Amino Acid Residues Gln\textsuperscript{4020} and Lys\textsuperscript{4021} of the Ryanodine Receptor Type 1 Are Required for Activation by 4-Chloro-\textit{m}-cresol\textsuperscript{*}

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The Ryanodine Receptor Type 1 (RyR1) and Type 2 (RyR2) Are Required for Activation

Striated muscle contraction is triggered by Ca\textsuperscript{2+} release from intracellular stores through the Ryanodine receptor (RyR), large homotetrameric channel protein embedded in the membrane of the sarcoplasmic reticulum (SR). In skeletal muscle, RyR1 is functionally associated with the \(\alpha\)1 subunit of the dihydropyridine receptor, a voltage-gated Ca\textsuperscript{2+} channel that activates RyR1 in response to muscle cell depolarization in a process known as excitation-contraction (EC) coupling. Distinct RyR isoforms expressed in either skeletal (RyR1) or cardiac muscles (RyR2) mediate EC coupling in these muscle types (1, 2). The third RyR isoform (RyR3) is expressed at low levels in many tissues but has not been shown to participate in EC coupling in skeletal or cardiac muscle (3, 4).

Many pharmacological compounds activate RyR1 including the methylxanthine derivative, caffeine (5), and the phenol-based compound, 4-chloro-\textit{m}-cresol (6). Both of these compounds are thought to directly activate RyR1 by increasing the affinity of Ca\textsuperscript{2+} binding to its activator site but these compounds differ in that 4-CmC is 25 times more potent in activating RyR1 compared with caffeine. In addition, these two compounds have distinct RyR isoform-dependent activation profiles: caffeine is much more potent in activating RyR2 and RyR3 compared with RyR1 (3, 7), whereas 4-CmC is a much more effective activator of RyR1 and RyR2 compared with RyR3 (3, 8).

This isoform-specific sensitivity to activation by caffeine and 4-CmC has made it possible to localize the activation sites of these compounds using chimeric RyR proteins. Substitution of amino acids 4187–4628 of RyR1 with the corresponding sequence from RyR2 increases the caffeine sensitivity of the resulting chimera compared with wtRyR1 (7, 9), whereas substitution of amino acids 4007–4180 of RyR1 into RyR3 enhances 4-CmC sensitivity in RyR3 (10), thus suggesting that determinants for caffeine or 4-CmC activation of the channel reside in these respective regions. In addition, performing the reverse substitution by replacing amino acids 4007–4180 in RyR1 with the equivalent RyR3 sequence greatly diminishes the 4-CmC sensitivity of the resulting chimeric protein (10), further strengthening the hypothesis that amino acid determinants required for full activation of RyR1 by 4-CmC reside within this 173-amino acid segment.

The amino acid sequence within the 4-CmC sensitivity region is highly conserved among all three RyR isoforms with over 66% identity and 87% similarity. This observation suggests that the isoform-specific differences in 4-CmC sensitivity are mediated by only a few amino acids that are divergent between the isoforms. To identify these amino acids, we performed site-
directed mutagenesis on 11 divergent amino acids within the 173 amino acid RyR1 4-CmC sensitivity region and tested the resulting constructs for 4-CmC sensitivity. Our results demonstrate that the differential sensitivity to 4-CmC between RyR1 and RyR3 can be attributed to 2 amino acid residues in RyR1, Gln4020 and Lys4021 and their RyR3 counterparts Leu3873 and Gln3874. These findings further suggest that these residues mediate 4-CmC activation of RyR1.

EXPERIMENTAL PROCEDURES

Construction of Mutated RyR1 and RyR3 cDNAs—cDNAs encoding the rabbit isoforms of RyR1, RyR2, and RyR3 have previously been cloned into pHSVprPUC thus enabling the generation of HSV amplicons required for transduction of 1B5 myotubes (11). Site-directed mutagenesis was performed using the QuikChange multi-mutagenesis Kit (Stratagene Inc., La Jolla, CA) on a RyR1 subclone created via the introduction of RyR1 sequence extending from the natural XhoI-HincII sites, located at positions 12,018 and 12,666, respectively, into the pGEM 3zf cloning vector (Promega Inc; Madison, WI). A silent mutation at position 12543 was added resulting in a unique AvrII restriction site that aided in the construction of full-length RyR1 cDNAs containing the mutations. Primers for mutagenesis, along with their reverse complements (not shown), were synthesized with the following sequence (nucleotides resulting in point mutations are indicated in italics): Mut 1 (Q4020L), 5’-GGAGCTGCTGGACCTGCA
GAGACATGGTGG-3’; Mut2 (K4021Q), 5’-GGAGCTGCTG
GACCTGCA
GAGACATGGTGG-3’; Mut1,2 (Q4020L and K4021Q), 5’-GGAGCTGCTGGACCTGCA
GAGACATGGTGG-3’; Mut3 (M4047T), 5’-GGCAGATGGTGGACCA
CGAGACATGGTGGAGTCCTCG-3’; Mut4 (S4099G), 5’-CCAGAAGGCCAT
GACATGGTGGAGTCCTCG-3’; Mut 5,6 (E4126I, E4127D), 5’-GATGATCAACTTCA
GAGACATGGTGGAGTCCTCG-3’; Mut7,8 (R4161K,N4162C), 5’-GCACGAC
CCGCGCCTG
AAGTGCTTCCCTGGAGCTGGC-3’; Mut9,10 (F4163L and L4166P): 5’-GCGCCTGAAGTGCTTCCCTGGAGCTGGC-3’; Mut11 (R4175E), 5’-CCTTG
AGTACTTC
GCCCTACCTAGGCCGCATGG-3’.

The introduction of each mutation was confirmed using DNA sequencing analysis.

RyR3 mutagenesis was performed using the primer extension method on a subclone comprised of RyR3 sequence between a previously introduced (10) AscI site at position 12,018 and 12,666, respectively, into the pGEM 3zf cloning vector (Promega Inc; Madison, WI). A silent mutation at position 12543 was added resulting in a unique AvrII restriction site that aided in the construction of full-length RyR3 cDNAs containing the mutations. Primers for mutagenesis, along with their reverse complements (not shown), were synthesized with the following sequence (nucleotides resulting in point mutations are indicated in italics): Mut 1 (Q4020L), 5’-GGAGCTGCTGGACCTGCA
GAGACATGGTGG-3’; Mut2 (K4021Q), 5’-GGAGCTGCTG
GACCTGCA
GAGACATGGTGG-3’; Mut1,2 (Q4020L and K4021Q), 5’-GGAGCTGCTGGACCTGCA
GAGACATGGTGG-3’; Mut3 (M4047T), 5’-GGCAGATGGTGGACCA
CGAGACATGGTGGAGTCCTCG-3’; Mut4 (S4099G), 5’-CCAGAAGGCCAT
GACATGGTGGAGTCCTCG-3’; Mut 5,6 (E4126I, E4127D), 5’-GATGATCAACTTCA
GAGACATGGTGGAGTCCTCG-3’; Mut7,8 (R4161K,N4162C), 5’-GCACGAC
CCGCGCCTG
AAGTGCTTCCCTGGAGCTGGC-3’; Mut9,10 (F4163L and L4166P): 5’-GCGCCTGAAGTGCTTCCCTGGAGCTGGC-3’; Mut11 (R4175E), 5’-CCTTG
AGTACTTC
GCCCTACCTAGGCCGCATGG-3’.
Cell Culture—1B5 myotubes were prepared as described (3, 12). Briefly, 1B5 myoblasts were propagated in Dulbecco’s modified Eagle’s medium (Invitrogen Laboratories) supplemented with 20% fetal bovine serum (v/v) (Hyclone; Logan, UT), 100 units/ml penicillin-G, 100 μg/ml streptomycin sulfate, 2 mM l-glutamine at 37 °C at 10% CO2. Cells were seeded onto a 96-well plate and cultured until they attained 50% confluency. 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 above at 37 °C at 18% CO2. After 7 days, differentiated myotubes were transduced with the RyR constructs using equivalent amounts of HSV virions (11) that contained the cDNAs encoding the RyRs. After 2 h at 37 °C in antibiotic-free medium, virus was removed and the myotubes were incubated for 2 days to allow full expression of the RyRs before testing.

Calcium Imaging—RyR-expressing myotubes were challenged with successive additions of 80 mM KCl, 40 mM caffeine, and 0.5 mM 4-Cmc dissolved in imaging solution (25 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 6 mM glucose, 2 mM CaCl2, 25 mM HEPES, pH 7.4 adjusted with NaOH). Note: for the 80 mM KCl solution, the NaCl concentration was reduced to 45 mM to maintain osmolarity. Changes in intracellular Ca2+ in 1B5 myotubes were measured as described previously (10) using the Ca2+ indicator dye Fluo-4. Record traces from individual cells were stored and analyzed using Microsoft Excel as follows.

Quantification of Ca2+ Transients—The relative amount of Ca2+ released in response to each RyR agonist was estimated by calculating the average change in Fluo-4 fluorescence during application of the agonist. This mean value was then normalized to the resting fluorescence of the cell obtained 10 s prior to the addition of the agonist. Comparison of 4-Cmc-elicited responses from cells transduced with various wt and mutant RyR constructs was accomplished by expressing the magnitude of each 4-Cmc-elicited response as a percentage of the magnitude of the 40 mM caffeine-elicited Ca2+ transient obtained from the same cell. These normalized values from each RyR mutant were then compared with the values obtained from the 4-Cmc induced transients from wtRyR1 and wtRyR3-expressing cells. This comparison was made using 2-way analysis of variance (ANOVA) with a Dunnett’s post test. A significant difference was inferred at p < 0.05.

Measurements of Single Channel Activity in Lipid Bilayer Membranes—Bilayers were composed of phosphatidylethanolamine:phosphatidylserine:phosphatidylcholine (5:3:2 w/w) dissolved in decane at a final concentration of 50 mg/ml across a 200 μm aperture on a polysulfone cup (Warner Instrument Corp. CT). The bilayer partitioned two chambers (cis and trans), which contained the following buffer solutions (in mM): cis, 500 CsCl, 0.9 free Ca2+, and 20 Hepes-Tris (pH 7.4); trans, 500 CsCl and 20 Hepes-Tris (pH 7.4). The free Ca2+ concentration was adjusted by addition of CaCl2 and EGTA based on calculations from the “Bound and Determined” software (13). The cis chamber was virtually grounded, whereas the trans chamber was connected to the head stage input of an amplifier (Bilayer Clamp BC 525C, Warner Instrument, Hamden, CT). Single channels were obtained by introducing rabbit skeletal muscle junctional SR vesicles to the cis chamber to induce fusion with the bilayer membrane. Immediately after incorporation of RyR1 single channels into the lipid bilayer, the SR vesicles were perfused

FIGURE 2. A, diagram depicts the chimera 1 region of RyR1 (black bar: amino acids 3768–4180) and the 1 CD region (amino acids 4007–4180), which mediates 4-Cmc sensitivity in RyR1. B, peptide sequence comparison of the 1 CD region from rabbit RyR1, RyR2, and RyR3. Boxed residues indicate the position of the point mutations created in this study. Gray bar depicts the location of putative membrane-spanning segment M2 (26).
out of the cis chamber to prevent multiple vesicle fusions. Single channel gating was monitored and recorded at a holding potential of −40 mV (applied to the trans side). The amplified current signals, filtered at 1 kHz (Low-Pass Bessel Filter 8 Pole, Warner Instrument) was digitized and acquired at a sampling rate of 10 kHz (Digidata 1320A, Axon-Molecular Devices, Union City, CA). Most of the recordings were made for at least 3 min before the contents of the cis and/or trans chambers were changed. The channel open probability (P_o), as well as the open and closed dwell times were calculated using pClamp software 9.0 (Axon-Molecular Devices). Junctional SR vesicles used in this work for single channel studies were from three different preparations.

Secondary Structure Prediction—The secondary structure predictions were performed using the Chou-Fasman method for α-helical predictions (14), the Kyte-Doolittle method for hydropathy predictions (15), and the method of Emini et al. (16) for surface probability prediction.

RESULTS

Although 4-CmC can activate all three RyR isoforms (6, 8, 17), it activates RyR1 and RyR2 much more effectively compared with RyR3. A primary determinant on RyR1 that mediates 4-CmC sensitivity resides between amino acid residues 3769 and 4180 (10). Substitution of this region into the corresponding region of RyR3 results in a RyR1-RyR3 chimera (chimera 1 rev) with higher sensitivity to 4-CmC activation relative to wtRyR3. To determine whether 4-CmC acts on the analogous region of RyR2 (amino acids 3730–4136), this segment was substituted into RyR3 to create chimera 1 rev RyR2 (Fig. 1A). When intracellular Ca^{2+} release experiments were used to compare the ability of these constructs to respond to 80 mM KCl (to test for depolarization-induced calcium release), 40 mM caffeine and 0.5 mM 4-CmC, wtRyR3 (Fig. 1B) responded to caffeine but not to KCl or 4-CmC, whereas chimera 1 rev RyR2 (Fig. 1C) and chimera 1 rev (Fig. 1D) both responded to caffeine and 4-CmC but not to KCl depolarization. This finding suggests that determinants required for 4-CmC activation of RyR1 and RyR2 are identical.

The amino acid sequences of the three RyR isoforms were compared within the 173-amino acid 1 CD region (Fig. 2A), a subregion of chimera 1 that is the smallest determinant of 4-CmC sensitivity we have previously defined in wtRyR1 (10). Between the three isoforms, 66% of the amino acids in this region are identical and 87% are highly homologous (Fig. 2B). We reasoned that amino acid residues involved in 4-CmC activation of RyR1 and RyR2 should be (a) either identical or highly homologous between these isoforms and (b) divergent in RyR3. Eight amino acid residues were identified that were identical between RyR1 and RyR2 but not RyR3 (positions 1–6, 9, and 10). In addition, 3 residues were
identified that were highly divergent between all three isoforms (positions 7, 8, and 11). Amino acid positions that were either identical or highly homologous between RyR3 and either RyR1 or RyR2 were not examined.

A RyR1 construct containing all 11 mutations (Mut1–11) was tested for activation by KCl, caffeine, and 4-CmC (Fig. 3A). Compared with wtRyR1 (Fig. 3B), responses to chemical depolarization and caffeine activation of the Mut1–11 construct

FIGURE 4. A, the following mutant RyRs were created containing mutations at positions 1, 2, 5–11 (Mut1,2,5–11), 1, 2, 7, 8 (Mut1,2,7,8), 1, 2, 5, 6 (Mut1,2,5,6) as indicated by the white vertical bars. B–E, agonist-induced changes in intracellular Ca²⁺ from a representative myotube expressing Mut 1, 2, 5–11 (B), Mut 1, 2, 7, 8 (C), and Mut 1, 2, 5, 6 (D) are indicated. Calibration bar = 0.5 F/F₀, arbitrary units versus 30 s. E, responses to 4-CmC were normalized to the response to caffeine as described under "Experimental Procedures" for the following numbers of myotubes: Mut1, 2, 5–11 (n = 39 myotubes), Mut 1, 2, 7, 8 (n = 69), Mut 1, 2, 5, 6 (n = 63). The asterisk indicates a statistically significant difference (p < 0.01) compared with wtRyR1 as determined using ANOVA with a Dunnett’s post test.
FIGURE 5. A, RyR1 constructs containing mutations at position 1, 2 (Mut1,2) or 3–11 (Mut3–11) were created. B and C, agonist-induced changes in intracellular Ca\(^{2+}\) from a representative myotube expressing Mut1,2 (B) or Mut3–11 (C) are indicated. Calibration bar = 0.5 F/F\(_o\), arbitrary units versus 30 s. D, responses to 4-CmC were normalized to the response to caffeine as described under “Experimental Procedures” for the following numbers of myotubes per construct: Mut1,2 (n = 32 myotubes) and Mut3–11 (n = 26). The asterisk indicates a statistically significant difference (p < 0.01) compared with wtRyR1 as determined using ANOVA with a Dunnett’s post-test.

FIGURE 6. A, RyR1 constructs containing mutations at position 1 (Mut1) or 2 (Mut2) were created. B and C, agonist-induced changes in intracellular Ca\(^{2+}\) from a representative myotube expressing Mut1 (B) or Mut2 (C) are indicated. Calibration bar = 0.5 F/F\(_o\), arbitrary units versus 30 s. D, responses to 4-CmC were normalized to the response to caffeine as described under “Experimental Procedures” for the following numbers of myotubes per construct: Mut1 (n = 60 myotubes) and Mut2 (n = 56). Asterisk indicates a statistically significant difference (p < 0.01) compared with wtRyR1 as determined using ANOVA with a Dunnett’s post-test.
were unaffected. However, activation by 4-CmC was significantly reduced (Fig. 3, C and D).

To identify which of these 11 mutated residues are responsible for the reduced 4-CmC sensitivity of the Mut1–11 construct, additional full-length RyR1 constructs containing smaller numbers of these mutations were tested (Fig. 4A). RyR1 constructs containing mutations at positions 1, 2, 5–11 (Fig. 4B), 1, 2, 7, 8 (Fig. 4C), or 1, 2, 5, 6 (Fig. 4D) all had significantly reduced 4-CmC-induced Ca$^{2+}$ transients compared with wtRyR1 (Fig. 4E), whereas their responses to KCl and caffeine were unaffected.

Since all mutated constructs with reduced sensitivity to 4-CmC contain mutations at positions 1 and 2, a RyR1 channel containing mutations of only these two amino acids was tested (Mut1; Fig. 5A). This construct also had significantly reduced 4-CmC-induced Ca$^{2+}$ transients compared with wtRyR1 (Fig. 5B). To determine whether positions 3–11 contribute to 4-CmC sensitivity, a RyR1 construct containing only mutations at these positions was tested (Mut3–11; Fig. 5A). The Mut3–11 construct could be activated by 4-CmC, although the 4-CmC-induced calcium transients were slightly reduced compared with wtRyR1 (Fig. 5, C and D). This finding leaves open the possibility that other residues in addition to positions 1 and 2 may be involved in 4-CmC activation of wtRyR1. However, the finding that mutations at positions 1 and 2 have a much more dramatic effect on 4-CmC sensitivity compared with mutations at positions 3–11 suggests that Gln$^{4020}$ and Lys$^{4021}$ constitute the primary determinant for activation of RyR1 by 4-CmC.

Full-length RyR1 constructs containing single mutations at either position 1 or position 2 were also tested (Fig. 6A). Activation of the Mut1 channel (Fig. 6B) with either caffeine or 4-CmC but not KCl resulted in repetitive oscillations in intracellular Ca$^{2+}$ in 55% of myotubes tested. These oscillations were not seen in myotubes expressing Mut2 (Fig. 6C), which responded to all three methods of activation similarly to wtRyR1. Furthermore, the normalized 4-CmC responses of myotubes expressing Mut1 were significantly reduced relative to wtRyR1 (Fig. 6D).

Substitution of RyR1 segments required for 4-CmC activation into RyR3 can restore 4-CmC sensitivity of this isoform (Fig. 1D). To test the hypothesis that positions 1 and 2 mediate RyR1 sensitivity to 4-CmC, we mutated these residues in RyR3 to their RyR1 counterparts (L3873Q/Q3874K, Fig. 7A). This construct (RyR3 Mut1,2) responded to 4-CmC (Fig. 7B) with Ca$^{2+}$ transients that were significantly larger than 4-CmC-induced Ca$^{2+}$ transients in RyR3 (Fig. 7C). These results further strengthen the hypothesis that these amino acid residues are the primary determinant of 4-CmC sensitivity in RyR1.

To determine whether 4-CmC preferentially activates either the cytoplasmic or luminal face of RyR1, single channel measurements were performed (Fig. 8). Consecutive additions of 4-CmC to the cytoplasmic (cis) face of RyR1 increased the Po from its resting level of 0.073 to 0.341 (25 μM 4-CmC) and 0.549 (50 μM 4-CmC; Fig. 8A). When applied to the luminal (trans) side of the channel, 4-CmC increased channel Po$^{\text{trans}}$ from a resting level of 0.106 to 0.431 (25 μM 4-CmC) and 0.636 (50 μM 4-CmC; Fig. 8B). Thus, under the experimental conditions used here, 4-CmC was equally potent and efficacious toward activating RyR1 channel activity from both cytoplasmic and luminal sides of the lipid bilayer. Moreover, addition of 4-CmC simultaneously to both cytoplasmic and luminal sides of the lipid bilayer produced an additive effect on channel activity, increasing a resting Po$^{\text{rest}}$ of 0.084–0.892 (25 μM 4-CmC each side) and 0.911 (50 μM 4-CmC; Fig. 8C). The dependence of RyR1 open probability on the concentration of 4-CmC applied to the cis, trans, or cis + trans sides of the bilayer is indicated in Fig. 9.

**DISCUSSION**

In this study, we have defined 2 amino acid residues of rabbit RyR1 (Gln$^{4020}$ and Lys$^{4021}$) that are required for 4-CmC activation. Mutation of these residues to their RyR3 counterparts selectively reduces 4-CmC activation of the mutant channel compared with wtRyR1. Conversely, mutation of these residues in RyR3 to their RyR1 counterparts imparts 4-CmC sensitivity to RyR3. Of these 2 residues, Gln$^{4020}$ appears to be more important in channel function since mutation of this residue in RyR1 results in repetitive Ca$^{2+}$ oscillations evoked by caffeine or 4-CmC.
Does 4-CmC Bind to the Gln4020-Lys4021 Dipeptide?—The 4-CmC binding site most likely is located between amino acids 3759–4544 of RyR1 since N-terminal deletion mutants of RyR2 that contain the corresponding sequence (3722–4485) can be activated by 4-CmC, whereas deletion mutants lacking this sequence cannot (18). Given that the Gln<sup>4020</sup>-Lys<sup>4021</sup> dipeptide is located within this region, this segment can be considered as a potential candidate for 4-CmC binding.

This hypothesis is strengthened by findings from our recent study on the structural determinants of the 4-CmC molecule that are required for activation of RyR1 (19). We found that the 1-OH of 4-CmC is critical for the ability of the compound to activate RyR1. Moreover, the phenolic proton appears to be shared between 4-CmC and a general base on RyR1, which has a pH profile consistent with it being either a histidine or a lysine residue. This residue could be the Lys<sup>4021</sup> defined in this study.

A second finding in our recent work is that hydrophobic groups at the 3- and 4- positions of 4-CmC are required for activation of RyR1. This result is consistent with our findings in the present study that indicate the proximity of a hydrophobic segment, possibly M2, to the QK dipeptide. One model consistent with both sets of results predicts that the QK dipeptide stabilizes the 1-OH moiety of 4-CmC, whereas the M2 segment stabilizes the 3- and 4-hydrophobic groups. This model provides a framework for additional experiments aimed at understanding the molecular mechanisms by which RyR1 responds to 4-CmC.

A separate but related question is whether the 4-CmC binding site resides on the luminal or cytoplasmic face of RyR1. Single channel studies (20) originally suggested a luminal binding site for 4-CmC since this compound preferentially activated RyR1 when it was applied to the luminal face of the channel. However, in our attempts to reproduce this data, we found that 4-CmC activates RyR1 with roughly equal potency when applied to either the cytoplasmic or luminal side. These results clearly indicate that elements of RyR1 structure essential for 4-CmC activation can be equally accessed from either the cytoplasmic or luminal sides of the lipid bilayer. The reason for the discrepancy between our results and those of Herrmann-Frank and co-workers (20) is not clear considering that the experimental protocols were nearly identical in the two studies. Nevertheless, a new finding in the present study indicates that when 4-CmC is applied simultaneously to both sides of the lipid bilayer, its effect on channel activity is essentially additive. These new data support a hypothesis in which the 4-CmC binding site most likely resides in close proximity to the lipid bilayer or even at the protein/lipid interface. This hypothesis is supported by studies on structural analogs of 4-CmC such as 4-alkyl phenols whose activity is directly proportional to the length of the 4-alkyl chain (21). These alkyl chains could anchor the compound to the membrane allowing the compound to diffuse to a binding site at the protein/lipid interface of RyR1. Presumably, 4-CmC acts similarly, which

![FIGURE 8. RyR1 single channel activity in the absence and presence of 4-CmC was measured as described under “Experiment Procedures.”](image-url)
could explain the lack of preferential activation we observe in our single channel measurements.

Location of the Gln\textsuperscript{4020}-Lys\textsuperscript{4021} Dipeptide within the RyR\textsubscript{1} Primary Structure—The Gln\textsuperscript{4020}-Lys\textsuperscript{4021} dipeptide is located proximally to a number of important functional elements in the RyR\textsubscript{1} primary structure. RyR\textsubscript{1} is thought to be comprised of two overall structural domains, a massive N-terminal cytoplasmic domain and a smaller C-terminal transmembrane domain that anchors the channel to the membrane and also contains the Ca\textsuperscript{2+} permeation pore (22). The Gln\textsuperscript{4020}-Lys\textsuperscript{4021} dipeptide is located at the beginning of this C-terminal channel domain. Directly adjacent to the dipeptide is the putative M2 transmembrane segment, which contains a highly conserved glutamate residue (Glu\textsuperscript{4032}) that has been implicated as a "Ca\textsuperscript{2+} sensor"; i.e. a residue required to translate Ca\textsuperscript{2+} binding to the channel into RyR activation (23). In addition, putative binding sites for calmodulin (amino acids 3614–3643 (22, 24)) and Ca\textsuperscript{2+} (amino acids 4079–4090 and 4116–4127 (25)) are located near the Gln\textsuperscript{4020}-Lys\textsuperscript{4021} dipeptide. Taken together, these observations suggest that the 4-C\textsubscript{m}C activation site and Ca\textsuperscript{2+} regulatory sites are proximal to each other. This arrangement could provide an attractive explanation for how localized conformational changes in RyR\textsubscript{1} structure induced by 4-C\textsubscript{m}C binding could be conveyed to nearby Ca\textsuperscript{2+} regulatory sites, thus increasing the sensitivity of RyR\textsubscript{1} to Ca\textsuperscript{2+} activation as has been reported (20).

Protein Modeling—To understand how the Q4020L and K4021Q mutations might alter the local structure of RyR\textsubscript{1}, we modeled the secondary structure between amino acids 4007 and 4037 and compared this predicted structure to the equivalent regions from RyR\textsubscript{2} and RyR\textsubscript{3} (Fig. 10, A–C). For all three isoforms, the predicted overall secondary structure is \( \alpha \)-helical. In wtRyR\textsubscript{1} and wtRyR\textsubscript{2}, the Gln\textsuperscript{4020}-Lys\textsuperscript{4021} dipeptide is adjacent to a highly hydrophobic segment (residues 4023–4041 in RyR\textsubscript{1}) originally predicted as the M2 transmembrane segment (26). In RyR\textsubscript{3}, this hydrophobic region is extended to include the RyR\textsubscript{3} equivalent of the 4-C\textsubscript{m}C activation dipeptide, Leu\textsuperscript{3873}-Gln\textsuperscript{3874}. When the Q4020L and K4021Q mutations are introduced into RyR\textsubscript{1} (Fig. 10D), this hydrophobic segment becomes extended as well, producing a similar hydrophathy profile to that of wtRyR\textsubscript{3}. These observations suggest that extending this hydrophobic segment to include the Gln\textsuperscript{4020}-Lys\textsuperscript{4021} dipeptide reduces 4-C\textsubscript{m}C sensitivity of RyR\textsubscript{1}.

A second major difference between RyR\textsubscript{1}/RyR\textsubscript{2} and RyR\textsubscript{3} in the predicted secondary structure is the presence of a putative surface-exposed region in RyR\textsubscript{1} and RyR\textsubscript{2} that is not present in RyR\textsubscript{3} (Fig. 10, A–C). This surface-exposed region lies just N-terminal to the Gln\textsuperscript{4020}-Lys\textsuperscript{4021} dipeptide of RyR\textsubscript{1} and to its
equivalent in RyR2. Thus, a hypothesis consistent with our experimental data and also the modeled structure of this area is that this surface-exposed region is required for 4-CmC activation of RyR1 and RyR2. This hypothesis is strengthened by the observation that the Q4020L/K4021Q mutant also lacks the predicted surface exposed region (Fig 10D). Indeed, the presence of this putative surface-exposed region is correlated well with 4-CmC sensitivity of the RyR. Mutation of Lys4021 alone (K4021Q) affects neither 4-CmC sensitivity nor the predicted surface exposed region (Fig. 10F), whereas mutation of Gln4020 alone (Q4020L) diminishes the probability of the surface exposed sequence (Fig. 10E) and also diminishes 4-CmC-induced Ca\(^{2+}\) transients.

CONCLUSION

In this report, we have defined a critical dipeptide segment of RyR1, Gln\(^{4020}\)-Lys\(^{4021}\), that is required for 4-CmC activation of the channel. Future work will be needed to understand the mechanism of channel activation by 4-CmC and how these residues are involved in this process.

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