Lobe-dependent Regulation of Ryanodine Receptor Type 1 by Calmodulin

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Calmodulin activates the skeletal muscle Ca\textsuperscript{2+} release channel RYR1 at nM Ca\textsuperscript{2+} concentrations and inhibits the channel at \mu M Ca\textsuperscript{2+} concentrations. Using a deletion mutant of calmodulin, we demonstrate that amino acids 2–8 are required for high-affinity binding of calmodulin to RYR1 at both nM and \mu M Ca\textsuperscript{2+} concentrations and are required for maximum inhibition of the channel at \mu M Ca\textsuperscript{2+} concentrations. In contrast, the addition of three amino acids to the N terminus of calmodulin increased the affinity for RYR1 at both nM and \mu M Ca\textsuperscript{2+} concentrations, but destroyed its functional effects on RYR1 at nM Ca\textsuperscript{2+}. Using both full-length RYR1 and synthetic peptides, we demonstrate that the calmodulin-binding site on RYR1 is likely to be noncontiguous, with the C-terminal lobe of both apocalmodulin and Ca\textsuperscript{2+}-calmodulin binding to amino acids between positions 3614 and 3643 and the N-terminal lobe binding at sites that are not proximal in the primary sequence. Ca\textsuperscript{2+} binding to the C-terminal lobe of calmodulin converted it from an activator to an inhibitor, but an interaction with the N-terminal lobe was required for a maximum effect on RYR1. This interaction apparently depends on the native sequence or structure of the first few amino acids at the N terminus of calmodulin.

The skeletal muscle calcium release channel RYR1 is regulated by calmodulin (CaM)\textsuperscript{1} in both its Ca\textsuperscript{2+}-free (apoCaM) and Ca\textsuperscript{2+}-bound (Ca\textsuperscript{2+}-CaM) states. ApoCaM is a partial agonist, whereas Ca\textsuperscript{2+}-CaM is an inhibitor of the channel (1). Both apoCaM and Ca\textsuperscript{2+}-CaM bind with nanomolar affinity to RYR1 (2–4). The ability of bound calmodulin to protect sites on RYR1 from tryptic cleavage was used to identify a site between amino acids 3614 and 3643 as a binding site for both apoCaM and Ca\textsuperscript{2+}-CaM (5). This site was predicted to be a CaM-binding site by Takeshima et al. (6). Subsequent studies demonstrated that both apoCaM and Ca\textsuperscript{2+}-CaM can bind to a synthetic peptide (R3614–43) representing amino acids 3614–3643 of RYR1. ApoCaM requires amino acids at the C-terminal part of this peptide, but Ca\textsuperscript{2+}-CaM does not, leading to the suggestion that CaM undergoes an N-terminal shift within the binding site upon binding Ca\textsuperscript{2+} (3).

In the course of analyzing the interaction of CaM with RYR1, we found that the addition of three amino acids (Gly-Ser-His) to the N terminus of Drosophila CaM (designated (N+3)CaM) increases its affinity for RYR1 compared with wild-type CaM. The additional amino acids do not significantly change the maximum binding at either nM or \mu M calcium concentrations (3). The ability of three amino acids to produce a large increase in affinity led us to investigate this phenomenon in greater detail. There are at least two possible explanations for the affinity increase: 1) the extension alters the conformation of the molecule; and/or 2) the N-terminal amino acids of CaM make specific and essential contacts with RYR1, and these residues are more accessible in the mutant.

Another type of mutant CaM involving the N terminus has also been studied. The deletion of seven amino acids (DQLTEEQ, residues 2–8) from the N terminus of mammalian CaM ((N–7)CaM) abolishes CaM-dependent activation of skeletal muscle myosin light chain kinase. This mutant CaM still binds the kinase with high affinity (7). Small-angle x-ray scattering structural analysis indicates that this mutant CaM has a different location in the complex with the kinase than native CaM (8).

These findings with the N-terminally altered CaMs suggest that the addition or deletion of a few amino acids at the N terminus can greatly change CaM interaction with a subset of targets. To determine the role for the N terminus of CaM in regulating RYR1, we analyzed the effects of addition and deletion of N-terminal amino acids of CaM on its interaction with RYR1 and the known CaM-binding peptide R3614–43, which represents the CaM-binding site on RYR1. Our findings suggest a complex role of both lobes of CaM in regulating the activity of RYR1.

EXPERIMENTAL PROCEDURES

Materials—Tran35S-labelTM (1175 Ci/mmol) was obtained from ICN Biomedicals, Inc. (Irvine, CA). [3H]Ryanodine (36 Ci/mmol) from PerkinElmer Life Sciences. Ryanodine was from BIOMOL Research Labs, Inc. Calcium calibration buffer was from Molecular Probes, Inc. (Eugene, OR). Fluorescent derivatives of CaM (F19W-CaM and F92W-CaM) were graciously provided by Dr. D. J. Black (Ohio State University Medical Center, Columbus, OH). Other chemicals were obtained from Sigma.

Peptide Synthesis—The peptide was synthesized in the core facility at Baylor College of Medicine under the direction of Dr. Richard Cook.
Sarcoplasmic Reticulum (SR) Membrane Preparation—SR membranes were prepared from rabbit hind leg and backstrap skeletal muscle and purified using a previous method (9). Protein concentrations were estimated by the method of Lowry et al. (10) using bovine serum albumin as the standard.

Expression and Purification of Mammalian (mCaM) and Drosophila (dCaM) Wild-type CaMs, (N-3)dCaM, (N-2)8mCaM, N-CaM, and C-CaM and [35S]Methionine Labeling of mCaM—The mCaM cDNA was graciously provided by Dr. Ruth Altschuld (Ohio State University). The mCaM and dCaM cDNAs were subcloned into pET3 vectors (Novagen, Madison, WI) for expression. The (N-2)-8mCaM DNA was subcloned into the pMCMII-26 vector for expression. The subcloning of the wild-type dCaM cDNA into the pET28a expression vector was used to produce and purify the dCaM expression vector and purified wild-type dCaM cDNA. (N-2)-8mCaM were carried out as described previously (11, 12). (N-3)dCaM was expressed and purified as described previously (5, 13). Expressed mCaM was metabolically labeled as described in the literature (14) and purified following the same method used for purification of wild-type CaM and (N-2)-8mCaM. Expression and purification of mCaM amino acids 1–75 (CaM-(1–75)), 1–80 (CaM-(1–80)), and 78–148 (CaM-(76–148)) were performed as previously described (15–17). Protein concentrations were determined by the Bio-Rad protein assay using purchased bovine brain CaM as the standard. To express and purify N-CaM and C-CaM, the cDNA fragments corresponding to amino acid residues 1–79 and 78–148, respectively, were individually PCR-amplified from the pAEDM vector containing the human liver CaM cDNA. Each amplified cDNA fragment was digested with NdeI and HindIII, gel-purified, and then ligated into the pET3 expression vector. N-CaM and C-CaM were expressed and purified following the same procedure used for wild-type CaM and (N-2)-8mCaM. Protein concentrations were estimated by the Bio-Rad protein assay using bovine brain CaM as the standard for the C-CaM construct and apritinin as the standard for the N-CaM protein.

Competitive Inhibition of [3H]Ryanodine Binding in the Presence of CaMs—SR membranes (10 μg/100 μl in each assay) were incubated with 5 nM [3H]ryanodine and 5 μM free Ca2+ in buffer containing 10 mM EGTA, 100 mM KCl, 5 mM MgCl2, 5 mM nitritotriacetic acid, and 1 μg/ml bovine serum albumin, 0.1% CHAPS, and 1 mM EGTA) in the absence (low Ca2+ buffer) or presence (high Ca2+ buffer) of 0.2 mM CaCl2 (high Ca2+ buffer) of 1.2 mM CaCl2 for binding at low or high Ca2+, respectively. Nonspecific binding is defined as binding in the presence of 5 μM unlabeled mCaM. Bound radioligand was separated from free radioligand by filtration through Whatman GF/F filters presoaked in binding buffer containing 0.3 mg/ml bovine serum albumin, and the filters were washed with 5 × 3 ml of ice-cold binding buffer. The equilibrium inhibition constant (Kd) was calculated with the equation described previously (11).

[3H]Ryanodine Binding in the Presence of CaMs—SR membranes (10 μg/200 μl in each assay) were incubated with 5 nM [3H]ryanodine and 10 μg (CaM-(1–75)) or 20 μg (CaM-(1–80)) CaM in buffer containing 10 mM EGTA, 100 mM KCl, 5 mM MgCl2, 5 mM nitritotriacetic acid, and 1 μg/ml bovine serum albumin, 0.1% CHAPS, and 1 mM EGTA) in the absence (low Ca2+ buffer) or presence (high Ca2+ buffer) of 0.2 mM CaCl2 (high Ca2+ buffer) or CaCl2 (low Ca2+ buffer) of 1.2 mM CaCl2 for binding at low or high Ca2+, respectively. Nonspecific binding is defined as binding in the presence of 10 μM unlabeled ryanodine. Bound [3H]ryanodine was separated from free ligand by the same methods used for [3H]labeled CaM binding.

Nondenaturing Gel Electrophoresis—To determine the interaction of CaM with the CaM-binding peptide R3614–43 on RYR1, 15 μl of 10 μM CaM was incubated with increasing concentrations of R3614–43 (0–100 μM) for 2 h at room temperature in the presence of 1 mM EGTA (low Ca2+) or 0.2 mM CaCl2 (high Ca2+). 15 μl of 2× nondenaturing sample buffer was added to the samples before loading onto a nondenaturing 8% polyacrylamide gel. To determine the interaction of CaM with mCaM and dCaM (N-CaM, amino acids 1–79; and C-CaM, amino acids 78–148) representing the N- and C-terminal lobes of CaM with R3614–43, the mCaM and dCaM were expressed and purified following the same procedure used for wild-type CaM and (N-2)-8mCaM. Protein concentrations were estimated by the Bio-Rad protein assay using bovine brain CaM as the standard for the C-CaM construct and apritinin as the standard for the N-CaM protein.

Fluorescence Spectroscopy—To determine the effects of the N-terminal mutation on the affinity of CaM for Ca2+, the Ca2+-dependent tryptophan fluorescence enhancement of R3614–43 bound to CaM was assayed. 3 μM CaM and 3 μM R3614–43 were incubated for 2 h with increasing concentrations of Ca2+ (0–20 μM) in a 90 μl of 10 mM Tris-HCl (pH 7.4) buffer containing 10 mM EGTA, 100 mM KCl, and 30 mM MOPS (pH 7.2) (Molecular Probes, Inc.). To monitor the effect of R3614–43 on the affinity of the CaM lobes for Ca2+, fluorescence changes in fluorescent derivatives of CaM substituted with tryptophan at either position 19 or 29 (F19W-CaM and F29W-CaM, respectively) were analyzed. 2.5 μM CaM fluorescence derivative and 10 μM R3614–43 were incubated as described above. The excitation and emission wavelengths were set at 280 and 328 nm, respectively. Spectra were collected on a SPECTRAmax GEMINI dual-scanning microplate spectrophotometer (Molecular Devices) as described previously (3, 19), and data were fit to a Hill equation as described by Murase and Iio (20). To determine the interaction of the N- and C-terminal CaM fragments (N2–75, 1–75, 1–80, and 76–148) with R3614–43, the fluorescence emission spectra of tropane 3620 in R3614–43 were monitored in the presence of these constructs and high and low calcium concentrations. 6 μM R3614–43 and variable concentrations of CaMs (mCaM, CaM-(1–75), CaM-(1–80), and CaM-(76–148)) were incubated at 22 °C in buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM EGTA, 5 mM nitritotriacetic acid, and 1 mM MgCl2 (low Ca2+) or 10 μM CaCl2 (high Ca2+). Multiple CaM/peptide ratios were used to determine the saturating effect of CaMs on R3614–43 fluorescence. Spectra were collected using an SLM-AMINCO Model 4800CTM, an excitation wavelength of 285 nm, a step size of 0.5 nm, and a band pass of 4 nm. Each spectrum was obtained by averaging three buffer corrected spectra and normalizing the average to the intensity at the maximum fluorescence emission wavelength of R3614–43 alone.

Circular Dichroism Spectroscopy—To elucidate the effect of N-terminal mutation on the secondary structures of CaM, CaM was analyzed by CD spectroscopy. Considering the far-UV transparency of buffers, the buffer conditions used for CD measurement were different from those used in other experiments in this study. 50 μg/ml CaM in 5 mM Tris-Cl (pH 7.9) and 0.1 mM EGTA (low Ca2+) or 0.1 mM CaCl2 (high Ca2+) was incubated for 2 h at room temperature. CD spectra were recorded with an Olis 1000 RSM spectropolarimeter (On-Line Instrument Systems, Inc., Bogart, GA) using a 1-mm cylindrical quartz cell as described previously (21). For each spectrum, a minimum of five scans was collected from 250 to 188 nm with a step resolution of 1 nm under constant nitrogen gas purge. All spectra were verified in three separately prepared samples. Base-line spectra of buffer and buffer + EGTA or CaCl2 were collected and subtracted from the experimental spectra where appropriate.

RESULTS

We have previously shown that [35S]-labeled (N+3)dCaM, derived from cleavage of the His tag when expressed in the pET28a vector, has an ~6-fold higher affinity for RYR1 than the non-extended CaM at both μM and μM Ca2+ concentrations (3). This finding led us to systematically analyze the interactions of N-terminal mutant CaMs with RYR1. We expressed and purified mCaM, (N-2)-8mCaM, dCaM, and (N+3)dCaM and compared the interactions of these CaMs with RYR1 and the calmodulin-binding peptide from RYR1 (R3614–43). Although there are three amino acid differences in sequence between mCaM and dCaM, they are all in the C-terminal lobe (positions 99, 143, and 147) and do not significantly alter the interactions with either RYR1 or the synthetic peptide R3614–43 (data not shown). For brevity, we will show mCaM rather than both mCaM and dCaM as a wild-type CaM control in all figures and tables.

Inhibition of [35S]-Labeled CaM Binding to RYR1 by N-terminal Mutant CaMs—To evaluate the effects of the mutations on their affinity for RYR1, we compared the ability of these CaMs to inhibit the binding of [35S]-labeled CaM to SR membranes. Significant differences in affinity were detected between wild-type and mutant CaMs at both low (Fig. 1A) and high (Fig. 1B) Ca2+ concentrations. The K values are summarized in Table 1. (N+3)dCaM had a 5-fold higher affinity at low Ca2+ concentrations and a 4-fold higher affinity at high Ca2+ concentrations than mCaM, whereas (N-2)-8mCaM had 8- and 3-fold lower affinities than mCaM at low and high calcium concentrations, respectively (Table 1). These effects of the N-terminal alterations on the overall affinity of CaM suggest that the N-terminal lobe of CaM is important in the interaction with RYR1.

The requirement of both lobes for binding is further supported by the findings that CaM fragments representing only
The N- or C-terminal lobe have dramatically reduced affinities for RYR1. The inhibition of \(^{35}\)S-labeled CaM by N-CaM (amino acids 1–78) and C-CaM (amino acids 79–148) at <10 nM and 200 \(\mu\)M Ca\(^{2+}\) is also shown in Fig. 1 (A and B), respectively. At <10 nM Ca\(^{2+}\), the apparent \(K_{i}\) for C-CaM is >7 \(\mu\)M, whereas the \(K_{i}\) for N-CaM is >120 \(\mu\)M. At 200 \(\mu\)M Ca\(^{2+}\), the apparent \(K_{i}\) for C-CaM is >3 \(\mu\)M, whereas the \(K_{i}\) for N-CaM is >60 \(\mu\)M (Table I).

**RYR1 Regulation by N-terminal Mutant CaMs**—The finding that the addition of three amino acids to the N terminus increased the affinity of CaM for RYR1, whereas the deletion of seven amino acids decreased its affinity, led us to examine the effects of these CaMs on \(^{3}H\)-ryanodine binding. \(^{3}H\)-Ryanodine binding is frequently used to screen for functional effects of agents on RYR1 (22–29). This is based on the finding that ryanodine binds preferentially to the open state of the channel; and agents that enhance \(^{3}H\)-ryanodine binding are generally channel activators, and agents that inhibit \(^{3}H\)-ryanodine binding are generally channel inhibitors (22, 26, 27, 29). \(^{3}H\)-Ryanodine binds with high affinity to one site per RYR1 tetramer. To determine the effect of N-terminal mutations on the functional interaction of CaM with RYR1, we analyzed \(^{3}H\)-ryanodine binding to SR membranes in the presence of the different CaMs at low and high Ca\(^{2+}\) concentrations (Fig. 2). Although (N+3)CaM had the highest affinity for RYR1 at low Ca\(^{2+}\), it had a very poor ability to enhance \(^{3}H\)-ryanodine binding at low Ca\(^{2+}\) (Fig. 2A). In contrast, it was as effective as mCaM in inhibiting \(^{3}H\)-ryanodine binding at high Ca\(^{2+}\) (Fig. 2B). Therefore, higher affinity does not correlate with increased efficacy.

The situation with (N2–8)mCaM was somewhat different. This mutant CaM had a lower affinity for RYR1 at both low and high Ca\(^{2+}\) concentrations and, at 5 \(\mu\)M, also showed decreased enhancement of \(^{3}H\)-ryanodine binding at low Ca\(^{2+}\) (Fig. 2A) and decreased inhibition of \(^{3}H\)-ryanodine binding at high Ca\(^{2+}\) compared with mCaM (Fig. 2B). The decreased activation by (N2–8)mCaM compared with mCaM at low Ca\(^{2+}\) is consistent with its lower affinity. However, the 5 \(\mu\)M (N2–8)mCaM used in the experiments at high Ca\(^{2+}\) should have been close to saturation (Table I). To determine whether this is really an issue with saturation, we examined \(^{3}H\)-ryanodine binding at low and high Ca\(^{2+}\) with increasing concentrations of (N2–8)mCaM (Fig. 2, C and D). At low Ca\(^{2+}\), (N2–8)mCaM enhanced \(^{3}H\)-ryanodine binding to the same extent as wild-type CaM, but the maximum enhancement required a much higher concentration (Fig. 2C). At high Ca\(^{2+}\), the inhibition of \(^{3}H\)-ryanodine binding by (N2–8)mCaM was biphasic (Fig. 2D), suggesting that 1) some of the (N2–8)mCaM is misfolded; 2) (N2–8)mCaM has reduced efficacy, but high concentrations have a nonspecific effect on the channel; or 3) a second molecule of CaM binds at the higher concentrations of CaM. Misfolding of a large fraction of (N2–8)mCaM at high Ca\(^{2+}\) seems unlikely based on the CD data described below. This type of biphasic inhibition curve was not seen with any of the other mutant CaMs.

The CaM fragments had a greatly reduced affinity for RYR1 at both high and low Ca\(^{2+}\) concentrations. The low affinity of N-CaM (representing amino acids 1–78) precludes its use in \(^{3}H\)-ryanodine binding assays. However, C-CaM (representing amino acids 79–148) at 10 \(\mu\)M did produce some enhancement of \(^{3}H\)-ryanodine binding at low Ca\(^{2+}\) and some inhibition of binding at high Ca\(^{2+}\), but the effect was much less than that seen with mCaM and mutant CaMs (data not shown). These findings again argue that both lobes of CaM are required for high affinity binding at both low and \(\mu\)M Ca\(^{2+}\) concentrations.

**Alterations in the N-terminal Amino Acids of CaM Do Not Significantly Alter Its Binding to R3614–43**—Our data with \(^{35}\)S-labeled CaM and \(^{3}H\)-ryanodine binding indicate that the addition or deletion of amino acids at the N terminus of CaM can alter affinity, efficacy, or both for interaction with RYR1. We have previously identified a CaM-binding site on native RYR1 (5) and have shown that a synthetic peptide matching the sequence of this site can bind both apoCaM and Ca\(^{2+}\)-CaM (3). We examined the effects of the N-terminal alterations in CaM on its interactions with this synthetic peptide. The interaction of the mutant CaMs with the synthetic peptide matching amino acids 3614–3643 on RYR1 was assessed by non-denaturing polyacrylamide gel electrophoresis. We found that addition or deletion of amino acids at the N terminus of CaM did not significantly alter the binding to R3614–43. The data obtained at high and low Ca\(^{2+}\) concentrations are shown in Fig. 3. In this gel system, the peptide alone did not enter the gel due to its positive charge. On the high Ca\(^{2+}\) gels, the complex of the peptide and CaM can be seen above the free CaM band. However, on the low Ca\(^{2+}\) gels, the CaM-peptide complex did not migrate as a discrete band; and therefore, the extent of interaction was assessed by the disappearance of the free CaM band. Representative gels using low and high Ca\(^{2+}\) concentrations are shown in the insets in Fig. 3 (A and B, respectively). The summarized data (\(I_{pK}\), the ratio of the intensity of the CaM band in the presence of the peptide to that of CaM alone) from the densitometric analysis of the CaM band at low and high Ca\(^{2+}\) with increasing peptide/CaM ratios are shown in Fig. 3 (A and B). The intensity of the CaM band decreased to a constant level that was independent of the concentration of R3614–43. This residual CaM may arise from dissociation of the complex on the gel during electrophoresis or from limited solubility of the peptide. All of the CaMs behaved the same as mCaM at both high and low Ca\(^{2+}\) .
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### Table 1

|                  | mCaM (N = 3)CaM | (N2–8mCaM) | N-CaM | C-CaM |
|------------------|------------------|------------|-------|-------|
| K_{(low Ca^{2+})} | 54 ± 4 nM        | 11 ± 1 nM  | 446 ± 25 nM | ≥120 μM | ≥7 μM |
| K_{(high Ca^{2+})} | 41 ± 2 nM        | 10 ± 1 nM  | 106 ± 5 nM | ≥60 μM  | ≥3 μM |

**Fig. 2. Effects of mCaM and mutant CaMs on [³H]ryanodine binding to SR membranes at low and high Ca^{2+} concentrations.** SR membranes (10 μg) in 200 μl of low Ca^{2+} buffer (A) or high Ca^{2+} buffer (B) were incubated with 5 nM [³H]ryanodine and 5 μM mCaM, (N+3)CaM, or (N2–8)mCaM as described under “Experimental Procedures.” *+, statistical significance compared with controls (p < 0.05 by t test). SR membranes (10 μg) in 200 μl of low Ca^{2+} buffer (C) or high Ca^{2+} buffer (D) were incubated with 1 nM [³H]ryanodine and increasing concentrations of mCaM (●) and (N2–8)mCaM (▲).

**Interaction of Peptide R3614–43 with CaM Fragments Representing the N- and C-terminal Lobes of CaM**—The lack of an effect of the mutations on the interactions of CaMs with the peptide suggested that the N-terminal lobe of CaM was not interacting with this sequence. To test this, we expressed two constructs representing amino acids 1–79 and 78–148 and tested the ability of these half-CaMs to interact with peptide R3614–43 at high and low Ca^{2+} concentrations (Fig. 4). N-CaM (amino acids 1–79) had a very low affinity for R3614–43 under low Ca^{2+} conditions, but some binding could be detected at high peptide/CaM ratios under high Ca^{2+} conditions. In contrast, C-CaM bound well with R3614–43 peptide (Fig. 4), but with less enhancement with N-CaM compared with C-CaM (Fig. 5B). The affinity of N-CaM for R3614–43 at high Ca^{2+} was lower than that of C-CaM, but nonetheless, binding was definitely detected (Fig. 5B, inset). Combining the results of Figs. 3–5, we conclude that the C-terminal lobe of CaM binds constitutively to the R3614–43 peptide, but the N-terminal lobe requires Ca^{2+} binding for peptide association. The interaction of the Ca^{2+}-bound N-terminal lobe with R3614–43 appears to be a low affinity interaction.

**Effect of Binding of R3614–43 on the Ca^{2+} Affinity of CaM**—The affinity of CaM for Ca^{2+} often increases when it is complexed with its target site (30). Because the R3614–43 peptide contains a tryptophan at residue 3620, we were able to examine the intrinsic tryptophan fluorescence of R3614–43 complexed with CaM as a function of Ca^{2+} concentration (3). To determine whether N-terminal mutations alter the Ca^{2+} dependence of the interaction of CaM with the peptide, we measured the tryptophan fluorescence of a mixture of CaM and R3614–43 at different Ca^{2+} concentrations. These data are shown in Fig. 6A.
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Fig. 3. Addition and deletion of amino acids at the N terminal of CaM do not alter CaM binding to R3614–43. 15 μl of 10 μM mCaM (●), (N+3)dCaM (■), or (N2–8)mCaM (▲) was incubated with increasing concentrations of R3614–43 (0–100 μM) for 2 h at room temperature in the presence of 1 mM EGTA (A) or 0.2 mM CaCl2 (B). III designate the ratio of the intensity of the CaM band in the presence of the peptide versus that of CaM alone, and intensities were obtained by densitometric analysis of Coomassie Blue-stained CaM bands from three independent nondenaturing gels. The insets are representative non-denaturing 20% polyacrylamide gels of CaM with increasing concentrations of R3614–43. Lanes 1–9 have peptide/CaM ratios of 0, 0.5, 1, 2, 3, 4, 5, 8, and 10, respectively. At low calcium (A), the complex of CaM and the peptide did not enter the gel, and the bands shown are the CaM bands. At high calcium (B), the upper bands are the complexes of CaM and the peptide, and the lower bands are free CaMs.

R3614–43 showed a Ca2+-dependent fluorescence (relative fluorescence units) enhancement in the presence of all three CaMs. No significant differences in fluorescence properties and calcium binding parameters were observed between the mutant CaMs and mCaM (Table II). These results further confirm that alteration of the N-terminal amino acids of CaM does not significantly alter its binding to R3614–43 and its affinity for calcium.

Two fluorescent derivatives of CaM have been previously used to assess the effects of peptides on the Ca2+ affinity of CaM. These CaMs have tryptophan engineered at position 19 or 92 such that changes in tryptophan fluorescence can be used to monitor Ca2+ binding to the lobes of CaM (31). The interactions of these CaMs with R3614–43 are shown in Fig. 6B. As shown in Fig. 6B, the increase in intensity between pCa 8 and 6.5 for mCaM and the peptide is attributable solely to a change in the environment of the tryptophan in the peptide. The shape of the curve observed for F19W-CaM in the presence of peptide (Fig. 6B) is well represented by the sum of the mCaM + R3614–43 curve and a calcium titration of F19W-CaM alone (Fig. 6B, dotted line). The peptide has only minor effects on the affinity of the N-terminal lobe for Ca2+. For F92W-CaM, the calcium-dependent increase in fluorescence intensity in the presence of R3614–43 took place at a much lower concentration of calcium compared with that for F92W-CaM alone. This finding reflects that R3614–43 greatly increases the affinity of the C-terminal lobe for Ca2+.

Thus, the Ca2+-dependence of the interaction of the peptide with CaM correlates with Ca2+ binding to the C-terminal lobe (but not the N-terminal lobe) of CaM.

Effect of Addition or Deletion at the N terminus on the Secondary Structures of CaM—To determine whether the N-terminal mutation has effects on the secondary structures of CaM, we analyzed the CD spectra of the CaMs. In studies from other laboratories, the wavelength was set above 200 nm for the far-UV CD spectra of CaM, and therefore, these spectra have only negative peak information on α-helices and β-sheets (32, 33). To find structural differences among mCaM, (N+3)dCaM, and (N2–8)mCaM, our CD spectra were collected down to 188 nm, allowing us to analyze both the positive and negative peaks for α-helices, β-sheets, and random coils. Positive peaks of α-helices and β-sheets in CD spectra are much stronger and sharper than negative peaks, allowing us to more readily determine whether the changes at the N terminus produced wavelength shifts and intensity changes in the CD spectra of CaM. The far-UV CD spectra of different CaMs at low and high Ca2+ concentrations are shown in Fig. 7 (A and B, respectively). At high calcium (Fig. 7B), all three CaMs have a prominent positive CD maximum at 192 nm and two negative CD maxima centered at 208 and 222 nm. These maxima are diagnostic of proteins with high α-helical content (34). No significant differences in spectrum shapes were found among these three CaMs, indicating that they have similar secondary structures and folding states. (N+3)dCaM did have a very small intensity increase (~10%) compared with the other two CaMs. At low calcium (Fig. 7A), the positive CD maximum is located at ~191 nm for all three proteins, and their intensities are 1:0.8:0.5 for mCaM/(N+3)dCaM/(N2–8)mCaM. mCaM has a negative peak...
at 206 nm, whereas (N+3)dCaM and (N2–8)sCaM have the peaks located at 205 nm. At 222 nm, the negative peak of mCaM is still evident, but that of (N2–8)sCaM has almost disappeared. The decrease in the negative peak at 222 nm indicates a small decrease in α-helical content. A decrease in α-helical content and an increase in random coil content were supported by the blue shift of the negative peak at 208 nm and the positive peak at 192 nm as well as the intensity decrease in the positive peak at 192 nm. Random coils usually have an intense negative peak at 198 nm. We conclude that the addition or deletion of N-terminal amino acids produces a decrease in α-helical content and an increase in the random coil content of apoCaM. High calcium, however, induces more α-helix formation and eliminates the effect of the N-terminal addition or deletion on the secondary structures of CaM.

DISCUSSION

The Ca\(^{2+}\) release channel RYR1 mediates rapid Ca\(^{2+}\) release into the cytoplasm from the SR during excitation-contraction coupling in skeletal muscle. About half of the ryanodine receptors are likely to be mechanically gated by a physical association with the transverse tubule dihydropyridine receptor and serve to trigger the Ca\(^{2+}\) response in response to muscle depolarization. The other half of the ryanodine receptors are likely to be more important in spreading the Ca\(^{2+}\) wave throughout the cell and are probably activated by Ca\(^{2+}\) released from neighboring channels. The sensitivity of these channels to Ca\(^{2+}\) may be regulated by the binding of CaM (3). CaM binds to specific high affinity binding sites on RYR1 (5, 35) and regulates RYR1 function by increasing channel activity at low Ca\(^{2+}\) concentrations and decreasing channel activity at high Ca\(^{2+}\) concentrations (1). At low Ca\(^{2+}\), CaM can best be classified as a partial agonist because it is not able to maximally activate RYR1. In addition to its direct effects on RYR1, CaM may also modulate the coupling of RYR1 and L-type calcium channels (19).

We had previously proposed (3) that Ca\(^{2+}\) binding to CaM bound to RYR1 produces an N-terminal shift in its binding site on RYR1. This hypothesis was based on the finding that the apoCaM- and Ca\(^{2+}\)-CaM-binding sites on RYR1 are overlapping (5) and the ability of CaM to bind differentially to a set of
synthetic peptides representing sequences in the binding site. Ca$^{2+}$-CaM binds to synthetic peptides matching amino acids 3614–3643 and 3614–34, whereas apoCaM binds only to the former peptide (3). Further clues about the determinants in CaM required for its functional effects on RYR1 came from studies with CaM with mutations in which the Ca$^{2+}$-binding sites were mutated so that Ca$^{2+}$ could not bind to these sites. Mutation of all four Ca$^{2+}$-binding sites makes CaM an activator of the channel at all Ca$^{2+}$ concentrations (3). CaM with binding sites 3 and 4 mutated is also an activator at all Ca$^{2+}$ concentrations (4). In contrast, CaM with the two N-terminal Ca$^{2+}$-binding sites destroyed activates and inhibits the channel, depending on the Ca$^{2+}$ concentration, to the same extent as mCaM. Because these mutations “knock out” Ca$^{2+}$ binding, these results demonstrate that Ca$^{2+}$ binding to the N-terminal domain is not necessary for inhibition of RYR1. An open question is whether Ca$^{2+}$ ever binds to the N-terminal lobe of CaM when it is bound to RYR1.

These studies seem to suggest that the C-terminal lobe of CaM plays the predominant role in the regulation of RYR1 by CaM. What role does the N-terminal lobe play in the partial activation of the channel at low Ca$^{2+}$ concentrations and the inhibition of the channel at high Ca$^{2+}$ concentrations? One of the expression vectors commonly used to express CaM in bacteria is pET28a, which adds three amino acids to the N terminus of CaM. We were surprised to find that these three amino acids dramatically increased the affinity of CaM for RYR1 at both nM and µM Ca$^{2+}$ concentrations. Even though the affinity was greater, this addition mutant was a very poor partial agonist of the channel at low Ca$^{2+}$, but was similar to wild-type CaM in its ability to inhibit the channel at high Ca$^{2+}$. In other words, the addition of three amino acids to the N-terminal lobe of apoCaM converts it from being a partial agonist to an antagonist of the channel at nM Ca$^{2+}$ concentrations. These findings led us to examine the role of the N-terminal amino acids of CaM in greater depth. We found that removal of amino acids 2–8 decreased affinity at both nM and µM Ca$^{2+}$ concentrations. Although lower in apparent affinity, the deletion mutant at higher concentrations was able to enhance [3H]ryanodine binding to the same extent as wild-type CaM; however, its ability to inhibit the channel at high Ca$^{2+}$ was biphasic and complete only at higher concentrations. These studies suggest an important role for the N-terminal lobe of CaM in regulating the activity of RYR1.

We next examined the ability of these mutants of CaM to bind to the synthetic peptide R3614–43. The mutations did not alter the interactions with this peptide. This finding suggested that this sequence was not the binding site for the N-terminal lobe of CaM. To confirm this, we examined the interaction of CaM fragments representing either the N- or C-terminal lobe. The C-terminal lobe of CaM bound to the peptide with higher affinity than full-length CaM at both nM and µM Ca$^{2+}$ concentrations. However, the N-terminal lobe did not bind to R3614–43 at nM Ca$^{2+}$ concentrations and bound with lower affinity than the C-terminal lobe at high Ca$^{2+}$ concentrations. More binding of the N-terminal lobe to the peptide was detected by fluorescence than on nondenaturing gels. This could

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**Fig. 7.** Effect of N-terminal mutations on CaM CD spectra at low and high Ca$^{2+}$ concentrations. 50 µg/ml calmodulin in 5 mM Tris-Cl (pH 7.9) was incubated with 0.1 mM EGTA (A) or 0.1 mM CaCl$_2$ (B). ---, mCaM; ---, (N+3)xCaM; ---, (N2-8)mCaM.

**Fig. 8.** Model for the interaction of apoCaM and Ca$^{2+}$-CaM with RYR1. C1, C2 and N1, N2 are the C and N lobe binding sites on RYR1, respectively. The red sphere represents the C lobe of apoCaM. The red square represents the C lobe of Ca$^{2+}$-CaM. The blue sphere represents the N lobe of apoCaM. The yellow sphere represents Ca$^{2+}$.
Ca²⁺ appears to be required for binding to either site N1 or N2. When the occupancy of the N1 binding site increases the activity of the channel. The movement of the C-terminal lobe (C1 to C2) and the subsequence 3614 (sites N1 and N2 and sites C1 and C2, respectively) on RYR1. C-terminal lobes of CaM each have two possible binding sites binding at a site(s) not yet identified.

An examination of the interaction of the N- and C-terminal lobe fractions with full-length RYR1 showed that the C-terminal lobe could bind with greatly reduced affinity at both N1 and C-terminal Ca²⁺ concentrations, but little or no binding of the bi-lobed R3643CaM could be detected. Although the C-terminal lobe was able to slightly enhance [³H]ryanodine binding at nM Ca²⁺ and slightly inhibit at μM Ca²⁺, the effects were minimal. These findings suggest that both lobes of CaM are necessary for high affinity binding to RYR1 and that both are likely to be involved in their functional effects on this channel. The N-terminal lobe of CaM did not bind to sequence 3614–3643 at nM Ca²⁺ and bound only with low affinity at high Ca²⁺. We have examined CaM binding to a series of overlapping peptides representing amino acids between positions 3609 and 3652 and have found a binding site for the N-terminal lobe of CaM (data not shown). Our data support a noncontiguous binding site for CaM on RYR1, with the C-terminal lobe binding between amino acids 3614 and 3643 and the N-terminal lobe binding at a site(s) not yet identified.

We propose the following model to explain the interaction of CaM with RYR1 (Fig. 8). We propose that the N- and C-terminal lobes of CaM each have two possible binding sites (sites N1 and N2 and sites C1 and C2, respectively) on RYR1. The binding sites for the C-terminal lobe (C1 and C2) are both found within sequence 3614–3643, and the site occupied depends on the Ca²⁺ occupancy of the two EF-hands of this lobe. The binding of Ca²⁺ induces an N-terminal shift (C1 to C2) in the site of interaction of the C-terminal lobe within this sequence. We propose that neither of the preferred N-terminal lobe-binding sites (N1 and N2) is located within sequence 3614–3643. Ca²⁺ binding to the N-terminal lobe does not appear to be required for binding to either site N1 or N2. When Ca²⁺ does bind to the N-terminal lobe, it has some affinity for sequence 3614–3643, which could either be nonspecific or represent a third interaction site for the N-terminal lobe. Which of the N-terminal lobe-binding sites is occupied is likely to depend on the location of the C-terminal lobe. We propose that occupancy of the N1 binding site increases the activity of the channel, whereas occupancy of the N2 binding site inactivates or inhibits the channel. At low Ca²⁺ concentrations, site N1 is favored because the C-terminal lobe is in its C1 site. When Ca²⁺ binds to the C-terminal lobe, this lobe shifts from C1 to C2, driven by the Ca²⁺-induced conformational change in the lobe. This shift facilitates the interaction of the N-terminal lobe with site N2. We propose that channel inhibition requires both the movement of the C-terminal lobe (C1 to C2) and the subsequence engagement of the N-terminal lobe at site N2. One possible explanation of the effect of the addition of amino acids to the N terminus of the N-terminal lobe is that these bulky amino acids increase the solvent exposure of the amino acids in the N-terminal sequence that are important for interaction with site N2, causing an N1 to N2 shift and a loss of activation at low Ca²⁺. The effect of deletion of amino acids 2–8 on affinity for both high and low Ca²⁺ suggests that the N-terminal lobe binding utilizes these amino acids at both high and low Ca²⁺. As a result, this deletion directly affects the affinities of the N-terminal lobe for its binding sites and its RYR1 regulatory abilities. Consistent with this, the C-terminal lobe fragment alone can bind and regulate RYR1, but the functional effect is much less than seen with mCaM.

In summary, we propose a noncontiguous CaM-binding site on RYR1, with the C-terminal lobe bound in both its Ca²⁺-bound and Ca²⁺-free states within the sequence between amino acids 3614 and 3643. The N-terminal lobe apparently binds at sites that are not immediately adjacent to this sequence in the primary structure of the channel. Both lobes of CaM appear to bind at both low and high Ca²⁺ concentrations, and both lobes appear to have at least two interaction sites. Our data support a model in which the N- and C-terminal lobes of CaM are required for the maximum functional effects at both nM and μM Ca²⁺ concentrations. The C-terminal lobe of CaM is likely to function as a Ca²⁺-driven switch, which, via changes in both the conformation of the CaM molecule itself and the location of its binding site, alters the accessibility of the N-terminal lobe to an inhibitory site.

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