Calmodulin in adult mammalian skeletal muscle: localization and effect on sarcoplasmic reticulum Ca\(^{2+}\) release

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Rodney George G. Calmodulin in adult mammalian skeletal muscle: localization and effect on sarcoplasmic reticulum Ca\(^{2+}\) release. Am J Physiol Cell Physiol 294: C1288–C1297, 2008. First published March 5, 2008; doi:10.1152/ajpcell.00033.2008.—Calmodulin is a ubiquitous Ca\(^{2+}\) binding protein that binds to ryanodine receptors (RyR) and is thought to modulate its activity. Here we evaluated the effects of recombinant calmodulin on the rate of occurrence and spatial properties of Ca\(^{2+}\) sparks as an assay of activation in saponin-permeabilized mouse myofibers. Control myofibers exhibited a time-dependent increase and subsequent decrease in spark frequency. Recombinant wild-type calmodulin prevented the time-dependent appearance of Ca\(^{2+}\) sparks and decreased the derived Ca\(^{2+}\) flux from the sarcoplasmic reticulum during a spark by \(~37\%. A recombinant Ca\(^{2+}\)-insensitive form of calmodulin resulted in an instantaneous increase in spark frequency as well as an increase in the derived Ca\(^{2+}\) flux by \(~24\%). Endogenous calmodulin was found to primarily localize to the Z-line. Surprisingly, removal of endogenous calmodulin did not alter the time dependence of Ca\(^{2+}\) spark appearance. These results indicate that calmodulin may not be essential for RyR1-dependent Ca\(^{2+}\) release in adult mammalian skeletal muscle.

Ryanodine receptor; calcium sparks; calcium-induced calcium release; calcium signaling; calcium imaging

IN VERTEBRATE SKELETAL MUSCLE, depolarization of the sarcolemma travels deep into the muscle fiber via sarcocemmal invaginations called the transverse tubules (t-tubules). Activation of the dihydropyridine receptor (DHPR), a voltage-dependent Ca\(^{2+}\) channel within the t-tubule, results in Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) through a process termed excitation-contraction coupling (ECC). Ca\(^{2+}\) is released from the SR through a large homotetrameric protein in the SR membrane, the ryanodine receptor (RyR). In mammals three RyR isoforms exist (RyR1–3), with the primary isoform in adult skeletal muscle being RyR1 (41) and a small amount (1–5%) of RyR3 in specific muscles such as the diaphragm and soleus (8, 21). In addition to the regulation by the DHPR, RyR1 also binds and is thought to be regulated by numerous endogenous molecules, including the Ca\(^{2+}\)-binding protein calmodulin (CaM).

One molecule of CaM is bound to each RyR1 monomer at both nonanomol and micromolar levels of Ca\(^{2+}\), for a total of four CaM molecules per RyR1 tetramer (29). In in vitro SR vesicle preparations CaM displays Ca\(^{2+}\) dependence in its functional effects on RyR1. At nanomolar levels of free Ca\(^{2+}\), Ca\(^{2+}\)-free CaM sensitizes RyR1 to activation, whereas at micromolar levels of free Ca\(^{2+}\), Ca\(^{2+}\) CaM inhibits RyR1 (35).

Using a mutant CaM that cannot bind Ca\(^{2+}\) (CaM123A), Rodney et al. (35) showed that Ca\(^{2+}\) binding to CaM converts CaM from an activator to an inhibitor of RyR1 in SR vesicles and planar lipid bilayers. These studies suggested that CaM does not simply sensitize RyR1 to Ca\(^{2+}\) but that CaM senses changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]), transducing these changes into functional alterations of RyR1. Recently, Yamaguchi et al. (48) have shown that similar to RyR1, RyR3 has a single conserved binding site for CaM. However, under low [Ca\(^{2+}\)], CaM activates RyR3 to a greater extent than RyR1; providing evidence for isoform-dependent regulation of RyR by CaM.

In muscle, localized discrete elevations in myoplasmic Ca\(^{2+}\) sparks (5, 25), arise from the opening of clusters of RyR channels. It is well established that these Ca\(^{2+}\) sparks provide a sensitive assay of the activation and modulation of RyR in a quasiphysiological setting. In permeabilized depolarized frog skeletal muscle, spontaneous Ca\(^{2+}\) sparks are initiated by ligand activation and are terminated by inactivation of RyR; with both being independent of the voltage sensor (25). Using Ca\(^{2+}\) sparks as an assay to monitor RyR function within frog skeletal muscle, we have previously reported that exogenously applied recombinant CaM localized to the triad and caused a highly cooperative dose-dependent increase in Ca\(^{2+}\) spark frequency (36). However, we did not observe any changes in the spatial and temporal properties of the release events, suggesting that Ca\(^{2+}\) binding to exogenous CaM does not participate in Ca\(^{2+}\) spark termination. To gain further insight into the CaM modulation of RyR-dependent SR Ca\(^{2+}\) release, we now extend our work to a mammalian preparation. Unlike the frog, intact adult mammalian myofibers do not produce voltage-activated Ca\(^{2+}\) sparks. This difference may be due to the presence of RyRβ (3) in the frog (27, 31, 46). It is well accepted, however, that although Ca\(^{2+}\) sparks occur less frequently in permeabilized mammalian myofibers, they remain a robust reliable assay of RyR1 function (10, 19, 24, 44, 45, 50). Therefore, we examined the effect of CaM on Ca\(^{2+}\) sparks in permeabilized adult mouse myofibers. Recombinant CaM resulted in a time-dependent alteration of Ca\(^{2+}\) sparks. Surprisingly, removal of endogenous CaM did not alter the occurrence of Ca\(^{2+}\) sparks, whereas immunofluorescence localization of endogenous CaM suggests that the triad may not be the predominant locus for CaM within the myofiber. These results indicate that CaM may not be essential for RyR1-dependent Ca\(^{2+}\) release in adult mammalian skeletal muscle.

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MATERIALS AND METHODS

Fiber preparation. Adult CD-1 mice (Charles River) were killed by CO₂ asphyxiation in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore, MD. Diaphragm and flexor digitorum brevis (FDB) muscles were dissected and enzymatically dissociated with either a combination of collagenase A (2 mg/ml, Roche) and Dispase II (6 mg/ml, Roche) (diaphragm) or collagenase A (2 mg/ml) alone (FDB) dissolved in essentially Ca²⁺-free HEPES-buffered rodent Ringer solution containing (in mM) 146 NaCl, 4.7 KCl, 0.6 MgSO₄, 1.6 NaCO₃, 0.13 NaH₂PO₄, 7.8 glucose, 20 HEPES, pH 7.3 supplemented with 10% fetal bovine serum (FBS, Biofluids) at 37°C for 1.5–2.5 h. Fiber bundles were then transferred to Ca²⁺-free HEPES-buffered Rodent Ringer solution containing (in mM) 90 K-sulfate, 10 creatine phosphate, 5 Na-ATP, 5 glucose, 0.1 EGTA, 7 Mg²⁺, 10 HEPES, and 0.05 Fluo-4 pentapotassium salt. Fibers were then exposed to an internal solution containing 0.05 mM Fluo-4 with or without CaM or CaM1234 (2 μM) before image acquisition. The free [Ca²⁺] was calculated to be 100 nM using MAXC v2.5 (1).

Fibers were imaged for spontaneous Ca²⁺ sparks on an inverted microscope (Olympus IX-71 with a ×60, 1.3 numerical aperture water-immersion objective) coupled to a Bio-Rad CellMap IC laser scanning confocal imaging system. A sequence of 40 XY images was collected over time after solution change to either internal solution without any added CaM (control) or an internal solution containing recombinant drosophila CaM (dCaM), either wild-type dCaM (2 μM), or CaM1234 (2 μM). The frequency of sparks was determined by using a modified automatic detection method as previously described (6). Briefly, an average fiber fluorescence image was generated by calculating the mean fluorescence pixel by pixel of all XY images. The region of the image corresponding to the fiber was manually defined as an area of interest, and potential local Ca²⁺ sparks were identified as contiguous pixels exhibiting fluorescence ≥ 1.5 SD above the mean fiber fluorescence. Regions selected as local Ca²⁺ events were identified in ΔF/F images as contiguous regions of pixels having fluorescence values ≥ 2 SD above the mean fluorescence and were selected by the criterion that at least 1 pixel within the 2 SD area must exceed 3 SD above the mean. Ca²⁺ sparks were characterized in the ΔF/F image by the measured parameters peak amplitude (peak ΔF/F) and full area integral (VIHM; μm²) and equivalent parameters of equivalent diameter (EDHM; μm) and equivalent volume integral (VIHM; μm³*ΔF/F) at half-maximal fluorescence as previously detailed by Chun et al. (6).

A

Control

Wild-type dCaM

dCaM1234

10 μm

B

Frequency (events/μm²s)

0.0 0.5 1.0 1.5 2.0

Time (seconds)

0 500 1000 1500 2000 2500 3000

C

Frequency (events/μm²s)

0.0 2.5 5.0 7.5 10.0

Control Wild-type CaM CaM1234

*P < 0.05 vs. control and wild-type dCaM.

Fig. 1. Effect of wild-type drosophila CaM (dCaM) and a mutant CaM that cannot bind Ca²⁺ (dCaM1234) on the appearance of Ca²⁺ sparks in diaphragm. A: representative ΔF/F images showing the occurrence of Ca²⁺ sparks (arrowheads). B: under control conditions (circles) Ca²⁺ spark frequency shows a time-dependent increase, peaking at 394 s. Wild-type dCaM (triangles) decreases the frequency of Ca²⁺ sparks. dCaM1234 (squares) results in an increase in the frequency of Ca²⁺ sparks and a shift in the time of peak frequency to 516 s. C: dCaM1234 increases the average frequency of Ca²⁺ spark occurrence by about 169% over the entire recording time. *P < 0.05 vs. control and wild-type dCaM.
Localization of recombinant CaM. Localization of recombinant CaM in saponin-permeabilized fibers was performed using Alexa Fluor 488 (Molecular Probes, Eugene, OR) labeled mammalian CaM (mCaM488, 1 μM) in conjunction with labeling of the actin cytoskeleton with Texas Red-X phalloidin (775 nM, Molecular Probes) for 20 min in internal solution as previously described (36). Alexa Fluor 488 is a 643-Da succinimidyl ester that is conjugated to the NH2-terminus of CaM.

Immunofluorescence localization of endogenous CaM. Myofibers were fixed with 4% paraformaldehyde in PBS for 10 min, washed three times with PBS, and permeabilized in 0.1% Triton for 10 min. Fibers were then incubated in 8% goat serum (Jackson Immunoresearch) for 1 h at 4°C. Fibers were incubated overnight at 4°C with a monoclonal antibody against CaM (mouse anti-calmodulin, Zymed) in 2% goat serum followed by labeling with an Alexa488-conjugated goat anti-mouse secondary antibody (Molecular Probes). Fibers were then washed three times with PBS and blocked with 8% donkey serum followed by incubation with the indicated primary antibody overnight. Myofibers were then washed three times with PBS containing 2% serum (donkey or goat) at room temperature, followed by incubation with either Cy5- or Alexa635-conjugated anti-mouse or anti-rabbit IgG overnight at 4°C. Images were acquired on either a Olympus FV500 or a Zeiss 510 fluorescent laser scanning confocal microscope. Secondary antibody labeling showed no detectable fluorescence pattern. For colocalization analysis, two-dimensional (X-Y) images were acquired sequentially so as to prevent spectral bleed through.

Data analysis. Data are reported as means ± SE, unless otherwise specified. Statistical analysis was performed in Sigma Stat (Jandel) with a significance level of P < 0.05. Colocalization analysis was performed in Volocity (Improvision).

RESULTS

The observation that spontaneous Ca2+ sparks do occur in permeabilized mammalian skeletal muscles (24) affords us the opportunity to study the effects of putative protein modulators of RyR1 on the SR Ca2+ release process in mammalian cells. In these studies Ca2+ sparks were used as a tool to assess the role of CaM in modulating RyR1 function in situ while immunofluorescence studies were undertaken to localize endogenous CaM in mammalian skeletal muscle.

Effect of dCaM on spontaneous Ca2+ sparks. Figure 1A shows representative ΔF/F images of Ca2+ sparks in saponin-permeabilized diaphragm myofibers in the presence of buffer (Control), wild-type dCaM, and dCaM1234. A detailed analysis revealed that peak Ca2+ spark occurrence was at 394 ± 28 s, followed by a decrease (Fig. 1B). Because of the time required for buffer exchange between control solutions and test solutions (e.g., wild-type dCaM or dCaM1234) and this time-dependent appearance of Ca2+ sparks, experiments were conducted under conditions in which the test solution was not paired with a control recording. In other words, after removal of the saponin solution the fiber was exposed to internal solution containing either control buffer, dCaM, or dCaM1234. Incubation of diaphragm fibers with wild-type dCaM (2 μM) effectively suppressed the time-dependent increase in spark frequency, the Ca2+ spark frequency remained relatively constant and low throughout the recording (Fig. 1B). dCaM1234 (2 μM), however, resulted in an increase in spark frequency at practically all time points compared with all other conditions tested. The time-to-peak Ca2+ spark occurrence was also shifted in the presence of dCaM1234 compared with controls (516 ± 44 vs. 394 ± 28 s, respectively, Fig. 1B). Figure 1C shows that on average over the entire recording time dCaM1234 increased Ca2+ spark frequency by 169% when compared with controls. The events that occur under these conditions result from voltage-independent SR Ca2+ release, and therefore, these data support the hypothesis that Ca2+-free CaM sensitizes RyR1 to Ca2+-induced Ca2+ release (CICR).

Assessment of the spatial parameters of Ca2+ sparks provides an estimate for the effect of wild-type dCaM and dCaM1234 on the amount of Ca2+ released during a spontaneous Ca2+ release event. When compared with controls, wild-type dCaM slightly increased (+4.5%) the peak amplitude (ΔF/F) but significantly decreased (−36.7%) the volume integral (i.e., mass) of Ca2+ release (Fig. 2A, Table 1). dCaM1234 increased the spatial and mass parameters of the release events compared with controls. There was a 24.3% increase in ΔF/F and a 24.4% increase in the volume integral (Fig. 2B, Table 1). Taken together, these data indicate that wild-type dCaM re-
roduces the likelihood of eliciting SR Ca\textsuperscript{2+} release, whereas dCaM\textsubscript{1234} sensitizes RyR1 to open. The observed changes in VIHM could represent alterations in the amount of Ca\textsuperscript{2+} released; i.e., wild-type dCaM decreasing and dCaM\textsubscript{1234} increasing the amount of Ca\textsuperscript{2+} released. Alternatively, the decrease in VIHM with wild-type dCaM may represent an increase in Ca\textsuperscript{2+} buffering within the myoplasm.

Previously, in amphibian skeletal muscle, wild-type dCaM increased Ca\textsuperscript{2+} spark frequency (36), just the opposite of what was observed in the present studies. Both amphibian skeletal muscle and mammalian diaphragm express RyR1 and RyR3, although diaphragm expresses RyR3 to a much lesser extent. To further investigate the differences observed between amphibian muscle and mammalian diaphragm, the effect of wild-type dCaM on Ca\textsuperscript{2+} spark frequency was examined in a mammalian fast-twitch muscle that expresses only RyR1 (e.g., FDB). Figure 3 shows that wild-type dCaM decreased Ca\textsuperscript{2+} spark frequency while dCaM\textsubscript{1234} increased Ca\textsuperscript{2+} spark frequency in FDBs. When compared with the diaphragm, wild-type dCaM was much more effective in inhibiting Ca\textsuperscript{2+} sparks, whereas dCaM\textsubscript{1234} was much less effective at activating Ca\textsuperscript{2+} sparks. Since amphibian skeletal muscle contains equal proportions of RyR1 and RyR3 (\alpha and \beta, respectively) while mammalian skeletal muscle expresses little (diaphragm) to no (FDB) RyR3, these data suggest that CaM shows isoform specificity in modulation of SR Ca\textsuperscript{2+} release.

Kinetics and localization of exogenous CaM. To assess the kinetics of CaM binding within the permeabilized myofiber, the diffusion of fluorescently labeled recombinant CaM (mCaM\textsubscript{488}) was assessed. Figure 4A shows the time course for washin of mCaM\textsubscript{488} (1 \textmu M) immediately after permeabilization. The data represent the fluorescence within the myofiber after subtraction of background fluorescence, which includes free mCaM\textsubscript{488}. These data were best fit by a double exponential, with a first phase time constant (\tau\textsubscript{1}) of 1.9 \pm 1.1 min and a second phase time constant (\tau\textsubscript{2}) of 12.0 \pm 2.6 min. The dissociation of mCaM\textsubscript{488} from the myofiber was also assessed. 

Table 1. Effect of wild-type dCaM and dCaM\textsubscript{1234} on the mean spherical and mass properties of Ca\textsuperscript{2+} sparks in diaphragm muscle

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<thead>
<tr>
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<th>Amplitude \Delta F/F</th>
<th>EDHM, \textmu m</th>
<th>VIHM, \textmu m\textsuperscript{3}</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.68 (0.54, 0.84)</td>
<td>0.85 (0.66, 1.1)</td>
<td>0.16 (0.07, 0.30)</td>
</tr>
<tr>
<td>Wild-type CaM</td>
<td>0.71 (0.60, 0.87)*</td>
<td>0.74 (0.59, 0.97)*</td>
<td>0.10 (0.05, 0.25)*</td>
</tr>
<tr>
<td>CaM\textsubscript{1234}</td>
<td>0.85 (0.73, 1.0)*,†</td>
<td>0.85 (0.72, 1.0)*</td>
<td>0.19 (0.11, 0.34)*,†</td>
</tr>
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Ca\textsuperscript{2+} spark spatial properties were obtained from xy images as described in MATERIALS AND METHODS. The values reported are the medians with 25 and 75 percentiles, respectively, in parentheses. CaM, calmodulin; EDHM, equivalent diameter at half-maximal fluorescence. \( P < 0.05 \) vs. \(*\)control or \(\dagger\)wild-type CaM.

Fig. 3. Effect of dCaM on Ca\textsuperscript{2+} sparks in flexor digitorum brevis myofibers. Wild-type dCaM decreases while dCaM\textsubscript{1234} increases average Ca\textsuperscript{2+} spark frequency in myofibers expressing only ryanodine receptor protein (RyR1). \( P < 0.05 \) vs. \(*\)control, vs. \(\dagger\)wild-type dCaM.

Fig. 4. Diffusion of mammalian CaM (mCaM\textsubscript{488}) within the diaphragm. A: time course for "wash-in" of mCaM\textsubscript{488} (1 \textmu M) immediately after fiber permeabilization. Data were best fit by a double exponential with time constants of 1.9 \pm 1.1 min and 12.0 \pm 2.6 min (\( n = 6 \) fibers). B: time course for "wash-out" of mCaM\textsubscript{488} (1 \textmu M). The binding of CaM\textsubscript{488} was allowed to reach equilibrium (45 min) before the start of the "wash-out." The data were best fit by a single exponential, with a time constant of 7.8 \pm 0.1 min and an offset of 0.55 \pm 0.002 (\( n = 4 \) fibers).
Permeabilized myofibers were incubated with mCaM488 (1 μM) for 45 min, without imaging, to reach steady state as determined in Fig. 4A. The bathing solution was then changed to an internal solution without mCaM488 every 5 min for 20 min. Images were acquired immediately after the first solution change and periodically for 35 min. Figure 4B shows the time course of washout of mCaM488. The data were best fit by a single exponential, with a time constant \( t_{\text{off}} \) of 7.8 ± 0.1 min and an offset of 0.55 ± 0.002, indicating that only 45% of the bound mCaM488 dissociates from the myofiber.

To assess the localization of recombinant CaM, permeabilized myofibers were double labeled with mCaM488 and Texas Red-X phalloidin. Figure 5 shows that the exogenously added mCaM localizes to the edge of the phalloidin band. Fourier analysis defined a repeating intensity with a long-axis spacing of 0.99 μm for mCaM488 and 1.98 μm for phalloidin. Colocalization analysis shows that mCaM488 and phalloidin do not colocalize within the muscle fiber (Table 2). Given that phalloidin localizes to the I-band in skeletal muscle (3), these data suggest that the exogenously added recombinant CaM localizes primarily to the triad, the sight of RyR-mediated SR Ca\(^{2+}\) release.

**Endogenous CaM localizes to the Z-line.** To determine the precise location of endogenous CaM within the muscle fiber, double immunofluorescence labeling was performed for CaM and for either α-actinin, RyR, or inositol trisphosphate receptors (IP₃R). Labeling of endogenous CaM showed a transverse sarcomeric pattern (Fig. 6a) with a long-axis spacing of 1.96 μm. The peak in the anti-CaM fluorescence aligned with the peak in the anti-α-actinin fluorescence (Fig. 6, b–d). Colocalization analysis confirms that endogenous CaM colocalizes with α-actinin (i.e., Z-lines) in mouse diaphragm muscle (Table 2). The peak in the CaM fluorescence pattern aligned with every other RyR fluorescence minima (i.e., between every other RyR doublet) in both diaphragm (Fig. 6B) and FDB (Fig. 6D) myofibers. Fourier analysis resulted in a long-axis spacing of 1.82 and 0.91 μm in the diaphragm and 2.03 and 1.10 μm in the FDB for CaM and RyR, respectively. Colocalization analysis indicates that the CaM does not colocalize with RyR (Table 2). Figure 6C shows that CaM colocalizes with IP₃R. The peak in the CaM fluorescence pattern aligns with the peak in the IP₃R fluorescence pattern, with a long-axis spacing of 1.82 μm for both CaM and IP₃R. Colocalization analysis

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<th>( R )</th>
<th>( mx )</th>
<th>( my )</th>
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<tbody>
<tr>
<td>CaM488 + phalloidin</td>
<td>0.0513</td>
<td>0.214</td>
<td>0.200</td>
</tr>
<tr>
<td>CaM + α-actinin</td>
<td>0.827</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>CaM + RyR</td>
<td>0.199</td>
<td>0.400</td>
<td>0.449</td>
</tr>
<tr>
<td>CaM + IP₃R</td>
<td>0.793</td>
<td>0.980</td>
<td>0.985</td>
</tr>
<tr>
<td>CaM + RyR (FDB)</td>
<td>0.285</td>
<td>0.328</td>
<td>0.368</td>
</tr>
<tr>
<td>CaM + α-actinin (permeabilized)</td>
<td>0.928</td>
<td>0.999</td>
<td>0.999</td>
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The Pearson’s correlation coefficient \( R \) and colocalization coefficients \( mx, my \) were obtained from immunofluorescence images colabeled with the indicated primary antibodies. RyR, ryanodine; IP₃R, inositol trisphosphate receptor; FDB, flexor digitorum brevis.

![Fig. 5. Recombinant CaM localizes to a region just outside the Z-line in adult diaphragm fibers.](http://ajpcell.physiology.org/) A: recombinant wild-type mCaM labeled with Alexa488. B: Texas Red phalloidin. C: overlay image. D: spatial profile of the fluorescence pattern indicated in the boxed region from A to C.
indicates that CaM and IP3R colocalize in adult mammalian skeletal muscle (Table 2). Since in mammalian skeletal muscle the triad is located at the end of the A-band (12, 42) and not at the Z-line, these data suggest that endogenous CaM is primarily located at sites away from the sites for RyR-dependent SR Ca2⁺ release, notably the Z-line.

The time-dependent increase in Ca²⁺ spark frequency and the inhibitory effect of wild-type recombinant dCaM may reflect dissociation of endogenous CaM upon permeabilization of the myofiber. Two approaches were taken to test this possibility. First, immunolocalization of CaM was performed in myofibers that had been saponinpermeabilized and bathed in internal control solution for 30 min before fixation. Figure 7A shows that the permeabilization protocol used for Ca²⁺ spark measurements does not result in an appreciable loss of endogenous CaM. Furthermore, immunolocalization of α-actinin in the same cell (Fig. 7B) shows that endogenous CaM colocalizes with α-actinin and thus remains localized to the Z-line (Fig. 7, C–D; Table 2). The second approach was to use the myosin light chain kinase (MLCK) CaM binding peptide to promote CaM dissociation. The MLCK CaM binding peptide (10 μM) promoted dissociation of CaM from the myofiber (Fig. 8, A–B); however, no significant effect on the time course of Ca²⁺ spark frequency was observed compared with controls (Fig. 8D). Taken together, these data indicate that dissociation of endogenous CaM does not play a significant role in the time course of Ca²⁺ spark appearance.

DISCUSSION

RyRs are a family of intracellular channels that regulate the release of Ca²⁺ from the SR. Over the last several decades there has been accumulating evidence that CaM is a potent modulator of RyR function. In vitro studies have suggested that CaM activates RyR1 and RyR3 at low Ca²⁺ concentrations (<1 μM) and inactivates these channels at elevated Ca²⁺ concentrations (>1 μM) (4, 35, 38). Both Ca²⁺-free CaM and Ca²⁺-CaM have been shown to bind to a single overlapping site within residues 3614–3643 of RyR1 (29) or residues 3467–3498 of RyR3 (48). Mutations within the 3614–3643 CaM binding site (L3624D and W3620A) disrupted the interaction of CaM with RyR1 (49) as assessed by in vitro binding assays. Interestingly, expression of L3624D or W3620A mutant RyR1 channels in dyspedic skeletal myotubes restored ECC (30), suggesting that CaM binding to the 3614–3643 CaM-binding region of RyR1 is not essential for voltage-activated SR Ca²⁺ release. More recently, using permeabilized adult frog skeletal muscle fibers, our group (36) has shown that exogenously applied recombinant dCaM activated RyR, as measured by an increase in Ca²⁺ spark frequency. Taken together, these studies indicate that the role of CaM in modulating SR Ca²⁺ release in skeletal muscle is equivocal.

In the present study, the role of CaM in modulating RyR-dependent SR Ca²⁺ release in mammalian skeletal muscle was investigated. To this end, we assayed spontaneous SR Ca²⁺ release (i.e., Ca²⁺ sparks) in adult permeabilized mammalian skeletal muscle fibers. Here we report that recombinant wild-type dCaM decreased the time-dependent increase in Ca²⁺ spark occurrence and the derived volume integral of Ca²⁺ released during a spark, whereas dCaM1234 (Ca²⁺-free CaM) increased Ca²⁺ spark frequency as well as the volume integral of Ca²⁺ released. These data support the premise that CaM is a modulator of RyR-dependent SR Ca²⁺ release.

Permeabilized myofibers showed a time-dependent increase in Ca²⁺ sparks that was blocked by recombinant wild-type
dCαM and enhanced by the Ca\(^{2+}\)-binding mutant dCαM1234 (Fig. 1). This transient nature of Ca\(^{2+}\) sparks in skeletal muscle following membrane permeabilization has been attributed to an excess production of reactive oxygen species (18, 19), with minor contributions coming from changes in SR Ca\(^{2+}\) content (26, 34). Whether the observed effects of dCαM on Ca\(^{2+}\) spark appearance in the present study result from alterations in the redox potential of the cell (e.g., CαM-dependent nitric oxide synthase) or through alterations in SR Ca\(^{2+}\) content are uncertain. Since in skeletal muscle, unlike cardiac muscle, store depletion appears to be a weak modulator of SR Ca\(^{2+}\) release (34), CaM-dependent changes in SR Ca\(^{2+}\) content are less likely.

The time-dependent increase in Ca\(^{2+}\) sparks might reflect dissociation of endogenous CαM from RyR1 upon permeabilization and that exogenous CαM may act by replacing endogenous CαM at the same inhibitory site. The action of dCαM1234 (Fig. 1) and the finding that the time course for the binding and dissociation of mCαM488 (Fig. 4) are on the same order of magnitude as the appearance of Ca\(^{2+}\) sparks are all consistent with this possibility. However, permeabilization of the myofiber did not result in an appreciable loss nor an alteration in the localization of endogenous CαM (Fig. 7), arguing against dissociation of endogenous CαM as the reason for the increase in Ca\(^{2+}\) sparks under these conditions. Furthermore, removal of endogenous CαM with MLCK CαM binding peptide did not significantly alter the time dependence of Ca\(^{2+}\) spark occurrence (Fig. 8). Thus the most likely explanation for the alteration of Ca\(^{2+}\) sparks in permeabilized mammalian skeletal myofibers is that the recombinant CαM is binding to sites not occupied by endogenous CαM, analogous to that proposed for permeabilized frog skeletal muscle (36).

Interestingly, in the present study wild-type dCαM decreased Ca\(^{2+}\) spark frequency, whereas in amphibian skeletal muscle wild-type dCαM activated Ca\(^{2+}\) sparks (36). Amphibian skeletal muscle expresses equal proportions of RyR1 and RyR3, whereas adult mammalian skeletal muscle expresses little, <1% of total RyR is composed of RyR3 (diaphragm), to no RyR3 (FDB). Thus the ability of CαM to activate Ca\(^{2+}\) sparks in amphibian skeletal muscle but inhibit Ca\(^{2+}\) sparks in adult mammalian skeletal muscle suggests a differential modulation of RyR1 versus RyR3 by CαM as previously suggested (13, 17, 27, etc.).

Fig. 7. Endogenous CαM remains localized to the Z-line after permeabilization of adult diaphragm fibers. Fluorescence confocal images of permeabilized mouse diaphragm fibers colabeled with anti-CαM (green, A) and anti-α-actinin (red, B) antibodies. Merged image is shown in C. D: spatial profile of the fluorescence pattern indicated in the boxed region from A to C.
The differential efficacy for inhibition of Ca\(^{2+}\)/H\(_{1001}\) sparks by wild-type dCaM and activation of Ca\(^{2+}\)/H\(_{1001}\) sparks by dCaM\(_{1234}\) between diaphragm and FDB myofibers (Fig. 3) could also be explained by an isoform-dependent modulation of SR Ca\(^{2+}\) release by CaM. Understanding the mechanisms for this differential regulation is important, as developing mammalian skeletal muscle expresses equal proportions of RyR1 and RyR3 and thus CaM may play a key role in Ca\(^{2+}\) signaling during myofiber development. Studies addressing this hypothesis are underway.

What might prevent endogenous CaM from associating with RyR in skeletal muscle? Recent data has suggested that the CaM binding site of RyR1 (amino acids 3614–3643) also interacts with a CaM-like domain of RyR1 (amino acids 4064–4210), suggesting that the CaM binding site is involved in an intramolecular interaction (14, 47). The CaM binding domain of RyR1 may also be a site for an intermolecular interaction with Ca\(_{1.1}\) (DHPR) in the t-tubule membrane (15, 39). Recently, Schneider and colleagues (33) have suggested that S100A1 modulates SR Ca\(^{2+}\) release. In those studies the authors show that in FDB fibers lacking S100A1, the voltage-activated global Ca\(^{2+}\) transient is depressed. Furthermore, they provide evidence that S100A1 binds to the amino terminal portion of the previously identified CaM binding site (29) and competes with CaM for this binding site. It is not known whether in the current study wild-type CaM displaces bound S100A1 from RyR1, resulting in inhibition of RyR1. The observation that wild-type recombinant dCaM reduced the volume integral of Ca\(^{2+}\) release in the present study would be consistent with this possibility. Additional studies are warranted to understand the interplay between CaM and S100A1 in the regulation of RyR-dependent SR Ca\(^{2+}\) release. Thus the ability of the CaM binding site of RyR1 to be a site for protein-protein interactions with proteins other than CaM...
might prevent CaM from associating with RyR in mature intact mammalian skeletal muscle. The finding that recombining CaM accumulates within the triad suggests that permeabilization of the myofiber disrupts any inter or intramolecular interactions that would normally prevent the association of CaM with RyR. Disruption of the DHPR-RyR1 intermolecular coupling is thought to be a prerequisite for the appearance of spontaneous Ca\(^{2+}\) sparks in mammalian skeletal muscle (2), allowing for binding of CaM to RyR1 and modulation of SR Ca\(^{2+}\) release. CaM may be localizing to regions away from the triad through its interactions with cytoskeletal proteins (e.g., dystrophin), IP\(_3\)Rs (Fig. 6), and phosphorylase b kinase (22). Harper and colleagues (16) have shown that digestion of glycogen granules with \(\alpha\)-amylase greatly reduced the striated staining pattern for CaM. Given that CaM is the \(\delta\)-subunit of phosphorylase b kinase (7), these data suggest that the localization of CaM may be primarily determined by glycogen.

The results reported in this study do not completely rule out a role of CaM in modulating skeletal muscle SR Ca\(^{2+}\) release. CaM may play a role in regulating SR Ca\(^{2+}\) release under conditions in which the intermolecular interactions between the DHPR and RyR1 have either not been formed or have been disrupted, such as during skeletal muscle development when the t-tubules have not formed contact with the SR or during times of fiber degeneration/regeneration when the t-tubules are disrupted (2). Strofiekeova (40) has recently shown, using skeletal muscle myotubes, that CaM is associated with the DHPR in vivo and mediates Ca\(^{2+}\)-dependent inhibition of DHPR current. In the present study, endogenous CaM was primarily found to reside at the Z-line; however, \(\sim 25\%\) of the peak CaM immunoreactivity can be seen at the level of the triad. It is not known whether this CaM is associated with the DHPR, but the data presented here strongly suggests that this CaM is not regulating RyR-dependent SR Ca\(^{2+}\) release. CaM has displayed Ca\(^{2+}\)-dependent translocation within cells (9, 11, 43); thus, CaM may respond to changes in intracellular [Ca\(^{2+}\)] and translocate to other regions of the cell. Stimulation of FDB myofibers at either 1 or 10 Hz did not result in nuclear translocation of CaM-yellow fluorescent protein (28). That study, however, did not look at higher frequencies of sustained activity. CaM may modulate SR Ca\(^{2+}\) release during levels of sustained activity (i.e., tetanic contractions) when the [Ca\(^{2+}\)], remains elevated. CaM may also regulate Ca\(^{2+}\) release via IP\(_3\)Rs. IP\(_3\)Rs have been localized to the Z-line in skeletal muscle (32, 37), and we now show that CaM colocalizes with IP\(_3\)Rs (Fig. 6, Table 2). IP\(_3\)Rs have been implicated in Ca\(^{2+}\) signals that regulate gene expression (20), and recent data suggest that CaM may be constitutively associated with the IP\(_3\)R and essential for proper IP\(_3\)R function (23). Therefore, it is possible that CaM is interacting with IP\(_3\)Rs and is regulating Ca\(^{2+}\) signaling involved in cellular functions other than contraction.

In conclusion, the results of this study show that under the conditions used in these experiments exogenous recombinant CaM can modulate RyR function; however, it appears that an alteration of the triadic architecture or an over abundance of CaM, or both, may be necessary to reveal such modulation. The results from O’Connel et al. (30), showing that deletion of the CaM binding region of RyR1 (amino acids 3614–3643) results in no overt change in voltage-dependent SR Ca\(^{2+}\) release, together with the results from this study leaves open the question as to what role CaM plays in modulating SR Ca\(^{2+}\) release in adult skeletal muscle. Future studies assessing the role of CaM in modulating SR Ca\(^{2+}\) release during skeletal muscle development, cycles of degeneration/regeneration, as well as IP\(_3\),R-dependent Ca\(^{2+}\) release are warranted and will provide critical information to our understanding of the role of CaM in modulating Ca\(^{2+}\) signaling in skeletal muscle.

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