Role of calmodulin methionine residues in mediating productive association with cardiac ryanodine receptors

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Balog, Edward M., Laura E. Norton, David D. Thomas, and Bradley R. Fruen. Role of calmodulin methionine residues in mediating productive association with cardiac ryanodine receptors. Am J Physiol Heart Circ Physiol 290: H794–H799, 2006.-Calmodulin (CaM) binds to the cardiac ryanodine receptor Ca2+-release channel (RyR2) with high affinity and may act as a regulatory channel subunit. Here we determine the role of CaM Met residues in the productive association of CaM with RyR2, as assessed via determinations of [3H]ryanodine and [35S]CaM binding to cardiac muscle sarcoplasmic reticulum (SR) vesicles. Oxidation of all nine CaM Met residues abolished the productive association of CaM with RyR2. Substitution of the COOH-terminal Mets of CaM with Leu decreased the extent of CaM inhibition of cardiac SR (CSR) vesicle [3H]ryanodine binding. In contrast, replacing the NH2-terminal Met of CaM with Leu increased the concentration of CaM required to inhibit CSR [3H]ryanodine binding but did not alter the extent of inhibition. Site-specific substitution of individual CaM Met residues with Gln demonstrated that Met124 was required for both high-affinity CaM binding to RyR2 and for maximal CaM inhibition. These results thus identify a Met residue critical for the productive association of CaM with RyR2 channels.

Cardiac contraction is triggered by Ca2+ influx through voltage-gated Ca2+ channels, which in turn initiates a much larger Ca2+ release from the sarcoplasmic reticulum (SR) via ryanodine receptor Ca2+-release channels (RyR2). The ~2,000-kDa RyR2 homotetramer consists of a COOH-terminal transmembrane assembly and a massive cytoplasmic NH2-terminal domain. Although RyR2 is activated by Ca2+, endogenous effectors and posttranslational modifications modulate Ca2+ activation of the channel. Indeed, the cytoplasmic domain of RyR2 acts as scaffolding to which accessory proteins, including the FKS056-binding protein and calmodulin (CaM), bind (14). At micromolar Ca2+, CaM inhibits both RyR2 and the skeletal muscle ryanodine receptor isoform (RyR1). However, at submicromolar Ca2+, CaM activates RyR1 but inhibits RyR2 (3). This isomer-specific effect of CaM may be attributed in part to differential tuning of the Ca2+ affinity of CaM on CaM binding to the different RyR isoforms (16). Thus, at submicromolar Ca2+, RyR2-bound CaM may be Ca2+-CaM, whereas RyR1-bound CaM may be Ca2+-free CaM (apo-CaM).

CaM is a 148-amino acid Ca2+-binding protein composed of NH2- and COOH-terminal globular domains connected by a flexible linker. High-affinity Ca2+-binding to two EF-hand Ca2+-binding motifs in each of the globular domains induces a conformational change from the more compact apo-CaM structure to the more open Ca2+-CaM structure. This conformational change also exposes hydrophobic target-binding surfaces in each of the globular domains (9). These hydrophobic patches and the conformational flexibility of CaM allow it to bind to and regulate numerous, structurally diverse targets. Indeed, CaM interactions are quite promiscuous in that CaM-binding domains share little sequence homology and the mode of CaM interaction with targets varies greatly (30).

CaM contains nine Met residues out of 148 amino acids. This is a much higher Met content than the statistical average for the occurrence of Met in other proteins (24). In mammalian CaM, four Met residues are clustered in each of the globular domains at residues 36, 51, 71, and 72 in the NH2 terminus and residues 109, 124, 144, and 145 in the COOH terminus. A ninth Met is located at position 76 in the linker region. As a result of this highly localized distribution, Met residues account for approximately half the surface area of the hydrophobic target-binding interface of Ca2+-CaM (24). The importance of the Met residues of CaM is indicated by their evolutionary conservation. For example, in CaMs from widely divergent organisms such as Tetrahymena and Dicystostelium, all nine Mts have been preserved (12). Two functions have been ascribed to the Met residues of CaM: stabilizing the open conformation of Ca2+-bound CaM (23) and providing a target-binding interface (24, 31). Whereas the high Met content of CaM contributes to effective target binding, the Met residues in Ca2+-CaM are surface exposed (35) and susceptible to oxidation. Oxidation of nonpolar Met to polar Met sulfoxide (MetO) decreases the efficacy of target regulation by CaM (5, 28, 33).

Site-specific replacement of CaM Met residues has been used to define the role of individual Met residues in the productive association of CaM with targets, i.e., both the binding of CaM to targets and the subsequent transduction of the functional effect of CaM to the targets. When compared with NH2-terminal mutations, site-specific replacement of COOH-terminal Met residues is more detrimental to the productive association of CaM with many (2, 8, 10, 32, 34) but not all targets (8, 19). However, even among the targets for which COOH-terminal CaM Mts are critical, the specific essential residue(s) vary (2, 10, 22). As such, the role of individual CaM Met residues in RyR2 regulation is undefined.

This work used three approaches to study CaM Met function: Met oxidation, Met-to-Leu substitution, and Met-to-Gln substitution. Results presented here identify a critical Met residue within the COOH-terminal domain of CaM as key in the regulation of RyR2 channels.

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METHODS

Materials. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Pigs were obtained from the University of Minnesota Experimental Farm. Tran[35S]Met and Cys were obtained from ICN Biochemicals (Costa Mesa, CA). [3H]Hypotanide was purchased from PerkinElmer (Boston, MA). Unlabeled ryanodine was obtained from Calbiochem (La Jolla, CA). Myosin light chain kinase-derived CaM-binding peptide was obtained from Peptide Technologies (Gaithersburg, MD). Disodium L-γ-methylenedenosenosine 5′-triphosphate (AMP-PCP) was from Sigma (St. Louis, MO).

Isolation of cardiac SR vesicles. Cardiac SR (CSR) vesicles were prepared from porcine ventricular tissue as previously described (15). Briefly, ventricular tissue was homogenized in 10 mM NaHCO3 and centrifuged for 5 min at 4,000 g. The supernatant was filtered through gauze, centrifuged for 20 min at 4,000 g, filtered a second time, and centrifuged 30 min at 80,000 g. Pelleted membranes were extracted in 0.6 M KCl and 20 mM Tris (pH 6.8) on ice for 45 min and then centrifuged 30 min at 120,000 g. The pellets were resuspended in 10% sucrose, centrifuged 30 min at 120,000 g, and resuspended in a minimal volume of 10% sucrose. CSR was frozen in liquid N2 and stored at −70°C. Endogenous CaM was removed from the SR by incubating CSR in 120 mM potassium propionate, 10 mM PIPES, pH 7.0, 100 μM Ca2+, and 1 μM myosin light chain kinase-derived CaM-binding peptide for 30 min at room temperature (4). The SR was then centrifuged through 15% sucrose in a Beckman 70.1 Ti rotor at 40,000 rpm for 30 min at 4°C to remove the CaM and CaM-binding protein. All isolation buffers contained 1 μM GSH, 0.05 μM aprotinin, and Ca-EGTA to oxidize a number of amino acids because the thioether group of Met residues were replaced with Gln. Mutants were constructed from the wild-type rat CaM cDNA by using primer-based site-directed mutagenesis following the protocol provided by the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). DNA sequence analysis confirmed the correct generation of each mutant.

CaM expression and purification. CaM was expressed in Escherichia coli [BL21(DE3)pLys5], purified via phenyl-Sepharose chromatography (18) and dialyzed overnight at 4°C against 2 mM HEPES, pH 7.0. CaM concentration was determined by using the published extinction coefficient ε292=3,029 M/cm (28).

[^35S]methionine incorporation. Wild-type rat CaM subcloned into the pET-7 vector was metabolically labeled with [35S]methionine and purified by phenyl-Sepharose chromatography (15). Briefly, bacterial growth was initiated in M9 media containing ampicillin. When the optical density value (measured at 600 nm) of the bacteria reached 0.5, the pelleted bacteria were resuspended in RPMI 1640 media containing ampicillin, one-fortieth of methionine, cysteine, and glucose concentration compared with the regular RPMI 1640 medium, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. A 2.5-SqM aliquot of [35S]methionine and [35S]cysteine was then added to the medium, and the bacteria were cultured for 5–6 h at the same conditions.

Analysis. The CaM concentration dependence of CSR vesicle [3H]ryanodine binding and the inhibition of [35S]CaM binding by unlabeled CaM were fit with a four-parameter Hill equation. The Ca2+ dependence of ryanodine binding was fit with an equation that assumes a high-affinity Ca2+-binding site, which when bound will activate the RyR, and a lower-affinity Ca2+-binding site, which when bound will inhibit channel opening (1). All curve fitting was performed with SigmaPlot 6.0 (Systat Software, Point Richmond, CA).

RESULTS

To assess the role of CaM Met residues in the productive association of CaM with RyR2, the Ca2+-dependence of CSR vesicle [3H]ryanodine binding in media containing either no CaM, 1 μM native CaM, or 1 μM oxidized CaM was compared (Fig. 1). The CaM oxidation protocol used here (incubation in 50 mM H2O2 for 24 h) was previously shown to selectively oxidize all nine Met residues of CaM to MetO (2). In the absence of CaM, CSR vesicle [3H]ryanodine binding exhibited a biphasic Ca2+-dependence (EC50 = 2.9 ± 0.3 μM; IC50 = 2,003 ± 503 μM). Native CaM (1 μM) depressed CSR [3H]ryanodine binding (EC50 = 10.1 ± 6.1 μM; IC50 = 1,073 ± 591 μM). In contrast, the Ca2+-dependence of CSR [3H]ryanodine binding determined in media containing 1 μM oxidized CaM virtually overlapped the Ca2+-dependence of CSR [3H]ryanodine binding determined in the absence of CaM (EC50 = 3.8 ± 0.7 μM; IC50 = 2,012 ± 398 μM).

[^35S]CaM binding to cardiac muscle SR vesicles. [35S]CaM binding to CSR vesicles was performed as previously described (4). CSR vesicles were incubated in 150 mM KCl, 20 mM PIPES, pH 7.0, 5 mM GSH, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 nM [35S]CaM. After 2 h incubation at 24°C, 0–5,000 nM unlabeled CaM was added and incubated for 30 min. Pellets were collected after centrifugation at 40,000 rpm for 20 min in a Beckman TLA-55 rotor at 20°C. Pellets were solubilized by overnight incubation in 10% SDS. The pellets were then diluted in 200 μl double-distilled H2O, and bound [35S]CaM was determined by scintillation counting. Nonspecific binding was determined by using 100-fold excess unlabeled CaM.

Oxidation of CaM. CaM (60 μM) was incubated in 50 mM homopiperazine-N,N′-bis-2-(ethanesulfonic acid), pH 5.0, 0.1 M KCl, 2.0 mM MgCl2, 50 mM H2O2 at room temperature for 24 h. The reaction was stopped by overnight dialysis (mol wt cutoff 3,500) at 4°C in distilled water (1 liter, for five times) buffered with 10 mM ammonium bicarbonate (pH 7.7). Although H2O2 can potentially oxidize a number of amino acids because the thioether group of Met residues was measured in the presence of 10 mM MgCl2 and 20°C in distilled water (1 liter, for five times) buffered with 10 mM PIPES, pH 7.0. CaM concentration was determined by using the published extinction coefficient ε292=3,029 M/cm (28).
SE of 3 experiments and are expressed as B/Bo, where B is 

\[ \frac{\text{bound}}{\text{total}} \] 

\[ \text{containing } 700 \] 

\[ \text{IC}_{50}: \text{No CaM} = 2.9 \pm 0.3 \mu M; \text{native CaM} = 10.1 \pm 6.1 \mu M; \text{oxidized CaM} = 3.8 \pm 0.7 \mu M. \] 

\[ \text{IC}_{50}: \text{No CaM} = 2.003 \pm 502 \mu M; \text{native CaM} = 1.073 \pm 591 \mu M; \text{oxidized CaM} = 2.012 \pm 398 \mu M. * \text{Significantly different from No CaM (} P < 0.05 \). 

To determine the effects of CaM oxidation on the affinity of CaM for RyR2, the dependence of CSR vesicle \[^{[3]H}\text{ryanodine binding on the concentration of native and oxidized CaM was compared. Native CaM inhibited CSR vesicle \[^{[3]H}\text{ryanodine binding with an IC}_{50} \text{ of } 27 \pm 1.8 \text{ nM (Fig. 2A). CSR vesicle ryanodine binding in media containing up to } 3 \mu M \text{ oxidized CaM did not differ from CSR vesicle ryanodine binding in the absence of CaM. Thus oxidation abolished CaM inhibition of RyR2. In competitive binding experiments (Fig. 2B), unlabeled native CaM completely displaced \[^{[35]S}\text{CaM previously bound to CSR. In contrast, unlabeled oxidized CaM (up to } 10 \mu M \) was unable to compete with \[^{[35]S}\text{CaM for CSR binding. Thus the loss of CaM regulation of RyR2 was the result of the inability of oxidized CaM to interact with the CaM binding site on the channel.} 

The functional role of the NH2- and COOH-terminal Met-rich patches of CaM in the regulation of RyR2 was examined by using two Met-to-Leu mutants. In the NH2-terminal Met-to-Leu mutant, the four NH2-terminal Met (residues 36, 51, 71, and 72) and Met76 in the linker region were changed to Leu. In the COOH-terminal Met-to-Leu mutant, the four COOH-terminal Met (residues 109, 124, 144, 145) were changed to Leu. Wild-type CaM (3 \mu M) and the NH2-terminal Met-to-Leu CaM mutant (3 \mu M) significantly (} P < 0.05 \) decreased CSR vesicle \[^{[3]H}\text{ryanodine binding compared with the absence of CaM (Fig. 3A). In contrast, CSR vesicle \[^{[3]H}\text{ryanodine binding in media containing 3 \mu M COOH-terminal Met-to-Leu CaM mutant was similar to CSR ryanodine binding in the absence of CaM. This suggests that the COOH terminus of CaM is critical for inhibition of RyR2. Thus the dependence of CSR vesicle \[^{[3]H}\text{ryanodine binding on the concentration Met-to-Leu mutants was compared with that of wild-type CaM (Fig. 3, B and C). Figure 3B shows that 3 \mu M NH2-terminal Met-to-Leu CaM mutant indeed inhibited CSR vesicle \[^{[3]H}\text{ryanodine binding to the same extent as wild-type CaM. However, a significantly higher concentration of the NH2-terminal Met-to-Leu mutant was required to half-inhibit CSR vesicle \[^{[3]H}\text{ryanodine binding (IC}_{50} \text{: wild type, } 9 \pm 1 \text{ nM; NH2-terminal Met-to-Leu mutant, } 111 \pm 40 \text{ nM; } P < 0.05 \). In contrast, the COOH-terminal Met-to-Leu CaM mutant inhibited CSR vesicle \[^{[3]H}\text{ryanodine binding to a significantly lesser extent than wild-type CaM (percent inhibition: wild type, 57 \pm 3; COOH-terminal Met-to-Leu mutant, 27 \pm 2; } P < 0.01 \).
Fig. 4. Inhibition of CSR vesicle $[^3H]$ryanodine binding by wild-type CaM and Met-to-Gln CaM mutants. $[^3H]$ryanodine binding to CSR vesicles was performed as described in METHODS in media containing 700 μM free Ca$^{2+}$. A: CaM NH$_2$-terminal Met-to-Gln substitutions. B: CaM COOH-terminal Met-to-Gln substitutions. Here $B_o$ is CSR vesicle $[^3H]$ryanodine binding in absence of CaM and $B$ is CSR vesicle $[^3H]$ryanodine binding in presence of indicated concentration of CaM. Data are means ± SE of 29 wild-type CaM experiments and 4–8 Met-to-Gln CaM experiments. $B_{max} = 1.0 ± 0.1$ pmol ryanodine/mg SR protein. Best fit parameters for each curve are presented in Table 1.

Thus the COOH-terminal Met residues of CaM are critical for CaM inhibition of RyR2.

To define the role of specific Met residues in the productive association of CaM with RyR2, individual Met residues were substituted with Gln (Fig. 4 and Table 1). Wild-type CaM depressed CRS vesicle $[^3H]$ryanodine binding 50% with an IC$_{50}$ of 37 ± 3 nM. Three Met-to-Gln mutations significantly altered CaM inhibition of RyR2. In the NH$_2$ terminus of CaM, the Met72Gln mutant lessened the extent of CaM inhibition to 68% of wild-type CaM but had a similar IC$_{50}$ as wild-type CaM. Two COOH-terminal mutants altered the productive association of CaM with RyR2. Like Met72Gln, Met144Gln inhibited CSR vesicle $[^3H]$ryanodine binding to a lesser extent than wild-type CaM (64% of wild type) but with a similar IC$_{50}$ as wild-type CaM. Met124Gln inhibited CSR vesicle $[^3H]$ryanodine binding to 57% of wild-type CaM and required a greater concentration to do so (IC$_{50}$ = 106 ± 21 nM). The Hill coefficients of SR vesicle $[^3H]$ryanodine did not differ between wild-type CaM and any of the Met-to-Gln CaM mutants (Table 1).

To determine whether the higher concentration of Met124Gln CaM required to inhibit CSR vesicle $[^3H]$ryanodine binding was due to a lower affinity of the mutant for RyR2, displacement of prebound wild-type $[^35S]$CaM by unlabeled wild-type and Met124Gln CaM was compared (Fig. 5). In a medium containing 700 μM Ca$^{2+}$, wild-type CaM displaced CSR vesicle-bound $[^35S]$CaM with an IC$_{50}$ of 598 ± 198 nM. In comparison, 2.7-fold more Met124Gln CaM was required to displace CSR-bound $[^35S]$CaM (IC$_{50}$ = 1,617 ± 441 nM). Thus the Met124Gln mutation decreased the affinity of CaM for RyR2.

Fig. 5. Inhibition of CSR vesicle $[^35S]$CaM binding by wild-type CaM and Met124Gln CaM. Binding was performed as described in METHODS in media containing 700 μM free Ca$^{2+}$. Data are means ± SE of 5 experiments. Wild-type: IC$_{50} = 598 ± 198$; Met124Gln: IC$_{50} = 1.617 ± 441$; *$P < 0.05$.

Previous work (2) demonstrated that substitution of Met109 with Gln abolished CaM activation of RyR1 at submicromolar Ca$^{2+}$ without affecting CaM inhibition of the channel at micromolar Ca$^{2+}$. Thus experiments examined the effects of the Met109Gln mutation on CaM regulation of RyR2 at submicromolar Ca$^{2+}$ (Fig. 6). To enhance cardiac SR vesicle $[^3H]$ryanodine binding at low Ca$^{2+}$ (1 mM EGTA, 0.023 mM CaCl$_2$; calculated free Ca$^{2+} = 10$ nM), 10 mM caffeine was included in the assay media. Under these conditions, high concentrations of wild-type CaM (>10 μM) enhanced CSR vesicle $[^3H]$ryanodine binding. In contrast, Met109Gln CaM, up to 30 μM, did not enhance CSR vesicle $[^3H]$ryanodine

![Graph showing inhibition of CSR vesicle $[^3H]$ryanodine binding by wild-type CaM and Met124Gln CaM.](image)

Table 1. Calmodulin concentration dependence of inhibition of CSR vesicle $[^3H]$ryanodine binding

<table>
<thead>
<tr>
<th>IC$_{50}$, nM</th>
<th>$n_H$</th>
<th>%Inhibition</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>37 ± 3</td>
<td>1.1 ± 0.9</td>
</tr>
<tr>
<td>M36Q</td>
<td>50 ± 6</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>M51Q</td>
<td>23 ± 7</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>M71Q</td>
<td>36 ± 11</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>M72Q</td>
<td>38 ± 9</td>
<td>2.6 ± 1.2</td>
</tr>
<tr>
<td>M76Q</td>
<td>36 ± 2</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>M109Q</td>
<td>42 ± 8</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>M124Q</td>
<td>106 ± 21*</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>M144Q</td>
<td>47 ± 5</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>M145Q</td>
<td>35 ± 4</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Half-inhibitory concentration (IC$_{50}$), Hill coefficient ($n_H$), and percent inhibition were derived from fits to Hill equation as described in METHODS and shown in Fig. 4. CSR, cardiac sarcoplasmic reticulum; WT, wild type. *Significantly different from wild-type calmodulin (P < 0.05). M, methionine; Q, glutamine.
binding. Thus the Met109Gln CaM mutation similarly impairs CaM activation of both RyR1 and RyR2 at submicromolar Ca\(^{2+}\).

**DISCUSSION**

In this study, oxidation and site-specific mutagenesis were used to identify CaM Met residues critical for inhibition of RyR2. CaM contains no cysteine residues, and under acidic conditions, oxidation can be specific for Met residues (7). The extensive in vitro oxidation protocol used here converted all nine Met residues of CaM to MetO (2). As a result, Met was changed from a nonpolar to a polar residue (17), the \(\alpha\)-helical content of CaM was decreased (20, 33), the cooperativeness between the Ca\(^{2+}\) binding sites of CaM was reduced (20), and the productive association of CaM with RyR2 was abolished (Fig. 2). Because less extensive oxidation produced CaM samples containing multiple oxiforms, (2) site-directed mutagenesis was used to selectively modify specific Met residues.

CaM function can be bifurcated, with the two lobes subserving different functions. A classic example is the two types of aberrant swimming behavior of *Paramecium* (26). One swimming behavior arises from a disregulation of a Ca\(^{2+}\)-activated \(K^+\) current as a result of mutations in the COOH-terminal lobe of CaM. The other behavior is the consequence of an altered Ca\(^{2+}\)-activated \(Na^+\) current due to mutations in the NH\(_2\)-terminal lobe of CaM. More recently, conversion of CaM from a RyR1 activator to an inhibitor has been attributed to Ca\(^{2+}\) binding to the COOH-terminal EF hands of CaM (16, 25). Thus the functional role of the NH\(_2\)- and COOH-terminal Met clusters of CaM was examined by replacing all the Met residues in each cluster with Leu. In comparison with Met, Leu has a similar volume, is slightly more hydrophobic, and has a reduced flexibility but a similar propensity to form \(\alpha\)-helices (17, 27). Through evolution, Leu is the most common substitute for Met (13). For instance, *Sarccharomyces cercvisiae* CaM has \(\sim\)60% identity with mammalian CaM, with only three of the nine Met residues having been retained. The other six Met residues have been replaced by Leu (12). Thus Leu is a relatively conservative substitution for Met. As such, substitution of the NH\(_2\)-terminal Met residues of CaM with Leu preserved CaM inhibition of RyR2. In contrast, replacement of COOH-terminal Met significantly decreased the extent of CaM inhibition of the channel (Fig. 3). This suggests that not only is Ca\(^{2+}\) binding to the COOH terminus of CaM a determinant of RyR inhibition (25) but that the resulting exposure of COOH-terminal Met is critical for RyR inhibition.

We next endeavored to determine the relative importance of specific Met residues in the productive association of CaM with RyR2. For this we made use of CaM Met-to-Gln mutants. A Gln substitution may be considered similar to the oxidation of Met to MetO in that an oxygen is located in the same position in the amino acid side chain. Although of similar size, this substitution replaces a hydrophobic residue with a polar one. Of the nine Met-to-Gln mutations examined, replacing Met124 with Gln was the most deleterious to CaM regulation of RyR2. This substitution decreased the extent of inhibition and increased by more than twofold the CaM concentration required for half-maximal inhibition. The substitution, however, had no detectable effect on the secondary structure of CaM as assessed by circular dichroism nor did it prevent the Ca\(^{2+}\)-induced mobility shift on SDS-PAGE or alter the Ca\(^{2+}\) affinity of CaM (2). Like its effect on RyR2 regulation, the Gln substitution for Met124 was the only mutation that altered Ca\(^{2+}\)-CaM inhibition of RyR1, increasing the CaM concentration required for half-maximal inhibition. The mutation also increased the apo-CaM concentration required for RyR1 activation (2). Thus Met124 is required for high-affinity binding of CaM to both RyR1 and RyR2. COOH-terminal Met124 has also been shown, along with Met109, to be critical for the high-affinity association of Ca\(^{2+}\)-CaM with a number of enzyme targets, including CaM kinases II and IV, smooth muscle myosin light-chain kinase (8), and *Bordetella pertussis* adenylate cyclase (32).

Although the Met109Gln mutation did not alter Ca\(^{2+}\)-CaM inhibition of either RyR1 or RyR2, this mutation abolished apo-CaM activation of RyR1. Therefore, an attempt was made to determine whether RyR1 and RyR2 share a requirement for Met109 in CaM activation at nanomolar Ca\(^{2+}\). With Ca\(^{2+}\) as the sole RyR2 activator, CaM was inhibitory (Fig. 1). However, increasing RyR2 activity at low Ca\(^{2+}\) via the addition of caffeine revealed apo-CaM activation of this isoform (Fig. 6). This is consistent with the previous reports using caffeine (15) and Ca\(^{2+}\)-insensitive CaM mutants (16). Thus the enhanced CSR vesicle \[^{3}H\]ryanodine binding by wild-type CaM but the inability of the Met109Gln mutant to similarly enhance CSR vesicle \[^{3}H\]ryanodine binding suggests that the two RyR isoforms do indeed share a requirement for a Met residue in CaM position 109 for CaM activation of the channels.

In summary, the present study demonstrated that extensive in vitro oxidation of CaM abolished the productive association of CaM with cardiac RyR2 channels. Met-to-Leu substitutions revealed that the COOH-terminal Met residues of CaM are required for CaM inhibition of RyR2. Furthermore, replacement of Met124 in the COOH terminus with Gln lowered the affinity of CaM for RyR2 and decreased the extent of channel inhibition. Thus Met124 is required for high-affinity productive association of CaM with both RyR1 (2) and RyR2.

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