 2-chlorotoluene was completely inactive (Fig. 1B).

These results indicate that all three chemical groups on the 4-C*m*C molecule contribute to its ability to activate RyR1. Next, we performed a systematic assessment of the functional significance of the chemical groups at each position of 4-C*m*C (1-, 3-, and 4-positions).

1-Position Modifications. Compounds containing chloro (compound **2b**) or methoxy (**2c**) groups in place of the 1-hydroxyl group of 4-C*m*C were poor activators of RyR1 compared with 4-C*m*C (Fig. 2A). The exchange of an amino group for the 1-hydroxyl group (**2a**) decreased the potency of the resulting compound compared with 4-C*m*C, whereas 4-C*m*C analogs containing either a chloro or methoxy group at the 1-position were completely inactive at concentrations up to 1 mM (Fig. 2B).

3-Position Modifications. Equivalent concentrations (500 μM) of compounds containing either a trifluoromethyl (compound **3a**), ethyl (**3b**), or chloro (**3c**) group in place of the 3-methyl group of 4-C*m*C stimulated [^{3}H]ryanodine binding to 112, 126, and 75% of levels of activation by 4-C*m*C, respectively, whereas an analog with a hydroxyl group at the 3-position (**3d**) was less active than 4-chlorophenol (**1c**), which

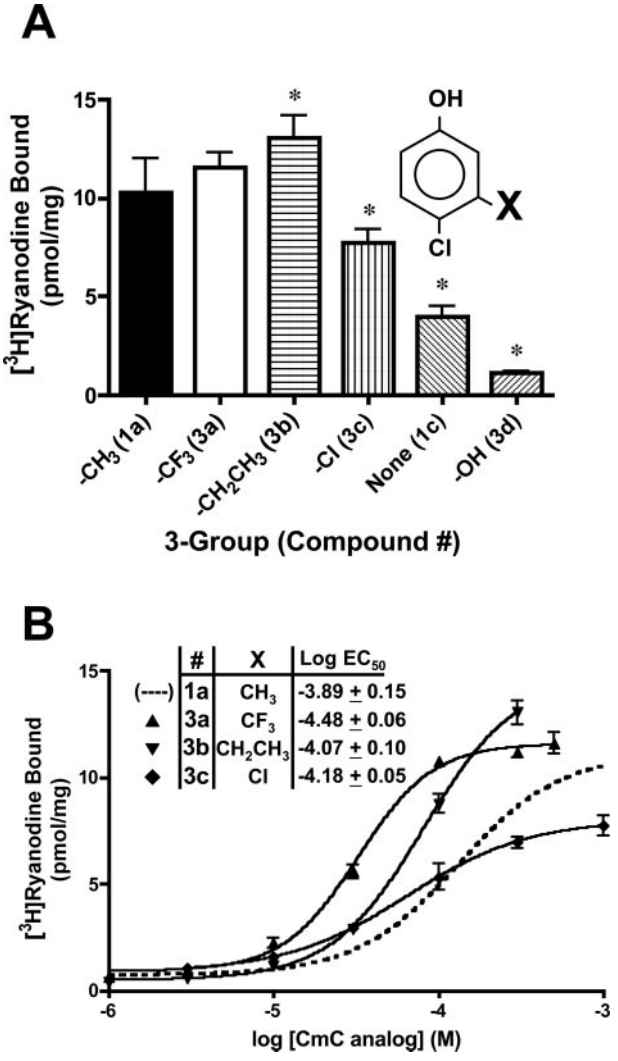


Fig. 3. A, equivalent concentrations (1 mM) of 4-C*m*C as well as analogs containing a 3-trifluoromethyl (**3a**), 3-ethyl (**3b**), 3-chloro (**3c**), no group (**1c**), or 3-hydroxyl group (**3d**) in place of the 3-methyl group of 4-C*m*C were tested for their ability to activate RyR1 in [^{3}H]ryanodine binding measurements as described under *Materials and Methods*. Values represent mean ± S.D. for $n = 8$ (4-C*m*C) or $n = 4$ measurements (all others). Asterisks indicate a significant difference ($p < 0.05$) compared with 4-C*m*C. B, concentration dependence of enhancement of [^{3}H]ryanodine binding was determined for each compound. Values represent mean ± S.D. for the number of determinations indicated in A. Log EC_{50} values were determined as described under *Materials and Methods*.

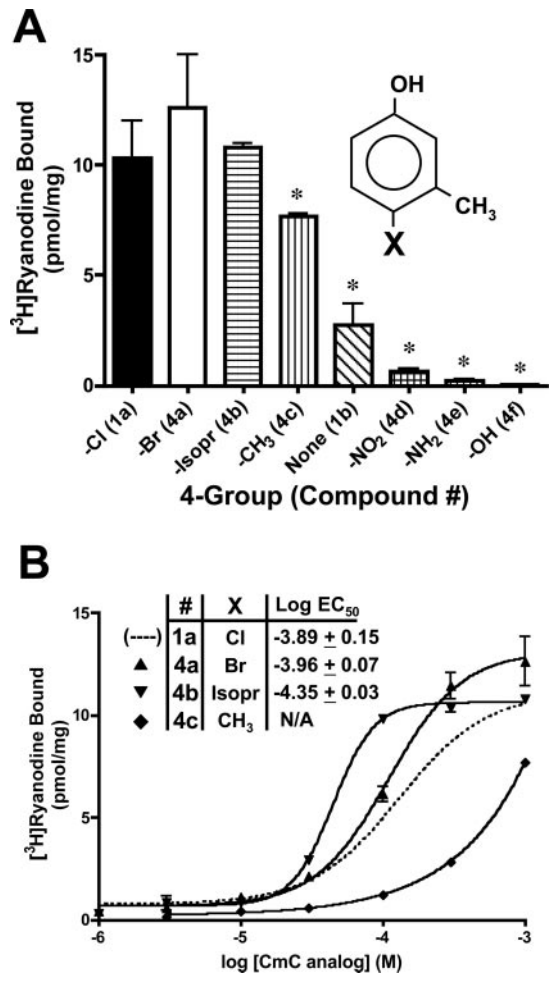


Fig. 4. A, equivalent concentrations (1 mM) of 4-C*m*C as well as analogs containing a 4-bromo (**4a**), 4-isopropyl (**4b**), 4-methyl (**4c**), no group (**1b**), 4-nitro (**4d**), 4-amino (**4e**), or 4-hydroxyl group (**4f**) in place of the 4-chloro group of 4-C*m*C were tested for their ability to activate RyR1 in [^{3}H]ryanodine binding measurements as described under *Materials and Methods*. Values represent mean ± S.D. for $n = 8$ (4-C*m*C) or $n = 4$ measurements (all others). Asterisks indicate a significant difference ($p < 0.05$) compared with 4-C*m*C. B, concentration dependence of enhancement of [^{3}H]ryanodine binding was determined for each compound. Values represent mean ± S.D. for the number of determinations indicated in A. Log EC_{50} values were determined as described under *Materials and Methods*.

lacks a chemical group at the 3-position (Fig. 3A). Of these compounds, **3a** was the most potent activator of RyR1 (EC_{50} = 33 μM compared with 129 μM for 4-C*m*C), although compounds **3b** and **3c** were also more potent activators than 4-C*m*C (EC_{50} = 85 and 66 μM, respectively; Fig. 3B).

4-Postion Modifications. Compounds containing hydrophobic [bromo (compound **4a**), isopropyl (**4b**), or methyl (**4c**)], hydrophilic [nitro (**4d**), hydroxyl (**4f**)], or charged [amino (**4e**)] groups in place of the 4-chloro group of 4-C*m*C were tested at equivalent concentrations (1 mM) in [^{3}H]ryano-

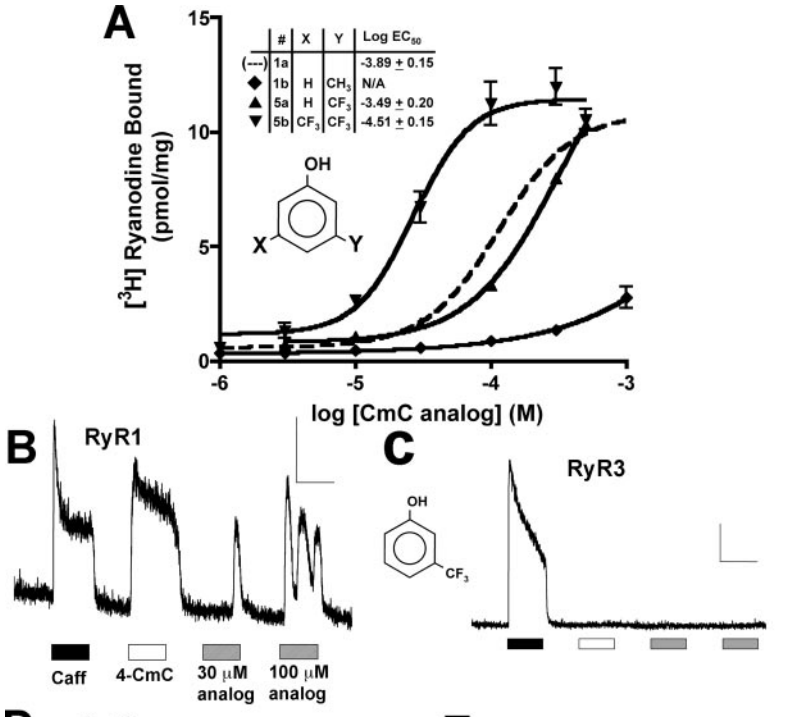


Fig. 5. A, concentration dependence of RyR1 activation by 4-C*m*C, compound **1b**, **5a**, and **5b** is indicated. The concentration dependence of enhancement of [^{3}H]ryanodine binding was determined for each compound as described under *Materials and Methods*. Values represent mean ± S.D. for n = 8 (4-C*m*C) or n = 4 measurements (all others). Log EC_{50} values were determined as described under *Materials and Methods*. B–E, changes in Fluo-4 fluorescence for a myotube expressing RyR1 (B and D) or RyR3 (C and E) challenged with 40 mM caffeine (black bar), 0.5 mM 4-C*m*C (white bar) as well as 30 and 100 μM **5a** (B and C) or **5b** (D and E) (gray bar) are indicated. Calibration bar, 0.2 F/F_o arbitrary units versus 30 s.

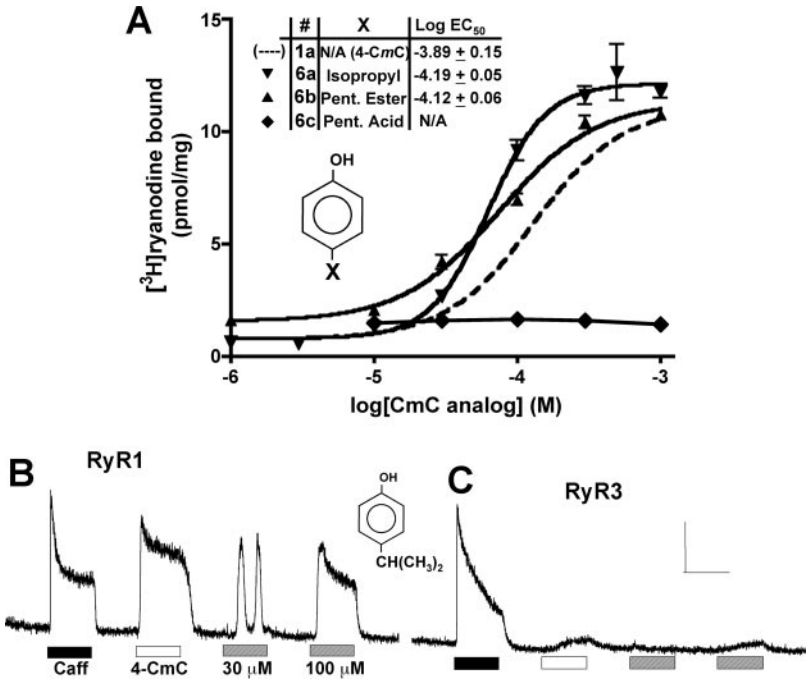


Fig. 6. A, concentration dependence of RyR1 activation by 4-C*m*C, **6a**, **6b**, and **6c** is indicated. The concentration dependence of enhancement of [^{3}H]ryanodine binding was determined for each compound as described under *Materials and Methods*. Values represent mean ± S.D. for n = 8 (4-C*m*C) or n = 4 measurements (all others). Log EC_{50} values were determined as described under *Materials and Methods*. Changes in Fluo-4 fluorescence for a myotube expressing RyR1 (B) or RyR3 (C) challenged with 40 mM caffeine (black bar), 0.5 mM 4-C*m*C (white bar) as well as 30 and 100 μM **6a** (C and D) (gray bar) are indicated. Calibration bar, 0.2 F/F_o arbitrary units versus 30 s.

dine binding assays. Compounds with hydrophobic groups at the 4-position activated RyR1 to the highest extent, whereas compounds with hydrophilic or charged groups at the 4-position were essentially inactive (Fig. 4A). Compounds with hydrophobic 4-groups (**4a**, **4b**, and **4c**) were further tested for their concentration dependence of RyR1 activation. Of these, compound **4a** (EC_{50} = 110 μM) and **4b** (EC_{50} = 44.7 μM) had the highest potency for RyR1 activation (Fig. 4B).

Structural Diversity and Isoform Specificity of 4-C*m*C Analogs. 4-C*m*C analogs that differ in the number of chemical groups attached to the benzene ring were also tested (Fig. 5). In [^{3}H]ryanodine binding assays, the 3-substituted phenol 3-(trifluoromethyl)phenol compound (**5a**), a structural analog of *m*-cresol (**1b**), was a potent activator of RyR1 with an EC_{50} = 323 μM. The presence of a second trifluoromethyl group at the 5-position ([3,5-bis(trifluoromethyl)phenol], **5b**) enhanced potency by an order of magnitude (EC_{50} = 31 μM; Fig. 5A) relative to **5a**.

To determine whether these compounds are selective for the 4-C*m*C binding site on RyR1, we measured their activation of RyR1 versus RyR3 expressed in cultured 1B5 myotubes using intracellular Ca^{2+} imaging. 4-C*m*C preferentially activates RyR1 compared with RyR3 (Fessenden et al., 2000), so 4-C*m*C analogs that display a similar isoform preference would be expected to interact at the same site as 4-C*m*C. 3-(Trifluoromethyl)phenol (**5a)** activated RyR1 with a threshold for activation of 30 μM (Fig. 5B), whereas this compound was inactive toward RyR3 at concentrations up to 100 μM (Fig. 5C). Likewise, the 3,5-disubstituted phenol (**5b)** also preferentially activated RyR1 (Fig. 5D) compared with RyR3 (Fig. 5E). The isoform specificity of these compounds suggests that they activate RyR1 via a similar mechanism as 4-C*m*C.

4-Alkyl phenols are strong activators of RyR1 whose potency is proportional to the length of the 4-alkyl chain (Beeler and Gable, 1993). We tested several 4-alkyl phenols to determine whether they could activate RyR1 and whether this activation was selective for the 4-C*m*C binding site. In [^{3}H]ryanodine binding assays, phenols containing isopropyl (compound **6a**) or pentanoic ethyl ester (**6b**) groups at the 4-position were potent activators of RyR1 (EC_{50} = 64.6 and 75.9 μM, respectively) compared with 4-C*m*C, whereas phenol with a 4-pentanoic acid group (**6c**) was inactive (Fig. 6A). Compound **6a** preferentially activated RyR1 (Fig. 6B) compared with RyR3 (Fig. 6C), indicating selectivity for the 4-C*m*C binding site on RyR1.

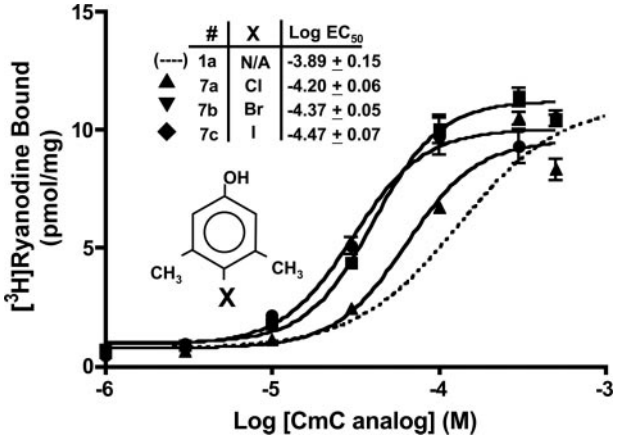


Fig. 7. Concentration dependence of RyR1 activation by 4-C*m*C, **7a**, **7b**, and **7c** was determined using [^{3}H]ryanodine binding measurements as described under *Materials and Methods*. Values represent mean ± S.D. for n = 8 (4-C*m*C) or n = 4 measurements (all others). Log EC_{50} values were determined as described under *Materials and Methods*.

Finally, tri-substituted phenols containing chemical groups at the 3,4,5-positions were tested in [^{3}H]ryanodine binding assays (Fig. 7). Addition of a second methyl group to the 5-position of 4-C*m*C (resulting in compound **7a**) increased the EC_{50} for activation of RyR1 to 63.1 μM (compared with 129 μM for 4-C*m*C). Likewise, 3,5-dimethylphenols containing a bromine (**7b**) or iodine (**7c**) atom at the 4-position also activated RyR1 (EC_{50} = 42.7 and 33.9 μM for bromine and iodine, respectively).

Discussion

4-Chloro-*m*-cresol is a potent activator of RyR1 and RyR2 relative to RyR3 (Fessenden et al., 2000). The structural determinants on RyR1 required for 4-C*m*C activation have been localized to a 173-amino acid region between amino acid residues 4007 to 4180 (Fessenden et al., 2003). To gain further insight into the 4-C*m*C binding site on RyR1, we have used a complementary approach to define the structural components on the 4-C*m*C molecule required for activation of the channel. Our work indicates that all three chemical groups on the 4-C*m*C molecule contribute to its ability to activate RyR1. In addition, we have described several distinct classes of phenols that also seem to activate RyR1 via the 4-C*m*C binding site based on the fact that they selectively activate RyR1 but not RyR3: 1) phenols with a single hydrophobic group at either the 3- or 4-positions; 2) phenols with two hydrophobic groups at the 3,4- or 3,5-positions; and 3) phenols with three hydrophobic groups at the 3,4,5-positions. These findings suggest that the 4-C*m*C binding pocket on RyR1 comprises both hydrophilic and hydrophobic elements that interact with the 1-hydroxyl group and hydrophobic groups at the 3-, 4-, and 5-positions, respectively.

Importance of the 1-Hydroxyl Group. Within the series of compounds we tested, those with a hydroxyl group at the 1-position activate RyR1 with highest potency. Replacement of the hydroxyl with a chlorine atom or removal of the hydroxyl group altogether, results in compounds with no measurable bioactivity toward RyR1. In addition, replacement of the phenolic proton with a methyl group (**2c**) also abolishes the ability of the compound to activate RyR1. This finding suggests that the phenolic proton is required for 4-C*m*C bioactivity.

When the 1-hydroxyl is replaced with an amino group, the resulting compound (**2a**) is significantly less active. This finding suggests that the recognition site for the OH group is not simply a hydrogen bonding group but rather a general base that requires a specific pK_a for the phenolic proton. The calculated pK_a values for the most active phenols tested in this study all fall within a range between 7.96 (**5b**) and 10.36 (**4b**), consistent with a lysine or possibly a histidine residue playing the role of the general base. For compound **2a**, however, the calculated pK_a is 4.08, suggesting that at pH 7, the amino group retains its protons and thereby cannot effectively interact with the putative lysine or histidine, resulting in weaker activation of RyR1.

Importance of the 3- and 4-Chemical Groups. 4-C*m*C analogs with more highly hydrophobic groups substituted for

the 3-methyl and 4-chloro groups are more effective activators of RyR1 compared with 4-C*m*C. For example, replacement of the 3-methyl group with a trifluoromethyl group (**3a**) enhances potency 4-fold. This chemical modification also increases the overall hydrophobicity of the compound. The partition coefficient of 4-C*m*C at pH 7.0 (log D) is 2.89 (Table 1), whereas the log D value for compound **3a** is 4.00. Likewise, substitution at the 3-position with chemical groups that enhance bioactivity such as an ethyl (**3b**) or chloro (**3c**) group also increase the overall hydrophobicity of the compound (log D values for these chemicals are 3.42 and 3.20, respectively). This trend is also evident at the 4-position whereby bioactivity-enhancing substitutions of a bromo (**4a**) or isopropyl (**4b**) group for the 4-chlorine atom also increase log D to 2.95 and 3.30, respectively.

Conversely, hydrophilic substitutions at the 3- and 4-positions decrease bioactivity. Replacement of the 4-chloro group with a nitro (**4d**), amino (**4e**), or hydroxyl (**4f**) group result in compounds that are inactive toward RyR1. Compounds **4e** and **4f** have log D values of 0.17 and 1.10 that are well below the corresponding value for 4-C*m*C (2.89). Likewise, replacement of the 3-methyl group with a hydroxyl group (compound **3d**) abolishes bioactivity and also lowers the log D value to 1.60.

The most convincing example of the importance of hydrophobicity is the comparison between phenol containing a 4-pentanoic acid group (**6c**) and its ethyl-esterified counterpart (**6b**). Addition of the ethyl ester increases the log D value from −0.30 to 3.10 while converting a compound with no detectable activation toward RyR1 to a compound with 2-fold higher potency compared with 4-C*m*C.

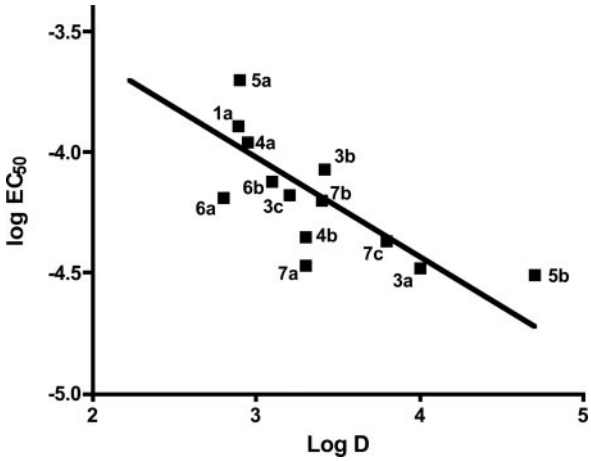


Fig. 8. Dependence of log EC_{50} on log D is indicated for all 4-C*m*C analogs with measurable EC_{50} values. Each point is labeled with the corresponding compound identifier number from Table 1. The data are fitted using linear regression analysis with a correlation coefficient of 0.69.

For the most potent 4-C*m*C analogs tested in this study, there is a linear dependence of the measured potency of each compound (as indicated by log EC_{50}) on its overall index of hydrophobicity (log D; Fig. 8). This relationship underscores the relationship between the hydrophobicity of the compound and its bioactivity. Thus, the 4-C*m*C binding site most likely contains a hydrophobic "pocket" to interact with groups on the 3,4- and/or 5-positions of 4-C*m*C and its analogs.

Changes in Efficacy of [^{3}H]Ryanodine Binding. 4-C*m*C enhances Ca^{2+} activation of [^{3}H]ryanodine binding via increasing both the apparent affinity of Ca^{2+} activation (increased potency) and the amount of [^{3}H]ryanodine bound at optimal [Ca^{2+}] (increased efficacy) (Herrmann-Frank et al., 1996b). Of the 4-C*m*C analogs we tested that exhibited enhanced potency relative to 4-C*m*C, only compounds with 3-substitutions of either an ethyl (**3b**) or chloro (**3c**) group had increased or decreased efficacies relative to 4-C*m*C, respectively. In contrast, compounds containing 4-substitutions that enhanced potency (**4a** and **4b**) had efficacies that were unchanged compared with 4-C*m*C. The significance of these observations is unclear, although it is tempting to speculate that chemical groups at the 3-position control the degree of efficacy of 4-C*m*C analogs. However, validation of this hypothesis will require further testing.

Noncanonical 4-C*m*C Analogs. In this study, we have identified a diverse set of phenols that are effective activators of RyR1. These compounds can be grouped into five structural classes: 3-monosubstituted (**5a**), 4-monosubstituted (**6a** and **6b**), 3,4-disubstituted (**1a**, **3a**, **3b**, **3c**, **4a**, and **4b**), 3,5-disubstituted (**5b**), and 3,4,5-trisubstituted phenols (**7a**, **7b**, and **7c**). Thus, the 4-C*m*C binding site can accommodate a variety of phenolic structures. This finding also suggests that long-chain 4-alkyl phenols, which have previously been shown to activate RyR1 (Beeler and Gable, 1993) most likely act on the 4-C*m*C binding site.

Hypothetical Model of 4-C*m*C Binding Site. Our results suggest that the 4-C*m*C binding site on RyR1 contains two critical components: 1) hydrophilic side chains or amino acids to stabilize the 1-hydroxyl on 4-C*m*C via hydrogen bonding and/or electrostatic interactions and 2) a hydrophobic surface to interact with hydrophobic groups on the 3,4,- and/or 5-positions on the benzene ring. It is difficult to predict the location of the hydrophilic residues interacting with the hydroxyl group given

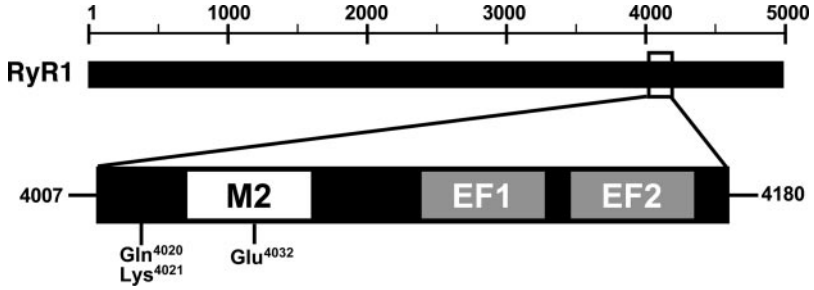


Fig. 9. 4-C*m*C sensitivity region previously identified using chimeric RyRs spans amino acids 4007 to 4180 (box) of the rabbit *wt*RyR1 primary structure (black bar). Within this segment are several proposed modulatory regions including a single conserved amino acid (Glu^{4032}) implicated in Ca^{2+} activation of the channel, two EF hands implicated in Ca^{2+} binding (EF1, 4081–4092; EF2, 4116–4127) and a hydrophobic region (M2, 4025–4041) originally suggested as a transmembrane sequence. In proximity to these regions is the $Gln^{4020}Lys^{4021}$ dipeptide segment that is required for 4-C*m*C activation of RyR1.

the ubiquitous nature of these types of residues in proteins. However, the narrow range of pK_a values of the most potent 4-C*m*C analogs suggests the involvement of a general base such as a lysine or histidine. This hypothesis is supported by recent work in our laboratory indicating that a mutation of a dipeptide segment of RyR1 ($Gln^{4020}Lys^{4021}$) selectively abolishes channel activation by 4-C*m*C (Fessenden et al., 2006). Thus, it is tempting to speculate that these residues may be involved in stabilization of the 1-hydroxyl of 4-C*m*C, although this hypothesis requires further testing.

Putative hydrophobic binding sites that may interact with chemical groups at the 3,4-positions of 4-C*m*C are more readily identified. Within the 173 amino acid region of RyR1 required for 4-C*m*C activation (Fessenden et al., 2003), the most hydrophobic region lies between amino acids 4025 to 4041 (Fig. 9). This segment is directly adjacent to the $Gln^{4020}Lys^{4021}$ dipeptide that is critical for activation of RyR1 by 4-C*m*C (Fessenden et al., 2006). In addition, this segment contains the putative "Ca^{2+} sensor" at residue Glu^{4032} (Chen et al., 1998) and also is in proximity to EF hand motifs implicated in Ca^{2+} regulation of RyR1 activity (Xiong et al., 1998, 2006; Fessenden et al., 2004) (Fig. 9). Thus, this hydrophobic segment is an attractive region to consider as a portion of the 4-C*m*C binding site, especially because 4-C*m*C activates RyR1 via modulation of the Ca^{2+} sensitivity of the channel (Herrmann-Frank et al., 1996b).

An alternate possibility is that the 4-C*m*C binding site on RyR1 lies near the lipid/protein interface and that the hydrophobic portions of the 4-C*m*C molecule anchor the compound to the sarcoplasmic reticulum membrane. This concept is supported by the fact that long-chain alkyl phenols are potent activators of RyR1 and increasing the length of the alkyl chain increases the potency of the compound (Beeler and Gable, 1993). The hydrophobic segment between amino acids 4025 to 4041 remains a good candidate for a 4-C*m*C binding site, because it was originally predicted as transmembrane helix (M2) of RyR1 (Zorzato et al., 1990). In this hypothetical case, the hydrophilic segments required to interact with the hydroxyl group on 4-C*m*C would be expected to be in proximity to this helix. However, it should be noted that recent experiments suggest that this segment does not constitute a transmembrane helix, but instead is cytosolic (Du et al., 2002). If this is the case, this region could still be involved in binding a lipid solvated 4-C*m*C molecule if it is in proximity to the surface of the lipid bilayer.

Conclusions

Our work suggests that the structural features of phenolic compounds required to activate RyR1 include an ionizable proton (pK_a = 8–10.5) at the 1-position and hydrophobic groups at the 3,4- and/or 5-positions of the benzene ring. In addition, phenols with highly hydrophobic groups are more potent activators of RyR1 compared with 4-C*m*C (the most potent compound we tested is compound **5b** (with an EC_{50} = 31 μM). Finally, the results with compound **6b** are encouraging in that this compound offers a chemical "handle" whereby a suitable reporter group can potentially be linked to identify the region(s) within the protein where the 4-C*m*C analogs bind.

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