A Calmodulin Binding Domain of RyR Increases Activation of Spontaneous Ca\(^{2+}\) Sparks in Frog Skeletal Muscle*

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The calmodulin C lobe binding region (residues 3614–3643) on the sarcoplasmic reticulum Ca\(^{2+}\) release channel (RyR1) is thought to be a region of contact between subunits within RyR1 homotetramer Ca\(^{2+}\) release channels. To determine whether the 3614–3643 region is a regulatory site/interaction domain within RyR in muscle fibers, we have investigated the effect of a synthetic peptide corresponding to this region (R3614–3643) on Ca\(^{2+}\) sparks in frog skeletal muscle fibers. R3614–3643 (0.2–3.0 \(\mu M\)) promoted the occurrence of Ca\(^{2+}\) sparks in a highly cooperative dose-dependent manner, with a half-maximal activation at 0.47 \(\mu M\) and a maximal increase in frequency of ∼5-fold. A peptide with a single amino acid substitution within R3614–3643 (L3624D) retained the ability to bind Ca\(^{2+}\)-free calmodulin but did not increase Ca\(^{2+}\) spark frequency, suggesting that R3614–3643 does not modulate Ca\(^{2+}\) sparks by removal of endogenous calmodulin. Our data support a model in which the calmodulin binding domain of RyR1 modulates channel activity by at least two mechanisms: direct binding of calmodulin as well as interactions with other regions of RyR.

The sarcoplasmic reticulum (SR)\(^{1}\) Ca\(^{2+}\) release channel (ryanodine receptor, RyR) is a homotetramer with a subunit molecular mass of ∼560 kDa. In mammalian tissue three isoforms have been identified, skeletal muscle (RyR1), cardiac muscle (RyR2), and brain (RyR3) ryanodine receptors. All three isoforms are structurally and functionally related. The majority of the protein (∼4/5) resides on the myoplasmic side of the SR where binding sites for endogenous modulators (e.g. Ca\(^{2+}\), Mg\(^{2+}\), FK506-binding protein, and calmodulin) are located (for review see Ref. 1). Recently, much attention has been focused on elucidating the role of calmodulin (CaM) in regulation of RyR function.

CaM is a ubiquitous Ca\(^{2+}\)-binding protein that plays an important role in Ca\(^{2+}\) signaling in many cell types by modulating the activity of numerous proteins, including ion channels. Each RyR1 subunit binds one molecule of CaM or four CaM molecules per RyR1 homotetramer, regardless of the cytosolic [Ca\(^{2+}\)] (2). In SR vesicle preparations CaM displays Ca\(^{2+}\) dependence in its functional effects on RyR1, at nanomolar [Ca\(^{2+}\)] Ca\(^{2+}\)-free CaM activates RyR1, whereas at millimolar [Ca\(^{2+}\)] Ca\(^{2+}\)-CaM inhibits RyR1 (3, 4). This Ca\(^{2+}\)-dependent bi-functional regulation of RyR function was found to require Ca\(^{2+}\) binding to CaM (3), in particular at the two C-terminal Ca\(^{2+}\) binding sites (3, 5).

Further characterization of the interaction of CaM with RyR1 has led to the localization of a single binding site for Ca\(^{2+}\)-free CaM or Ca\(^{2+}\)-CaM binding to a region between amino acids 3614 and 3643 (2, 6), which is within the large cytoplasmic domain of the channel. Alklylation and tryptic cleavage studies identified cysteine 3635, which lies within the 3614–3643 region, as being important for inter-subunit cross-linking (7), suggesting that the CaM binding region also lies at an inter-subunit contact site within RyR1. More recently, Zhang et al. (8) have shown that Cys\(^{3635}\) forms an inter-subunit disulfide bond with a cysteine residue between 2000 and 2401 while the N-terminal lobe of CaM interacts with a region around amino acids 1975 and 1999 of RyR1, suggesting that CaM not only binds at a site of inter-subunit contact but may also span the two subunits.

These previous studies have been conducted on isolated SR vesicles and/or purified RyR1 channels, which removes the channel from the complex environment of the triad. In attempts to gain further understanding of the role of CaM in regulating SR Ca\(^{2+}\) release in a more fully constituted setting we have recently reported on the effect of CaM on spontaneous Ca\(^{2+}\) sparks in permeabilized frog skeletal muscle (9). Ca\(^{2+}\) sparks are local, discrete elevations in myoplasmic [Ca\(^{2+}\)] due to the opening of RyR (10, 11). The measurement of Ca\(^{2+}\) sparks provides a convenient tool to assess the function and regulation of RyR in a more physiological setting within a living muscle cell. We found that exogenously applied CaM localized to the triad and caused a highly cooperative dose-dependent increase in Ca\(^{2+}\) spark frequency. Two possible mechanisms for these effects are that CaM promotes activation of RyR1 either by disrupting an inter-subunit interaction that stabilizes the closed state and/or by coordinating the movement of all four subunits within an RyR1 tetramer to the open state.

If the CaM binding region of RyR1 (amino acids 3614–3643) is indeed an inter-subunit interaction site then addition of an exogenous peptide corresponding to this sequence might disrupt a native interaction between RyR subunits and also possibly interfere with the interaction of CaM at this contact site, either of which might result in an alteration of RyR activity. Therefore, we tested the effects of a synthetic peptide corresponding to 3614–3643 of RyR1 (R3614–3643) on spontaneous

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§ The abbreviations used are: SR, sarcoplasmic reticulum; RyR, sarcoplasmic reticulum calcium release channel, ryanodine receptor; CaM, calmodulin; FDHM, temporal half-duration; FWHM, spatial half-width; Alexa488-CaM, Alexa Fluor® 488-labeled CaM; (N+5)CaM, dominant negative CaM; R3614–3643, synthetic peptide corresponding to the region 3614–3643.
Ca\(^{2+}\) sparks. We found that R3614–3643 increases Ca\(^{2+}\) spark occurrence in permeabilized frog skeletal muscle fibers in a highly cooperative dose-dependent manner. The maximum increase in Ca\(^{2+}\) spark frequency produced by R3614–3643 was about half that produced by exogenous recombinant CaM. A single amino acid mutation within R3614–3643 (L3624D) abolished the activating effect of the peptide. Both R3614–3643 and L3624D bind CaM with similar affinity. Thus, the spark-activating effect of R3614–3643 cannot be attributed to “stripping” endogenous CaM from the fiber. Interestingly, the maximum increase in Ca\(^{2+}\) spark frequency produced by exogenous recombinant CaM was the same in the presence or absence of R3614–3643, even though R3614–3643 binds to CaM. Our results support a model in which the CaM binding region of RyR1 is a site of inter-domain or inter-subunit contact within RyR that stabilizes the closed state of the channel. Addition of either exogenous R3614–3643 or endogenous CaM disrupts the native interaction, thereby destabilizing the closed state of the channel, with CaM having a stronger destabilizing effect than R3614–3643. Some of these data have been presented in abstract form (12, 13).

**EXPERIMENTAL PROCEDURES**

**Synthesis and Purification of Peptides and Recombinant Calmodulin**—Peptides were synthesized in the core facility at the University of Maryland School of Medicine (Baltimore, MD). CaM was expressed and purified as previously described (9).

**Preparation of Skeletal Muscle Fibers**—Frogs (Rana pipiens) were first placed in a cold-induced torpor (crushed ice-water slurry, 20 min) followed by rapid decapitation and spinal cord destruction according to the method of Cheng et al. (1024-ms acquisition time, 2 ms per line, 768 pixels per line, and 0.18 
μm per pixel). Line scan images were processed to identify and store potential spark locations by an automated computer detection routine using a relative threshold algorithm as described by Cheng et al. (18) and analyzed as previously described (9). For each selected event, the peak amplitude (ΔF/F), 100% rise time, temporal half-duration (full duration at half-max, FWHM), and spatial half-width (full width at half-max, FWHHW) were determined from the temporal and spatial fits. Events with a ΔF/F < 0.4 were excluded from data analysis post hoc. The frequency of events per sarcomere was calculated from the number of sparks per image divided by the number of sarcomeres along the line and by the image duration (1.024 s).

To account for the variability in the starting Ca\(^{2+}\) spark frequency among fibers, each data point was normalized to the average Ca\(^{2+}\) spark frequency for the same group of fibers prior to the addition of exogenous protein. For the dose-dependent effect of R3614–3643 the data were fit to Equation 1,

\[
f = f\text{max}(R - \frac{1}{x}(k\text{x} + x^2)) + f\text{min}
\]  

(Eq. 1)

where \(f\) is the event frequency normalized to the average event frequency in the same group of fibers prior to R3614–3643 application, \(R\) is the fractional maximal increase in spark frequency, \(f\text{min}\) is the Hill coefficient, and \(K\) is the concentration of R3614–3643 that elicits 50% of the increase in frequency (\(E_\text{C50}\)).

**Fluorescence Spectroscopy**—The change in intrinsic tryptophan fluorescence of R3614–3643 or L3624D upon binding CaM was monitored using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments). CaM and either R3614–3643 or L3624D were added to final concentrations of 2 and 1 μM, respectively. Tryptophan excitation was set at 295 nm, and emission spectra were recorded from 310 to 400 nm. Final fluorescence data were obtained by subtracting CaM and buffer effects from those of the CaM plus peptide and then normalized to the fluorescence for R3614–3643 or L3624D alone. Because CaM contains no tryptophan residues, the observed fluorescence is attributed to the single tryptophan residue in either R3614–3643 or L3624D.

To assess the relative affinity of R3614–3643 and L3624D for CaM the fluorescence emission spectra of Alexa Fluor® 488-labeled CaM (Alexa488-CaM, Molecular Probes, Eugene, OR) were collected by exciting at 485 nm and recording between 500 and 600 nm. Increasing amounts of either R3614–3643 or L3624D (0.25–200 nM) were titrated against 1 mM Alexa488-CaM in a buffer containing 90 mM potassium glutamate, 20 mM Tris maleate, 1 mM EGTA, 1 mM dithiothreitol, pH 7.0. Final fluorescence data were obtained by subtracting peptide and buffer effects from those of the Alexa488-CaM plus peptide and correcting for minor dilution during titration.

A variant of the Hill model (Equation 2) was used to resolve the change in Alexa Fluor® 488-conjugated CaM fluorescence as a function of peptide concentration ([P]), returning the Hill coefficient \(n\) and peptide concentration yielding half-maximal binding ([P]_1/2). Nonlinear regression was performed using PRISM version 3.0.3 software (GraphPad).

\[
F(P) = F_{\text{CaM}} + F_{\text{CaM-P}} (\frac{([P]/[P]_{1/2})^n}{1 + ([P]/[P]_{1/2})^n})
\]  

(Eq. 2)

To estimate the fractional concentrations of each CaM species ([CaM]_free, [CaM-P], and [CaM-P2]) for given concentrations of total calmodulin ([CaM]_total) and peptide ([P]_total), it was necessary to first estimate the macroscopic association constants (\(K_1\) and \(K_2\)) describing each stage of CaM-P assembly. For a positively cooperative model, this is impossible without knowing the intrinsic fluorescence of the intermediate (CaM-P) species. However, inserting an approximation of this value into Equation 3 allowed estimation of \(K_1\) and \(K_2\) from regression of F[518] versus [P] plots, assuming [CaM]_total ≪ 1/K_1 or 1/K_2.

\[
F(518) = F_{\text{CaM}} + F_{\text{CaM-K1}} + F_{\text{CaM-P1-K1}} (\frac{([P]/[P]_{1/2})^n}{1 + ([P]/[P]_{1/2})^n})
\]  

(Eq. 3)

Using values of \(K_1\) and \(K_2\) estimated using Equation 3, equilibrium concentrations of all reaction species were resolved for any combination of [P]_total and [CaM]_total by the system of equations in Equations 4–7.

\[
[P]_\text{free} = (\frac{[P]_\text{total}}{[P]_{1/2}}) + 2([CaM]_\text{total}) - ([CaM]_\text{total})^2
\]  

(Eq. 4)

\[
[CaM]_\text{total} = ([CaM]_\text{free} + [CaM-P] + [CaM-P_2])
\]  

(Eq. 5)

\[
[CaM-P] = K_2 [CaM]_\text{free} [P]_\text{free}^2
\]  

(Eq. 6)

\[
[CaM-P_2] = K_1 [CaM]_\text{free} [P]_\text{free}^2
\]  

(Eq. 7)

All real solutions of ([P]_free and [CaM]_free) (>0 and >0) for this system of equations were determined for given values of [P]_total, [CaM]_total, \(K_1\), and \(K_2\) using Mathematica version 4.1 (Wolfram Research). Calculated values of [P]_free and [CaM]_free were then used to derive [CaM-P] and [CaM-P_2] using Equations 6 and 7, which were then expressed as fractional concentrations of [CaM]_total.

**Data Analysis**—Unless otherwise stated, results are reported as means ± S.E. Statistical analysis for comparison of means was performed using analysis of variance with a significance level of \(p < 0.05\). The spatial and temporal properties of Ca\(^{2+}\) sparks (amplitude, rise time, FWHM, and FWHHW) were not normally distributed; therefore a non-parametric analysis of variance was performed (Dunn’s post hoc analysis). All statistical analysis was performed with SigmaStat (Jandel Scientific), and non-linear curve fitting was performed in SigmaPlot (Jandel Scientific), unless otherwise noted.
**FIG. 1.** R3614–3643 increases spontaneous Ca\(^{2+}\) spark frequency. A, sequence alignment of the CaM binding region (3614–3643) for rabbit RyR1, frog RyRα, and frog RyRβ. The **underlined** residues denote differences between isoforms in the primary structure. B, linescan images for Sham and 3 μM R3614–3643. Images display the fiber fluorescence as ∆F/F from fibers loaded with the Ca\(^{2+}\) indicator dye Fluo-3 (50 μM). Below each ∆F/F image is the temporal time course for the line indicated by the arrow. Addition of 3 μM R3614–3643 increased the number of spontaneous Ca\(^{2+}\) sparks when compared with Sham controls. C, dose-dependent effect of R3614–3643 on spontaneous Ca\(^{2+}\) spark frequency. The frequency of spontaneous Ca\(^{2+}\) sparks before and after the addition of various concentrations of R3614–3643 (0.2–3.0 μM) at a [Mg\(^{2+}\)]\(_{100}\) of 0.65 mM. To control for variability in the resting Ca\(^{2+}\) spark frequency, the frequency of each fiber in the presence of the indicated R3614–3643 was normalized to the average frequency for that group of fibers prior to addition of R3614–3643. The data for no added R3614–3643 are buffer and time controls (sham) as described under “Experimental Procedures.” The **solid line** was obtained by fitting the data to Equation 1. The fractional maximal increase in spark frequency was 5.3 ± 1.4, the EC\(_{50}\) was 0.47 ± 0.02 μM, and the Hill coefficient was 8.2. All data points are presented as mean ± S.E. for at least three fibers at each R3614–3643 concentration.
results

R3614–3643 Increases the Frequency of Occurrence of Spontaneous Ca\textsuperscript{2+} Sparks—A sequence alignment of the CaM binding sequence (3614–3643) from rabbit RyR1 with that of RyR\textalpha{} and RyR\textbeta{} from frog shows that this region is highly conserved (Fig. 1A). Therefore, we assessed the effects of a synthetic peptide representing amino acids 3614–3643 of the rabbit RyR1 sequence on RyR function in frog skeletal muscle. The frequency of occurrence of spontaneous Ca\textsuperscript{2+} sparks provides a measurement of the activation state of the RyR channels that give rise to the Ca\textsuperscript{2+} spark (19, 20). Application of R3614–3643 resulted in an increase in the frequency of spontaneous Ca\textsuperscript{2+} sparks, indicating an increase in the rate of activation of RyR. Representative \Delta F/F\textsubscript{0} linescan images from control conditions and after application of R3614–3643 (3.0 \mu M) are shown in Fig. 1B. The concentration dependence of the activation of Ca\textsuperscript{2+} sparks is shown in Fig. 1C. Addition of R3614–3643 (0.2–3.0 \mu M) resulted in a highly cooperative, dose-dependent increase in Ca\textsuperscript{2+} spark frequency. Fitting the data to Equation 1 resulted in a fractional maximal increase (R) of 5.3 \pm 1.4, with a half-maximal activation (EC\textsubscript{50}) of 0.47 \pm 0.02 \mu M and a Hill coefficient (n) of 8.2.

The spatial and temporal properties of Ca\textsuperscript{2+} sparks reflect the underlying channel kinetics and resulting Ca\textsuperscript{2+} flux, which underlie the Ca\textsuperscript{2+} spark. To assess whether R3614–3643 alters the kinetics of the Ca\textsuperscript{2+} release event we measured the spatial and temporal properties of the Ca\textsuperscript{2+} sparks initiated by R3614–3643. The effect of R3614–3643 (3 \mu M) on the spatial and temporal properties of Ca\textsuperscript{2+} sparks is shown in Fig. 2. Ensemble averages and surface plots of identified Ca\textsuperscript{2+} spark events for Sham and 3 \mu M R3614–3643 are shown in Fig. 2A. Despite an approximate 5-fold increase in Ca\textsuperscript{2+} spark frequency only small differences in the spatial and temporal properties were observed (Fig. 2B). Median values of the population distribution for 3 \mu M R3614–3643 showed a small but statistically significant increase for FWHM and FWHM, no differences were observed for amplitude or rise time.

A Single Amino Acid Substitution Abolishes the Activating Effect of R3614–3643—Given that the 3614–3643 region of RyR lies at an inter-subunit contact site (7, 8) and is also a binding site for CaM (21), it is conceivable that the increase in the rate of activation of RyR observed with R3614–3643 could be due to the peptide binding to either the inter-subunit contact site on RyR and/or binding to native CaM. In an effort to distinguish these possibilities we made the synthetic peptide R3614–3643 with a single amino acid mutation, L3624D. This mutation, when made in full-length RyR1, resulted in the loss of both CaM binding and functional regulation of RyR1 by CaM in SR vesicle preparations (6). Application of L3624D (2 \mu M and 10 \mu M) did not result in an increase in the frequency of occurrence of spontaneous Ca\textsuperscript{2+} sparks (Fig. 3). When added in combination with R3614–3643, excess L3624D (10 \mu M) attenuated the activation of Ca\textsuperscript{2+} sparks observed with R3614–3643 (2 \mu M, Fig. 3), suggesting competition for a similar binding site.

R3614–3643 and L3624D Bind CaM with Similar Affinity—It is conceivable that the observed increase in Ca\textsuperscript{2+} spark frequency produced by R3614–3643 might be due to either: 1) a direct effect of R3614–3643 on RyR, 2) an indirect effect produced by R3614–3643 binding endogenous CaM and thereby removing CaM from the fiber, or 3) a combination of the two effects. In efforts to distinguish these mechanisms we assessed the binding of CaM to R3614–3643 and L3624D. Intrinsic tryptophan fluorescence of R3614–3643 and L3624D was monitored under saturation binding conditions (Fig. 4). CaM (2 \mu M) binding to R3614–3643 (1 \mu M) resulted in an increase and a 10 nm blue shift in the peak intrinsic tryptophan fluorescence of R3614–3643 (Fig. 4A). Upon binding CaM (2 \mu M), the peak intrinsic tryptophan fluorescence of L3624D (1 \mu M) also increased in a similar fashion to that for R3614–3643; however, there was little to no shift in the wavelength at which the peak tryptophan fluorescence occurred (Fig. 4B). These data indicate that CaM is able to bind both R3614–3643 and L3624D, but the lack of blue shift observed in the CaM-L3624D interaction suggests that there is some difference in the mechanism or structural consequence of the interaction of CaM with these two peptides.

We next assessed the relative affinity of both R3614–3643 and L3624D to bind CaM. Both R3614–3643 and L3624D (0.25–200 nM) quenched the fluorescence of Alexa488-CaM in a dose-dependent manner (Fig. 5, A and B, respectively). A plot of the fluorescence intensity at 518 nm as a function of the concentration of peptide added (Fig. 5C) shows that each peptide virtually fully quenched the Alexa488-CaM fluorescence. The concentration of R3614–3643 for a half-maximal quenching of Alexa488-CaM fluorescence is 22 \pm 2 nM, with a Hill coefficient of 1.6 \pm 0.2. The concentration of L3624D needed for half-maximal quenching of Alexa488-CaM fluorescence is 33 \pm 2 nM, with a Hill coefficient of 1.5 \pm 0.1. The apparent affinity and Hill coefficients of R3614–3643 and L3624D for CaM are on the same order of magnitude as previously reported for R3614–3643 (21). Also, the apparent affinity of CaM for either R3614–3643 or L3624D is similar to the affinity of CaM for RyR1 (21). To ensure that the quenching of the Alexa488-CaM fluorescence was not due to nonspecific effects of the peptides we monitored the fluorescence of Alexa488-CaM in the presence of insulin \beta chain (3.5 kDa), which has a molecular mass comparable to R3614–3643 and L3624D (3.7 kDa). Insulin \beta chain (150 nM) resulted in less than a 5% quenching of the Alexa488-CaM fluorescence (data not shown). Our finding that the L3624D mutant does not increase the occurrence of spontaneous Ca\textsuperscript{2+} sparks cannot be attributed to a loss in CaM binding to the mutated peptide, suggesting that this single amino acid mutation does not abolish the spark-activating effect by disrupting the ability R3614–3643 to bind CaM. Instead the mutation likely disrupts the association of the R3614–3643 peptide with its complimentary contact site within RyR. Therefore, removal of endogenous CaM from RyR by R3614–3643 is unlikely to contribute to the increase in Ca\textsuperscript{2+} spark frequency.

The Effects of R3614–3643 and Recombinant CaM on Ca\textsuperscript{2+} Sparks Are Not Additive—We have previously shown that recombinant CaM increased the frequency of spontaneous Ca\textsuperscript{2+} sparks in frog skeletal muscle fibers (9). We next set out to determine whether the increase in Ca\textsuperscript{2+} spark frequency observed with either R3614–3643 alone (Fig. 1) or CaM alone (see Fig. 7 of Ref. 9) are additive. If R3614–3643 and CaM were activating RyR by different independent mechanisms, we might expect to observe some degree of additive effect upon the application of both ligands. However, in light of the fact that these two ligands bind each other in a cooperative manner (see Fig. 5C) it is also conceivable that the combination of R3614–3643 and CaM might have no effect on RyR channel activation. Addition of R3614–3643, at either 2 or 4 \mu M, in combination with recombinant wild type CaM (2 \mu M) resulted in an increase in Ca\textsuperscript{2+} spark frequency that was not different from CaM (2 \mu M) alone (Fig. 6A). One possible explanation of this finding might be that in the presence of bound exogenous CaM, R3614–3643 cannot bind to RyR. To test this possibility we made use of the dominant negative CaM ((N\textsuperscript{+3}CaM). Previous studies have demonstrated that (N\textsuperscript{+3}CaM binds to isolated RyR1 with ~5-fold higher affinity than wild type CaM, binds R3614–3643 similar to wild type CaM, prevents the localization of fluorescently labeled wild type CaM (Alexa488-CaM) in
Fig. 2. Effect of R3614–3643 on the spatial and temporal properties of spontaneous Ca
sparks. A, average spark image and the spatial and temporal profiles through the peak \( \Delta F/F \) for Sham (left, \( n_{\text{events}} = 107 \)) and 3 \( \mu \text{M} \) R3614–3643 (right, \( n_{\text{events}} = 836 \)). The surface plot for the average event is displayed below. B, distribution of the spatial (amplitude and FWHM) and temporal (rise time and FFDHM) properties of Ca
sparks for Sham (open bars) and R3614–3643 (hatched bars). The box plots represent the 25th, 50th (median), 75th percentiles, whereas the error bar represents the 10th and 90th percentiles. \( p < 0.05 \) versus Sham (a).
permeabilized muscle fibers, and prevents the increase in spark frequency due to exogenous wild type CaM in permeabilized muscle fibers, but it does not itself increase the activation rate of RyR (9, 22). Fig. 6B shows that in the presence of (N+3)CaM (2 μM), R3614–3643 (2 μM) still increased Ca\(^{2+}\) spark frequency to a level that is not different from R3614–3643 alone.

To determine which ligand species might be leading to the activation of Ca\(^{2+}\) sparks during simultaneous application of CaM and R3614–3643, we used the binding data obtained in Fig. 5C and Equations 3–7 to simulate the fractional concentrations of each CaM species ([CaM\(_\text{free}\)], [CaM-P]), and [CaM-P\(_2\)] for the given concentrations of total CaM ([CaM]\(_\text{total}\)) and R3614–3643 ([P]\(_\text{total}\)). Data for R3614–3643 are the same as presented in Fig. 1C. Bars represent mean ± S.E. p < 0.05 versus Sham (a), R3614–3643 (b), or L3624D (c).}

**Fig. 3.** The L3624D mutation within R3614–3643 does not increase Ca\(^{2+}\) spark frequency. Frequency was determined as the mean number of events identified in each fiber and normalized to the mean frequency of that group of fibers prior to the addition of either R3614–3643 (2 μM), L3624D (2 μM), L3624D (10 μM), or the combination of R3614–3643 (2 μM) plus L3624D (10 μM). Data for R3614–3643 are the same as presented in Fig. 1C. Bars represent mean ± S.E. p < 0.05 versus Sham (a), R3614–3643 (b), or L3624D (c).
FIG. 4. Changes in the intrinsic tryptophan fluorescence of R3614–3643 and L3624D upon CaM binding. Intrinsic tryptophan fluorescence was monitored in the absence (open circle) and presence (closed circle) of CaM (2 μM) for R3614–3643 (A, 1 μM) or L3624D (B, 1 μM) in internal solution used for Ca^{2+} sparks studies. Data were normalized to the peak fluorescence in the absence of CaM and are plotted as mean ± S.E.

FIG. 5. Changes in the Alexa488-CaM fluorescence intensity as a function of concentration of the peptides R3614–3643 or L3624D. The fluorescence emission spectra of Alexa488-CaM (1 nM) were recorded in the presence of increasing amounts of either R3614–3643 (A) or L3624D (B). The fluorescence intensity at 518 nm of each spectrum was plotted as a function of [peptide] (C) for R3614–3643 (closed circles) and L3624D (open circles). The solid lines were obtained by fitting the data to Equation 2. The peptide concentrations yielding half-maximal binding were 22 ± 2 nM and 33 ± 2 nM for R3614–3643 and L3624D, respectively. The Hill coefficients for R3614–3643 and L3624D were 1.6 ± 0.2 and 1.5 ± 0.1, respectively. All data points are presented as mean ± S.E. for three independent titrations.
neously interact with RyR, but at different sites, and that with respect to the functional state of RyR this interaction is not additive. Future structural studies probing the conformational state of RyR in the presence of single and multiple ligands will provide valuable information for our understanding of the mechanisms of RyR channel regulation and thus regulation of SR Ca$^{2+}$/H$^{+}$ release.

Valdivia and colleagues (25) have suggested that the activation of RyR1 by the CaM-binding peptide in their isolated SR vesicle experiments may be due to formation of a disulfide bond between the cysteine residue located at position 22 of the synthetic peptide, corresponding to Cys$^{3635}$ of RyR1, and a highly reactive cysteine residue in another region within RyR1. This highly reactive cysteine within RyR could be either Cys$^{3635}$ or one of the other cysteine residues thought to be involved in an inter-subunit disulfide bond formation within RyR1, located somewhere between amino acids 2000 and 2401 (8). In our experiments the internal solution used for dilution of stock peptide as well as to record Ca$^{2+}$/H$^{+}$ sparks contains 3 mM dithiothreitol (see "Experimental Procedures"). This concentration is likely sufficient to maintain reducing conditions within the muscle fiber. Increasing the concentration of dithiothreitol up to 8 mM did not alter the ability of R3614–3643 to increase Ca$^{2+}$/H$^{+}$ spark frequency (data not shown). Furthermore, the mutant peptide L3624D also contains the cysteine at position 22, and if disulfide bond formation between the synthetic peptide and a region of RyR was required for the activating effect of R3614–3643, then L3624D should have also increased Ca$^{2+}$/H$^{+}$ spark frequency. However, L3624D had no effect on Ca$^{2+}$/H$^{+}$ spark frequency. In addition, Meissner and colleagues (26) have suggested that Cys$^{3635}$ of RyR1 does not sig-

![Fig. 6. R3614–3643 does not alter the CaM effect on Ca$^{2+}$/H$^{+}$ spark frequency. Frequency was determined as the mean number of events identified in each fiber and normalized to the mean frequency of that group of fibers prior to the addition of either CaM plus R3614–3643 (A) or (N+3)CaM plus R3614–3643 (B). Ligand combinations were mixed prior to application to the muscle fiber. Data for R3614–3643 are the same as that presented in Fig. 1C. Bars represent mean ± S.E. p < 0.05 versus Sham (a), R3614–3643 (b), and (N+3)CaM (c).]
acids mutations within RyR1 can lead to altered SR Ca2⁺ release. Our findings that a single amino acid mutation within domain peptide 4 prevents the peptide from disrupting the domain interaction within RyR1 and thereby abolishing the channel activity. The CaM binding region of RyR interacts with another subunit within RyR, stabilizing a closed state of the channel. Addition of R3614–3643 disrupts this native subunit-subunit interaction, promoting opening of the channel by destabilizing the closed state. 

A region between 1393 and 1527 of the carboxytail of the α1s subunit of the DHPR may also interact with the 3614–3643 region of RyR, independent of CaM (27). Hamilton and colleagues (28) have also previously shown that a synthetic peptide within this region (amino acids 1487–1506) inhibited [3H]ryanodine binding to SR vesicles and decreased single channel activity of RyR1 channels incorporated into planar lipid bilayers. Therefore, it is possible that an interaction between the 3614–3643 region of RyR and the 1487–1506 region of the DHPR stabilizes a closed state of RyR. Addition of R3614–3643 may disrupt this interaction, destabilizing a closed state of RyR and thereby increasing the frequency of Ca2⁺ sparks. At present, we cannot rule out this possibility, but future studies using these regions of the carboxy-tail of the α1s subunit of the DHPR should help provide further insight into these possible mechanisms.

**Table 1**

| Efficiency (%) | $K_1$ (×10⁻⁷ M⁻¹) | $K_2$ (µM) | [CaM]₀ (µM) | [P]₀ (µM) | [CaM-P]₀ (µM) | [CaM-P₂] (µM) | Fractional concentrations |
|----------------|------------------|-----------|-------------|-----------|----------------|----------------|-------------------------|
| 20             | 3.180            | 9.05      | 781         | 18.4      | 457            | 761            | 0.391 0.228 0.380       |
| 50             | 2.300            | 8.49      | 781         | 21.6      | 416            | 784            | 0.402 0.208 0.392       |
| 80             | 1.910            | 8.49      | 803         | 24.5      | 354            | 798            | 0.410 0.192 0.399       |

| Efficiency (%) | $K_1$ (×10⁻⁷ M⁻¹) | $K_2$ (µM) | [CaM]₀ (µM) | [P]₀ (µM) | [CaM-P]₀ (µM) | [CaM-P₂] (µM) | Fractional concentrations |
|----------------|------------------|-----------|-------------|-----------|----------------|----------------|-------------------------|
| 20             | 3.180            | 9.05      | 22.9        | 168       | 122            | 1860           | 0.011 0.061 0.930       |
| 50             | 2.400            | 8.72      | 28.3        | 177       | 120            | 1855           | 0.014 0.080 0.908       |
| 80             | 1.910            | 8.49      | 33.4        | 185       | 118            | 1854           | 0.017 0.059 0.927       |

*Quenching efficiency of the first peptide binding event (i.e. Alexa488-CaM + P ⇔ CaM-P).*

**Fig. 7. Proposed mechanism for how R3614–3643 increases channel activity.** The CaM binding region of RyR interacts with another subunit within RyR, stabilizing a closed state of the channel. Addition of R3614–3643 disrupts this native subunit-subunit interaction, promoting opening of the channel by destabilizing the closed state.

significantly contribute to redox modulation of RyR1 by O₂ tension and glutathione. Taken together, these data indicate that it is unlikely that disulfide bond formation is involved in the activation of RyR by R3614–3643. Our finding that a single amino acid mutation (L3624D) within the synthetic peptide destroys the ability of R3614–3643 to increase Ca2⁺ spark frequency may provide some insight into the conformation of the inter-subunit interaction within RyR. The loss of a functional effect upon mutating a single amino acid within a putative domain-domain interaction site is analogous to studies conducted by Yamamoto et al. (23) who showed that a single amino acid mutation within domain peptide 4 prevented the peptide from disrupting the domain-domain interaction within RyR1 and thereby abolishing the activating effect of domain peptide 4. Interestingly, the mutation made in domain peptide 4 corresponds to a mutation occurring in RyR1 that is associated with malignant hyperthermia (23), providing some indications as to how single amino acids mutations within RyR1 can lead to altered SR Ca2⁺ release. Our findings that the L3624D peptide had no effect on the functional state of the channel would support a model in which the L3624D mutation in the full-length RyR would destabilize this region of RyR, leading to an altered inter-subunit interaction. Although there are no known mutations that have been identified within the 3614–3643 CaM binding region that lead to an altered RyR channel, our finding that a single residue change within this region (L3624D) removes the effective-
region of RyR is involved in an inter-subunit interaction that stabilizes a closed state of the channel. The binding of peptide R3614–3643 to its corresponding contact site in RyR would disrupt this native interaction, destabilize the closed state of the channel, and lead to an increase in the rate of activation of RyR. A single amino acid mutation within this peptide (L3624D) prevents the R3614–3643 peptide from disrupting the inter-subunit interaction but does not appreciably alter the peptide binding to CaM. The results from this study provide further detail into intrinsic RyR interactions as well as RyR-ligand interactions and thus provide further insight into the basic mechanisms of SR Ca\(^{2+}\) release.

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