T-tubule Depolarization-induced Local Events in the Ryanodine Receptor, as Monitored with the Fluorescent Conformational Probe Incorporated by Mediation of Peptide A

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There is a considerable controversy about the postulated role of the Thr\(^{671}\)–Leu\(^{690}\) (peptide A) region of the dihydropyridine (DHP) receptor \(\alpha_1\) II-III loop. Here we report that peptide A introduced the fluorescence probe methyl coumarin acetamide (MCA) in a well defined region of the ryanodine receptor (RyR), A-site, in a specific manner. Depolarization of the T-tubule moiety of the triad induced a rapid increase of the fluorescence intensity of the MCA attached to the A-site. Other RyR agonists, which activate the RyR without mediation of the DHP receptor (e.g. caffeine, polylysine, and peptide A), induced Ca\(^{2+}\) release without producing such an MCA fluorescence increase. Both magnitudes of the fluorescence change and Ca\(^{2+}\) release increased with the increase in the degree of T-tubule depolarization. MCA fluorescence increase at the A-site and subsequent sarcoplasmic reticulum Ca\(^{2+}\) release were blocked by blocking of the DHP receptor-to-RyR communication. These results may be accounted for by two alternative models as follows. (a) Upon T-tubule depolarization a portion of the DHP receptor comes close to the RyR, forming a hydrophobic interface (within such an interface the A-site is located), or (b) T-tubule depolarization may produce a local conformational change in the A-site-containing region of the RyR that is not necessarily within the DHP receptor/RyR junction.

The concept that one of the cytoplasmic loops of the DHP \(\alpha_1\) receptor \(\alpha_1\) subunit (II-III loop) plays a critical role in skeletal muscle-type E-C coupling emerged from an earlier finding of Tanabe \textit{et al.} (1, 2) that replacement of the II-III loop of the cardiac DHP receptor with the skeletal muscle-type sequence conferred the skeletal muscle-type E-C coupling, whereas the skeletal muscle-type E-C coupling is conferred by the skeletal sequence in the II-III loop to the RyR, respectively (14, 16). According to the above view that the peptide A region plays an important role in the activation process of E-C coupling has been questioned by several investigators. According to Proenza \textit{et al.} (17), a moderate degree of scrambling of the amino acid sequence in the peptide A-10 region (see above) produced no detectable changes in E-C coupling in the dygenic myotubes, although the same scrambling produced a severe loss of the activating function of peptide A-10 in case of the \textit{in vitro} experiments (12). Furthermore, according to Wilkens \textit{et al.} (18) replacement of the Leu\(^{720}\)–Leu\(^{764}\) region of the housyflle II-III loop, which has the sequence structure highly dissimilar to the skeletal muscle \(\alpha_1\) II-III loop, with the skeletal muscle sequence-restored skeletal muscle-type E-C coupling. Furthermore, according to the
FIG. 1. A, site-specific labeling of the RyR moiety with the fluorescent conformational probe MCA by mediation of peptide A or peptide C. Note that photo-affinity cross-linking of the triad with the conjugate of SAED with peptide A or peptide C permitted specific MCA fluorescence labeling almost exclusively at the RyR out of the many proteins present in the triad preparation. Digestion of the RyR with calpain II produced two fragments with ~150 and 400 kDa. The 400-kDa fragment corresponds to the segment at the C-terminal side of the calpain cleavage site as evidenced by its reactivity with anti-residue 5029 antibody (Ab 5029), whereas the 150-kDa fragment corresponds with the segment at the N-terminal side as shown by its reactivity with anti-residue 416 antibody (Ab 416). The MCA incorporated by mediation of peptide A is almost exclusively in the 400-kDa fragment, whereas the MCA incorporated by mediation of peptide C is almost exclusively in the 150-kDa fragment. The whole set of the experiment shown in this figure was repeated at least five times for the reproducible results. B, cold chase experiments showing the competition of the un-conjugated (cold chase) peptide A (left panel) and peptide C (right panel) with the peptide-SAED conjugates. Sample 1 (lanes 1 and 1’), photo-affinity MCA labeling was performed by mediation of 5 μM peptide A-SAED conjugate. Sample 2 (lanes 2 and 2’), photo-affinity MCA labeling was performed by mediation of 5 μM peptide A-SAED conjugate in the presence of 500 μM peptide A. Sample 3 (lanes 3 and 3’), photo-affinity MCA labeling was performed by mediation of 10 μM peptide C-SAED conjugate. Sample 4 (lanes 4 and 4’), photo-affinity MCA labeling was performed by mediation of 10 μM peptide C-SAED conjugate in the presence of 500 μM peptide A. Lanes 1, 2, 3, and 4, Coomassie Blue-stained gels. Lanes 1’, 2’, 3’, and 4’, fluorescence gels. The density of the peptide A-mediated MCA fluorescence labeling in the presence of 500 μM peptide A was 23.1 ± 3.5% that of the control (without cold chase) (n = 4). The density of the peptide C-mediated MCA fluorescence labeling in the presence of 500 μM peptide C was 31.8 ± 5.2% that of the control (without cold chase) (n = 4).

more recent report of Ahern et al. (19) deletion of the Thr671-Leu690 peptide A region from the α1 subunit produced virtually no effect on Ca2+ conductance, charge movement, and Ca2+ transients. Thus, the in vivo evidence accumulated so far is inconsistent with the view that the peptide A region may play an active role in the in vivo E-C coupling.

To test the physiological significance of the information obtained from the in vitro studies with peptide A, we addressed in this study two key questions as follows. (a) Do these II-III loop peptides, peptide A and peptide C, bind to the RyR in a site-specific manner? (b) Can the fluorescence probe that is attached to the peptide A-binding site or to the peptide C-binding site permit the location of events that are relevant to the physiological coupling between the DHP receptor and the RyR? As shown in our recent publications (12, 14, 20, 21), the fluorescence conformational probe, MCA, can be incorporated into the designated site on the RyR in a site-specific manner using an appropriate RyR-specific ligand (e.g., the channel blocker, neomycin (14, 20), and an agonist of the RyR, polylysine (21)) as a site-directing carrier. Here we report that the site-directed fluorescence labeling technique using peptide A as a site-directing carrier permitted us to introduce the MCA probe into the 160-kDa segment at the C-terminal side of the amino acid residue 1400 of the RyR, indicating that peptide A binds to this region of the RyR in a specific manner. Furthermore, depolarization of the T-tubule moiety of the triad, but not any of chemical/pharmacological agonists of the RyR, produced a rapid increase in the fluorescence intensity of the MCA attached to the peptide A-binding site. The magnitude of the depolarization-induced Ca2+ release was approximately proportional to that of the MCA fluorescence change as determined at various degrees of T-tubule depolarization. Inhibition of T-tubule depolarization and T-tubule-to-RyR signal transmission resulted in the inhibition of both MCA fluorescence change and Ca2+ release. Various agonists of the RyR other than T-tubule depolarization, such as caffeine, polylysine, and peptide A, induced Ca2+ release but did not produce any appreciable change in the MCA fluorescence. These results suggest that depolarization in the T-tube produces dramatic changes either in the DHP receptor/RyR interface or in the
Fig. 2. Peptide mapping of the MCA-labeling sites (peptide A- and peptide C-binding sites). To produce shorter peptide fragments to further localize the labeling sites, the RyR that had been labeled with MCA by mediation of either peptide A, or peptide C was digested with trypsin at various trypsin/SR protein ratios (no digestion (1st lane), 4000:1 (2nd lane), 2000:1 (3rd lane), 1000:1 (4th lane), 500:1 (5th lane)) at 22 °C. Electrophoretically separated bands on the 6% SDS gel were transferred to Immobilon-P membrane. The blotted sample was reacted with various primary antibodies (Ab 416, Ab 1417, Ab 2727, and Ab 5029) overnight at 4 °C. The membranes were then incubated with the appropriate second antibodies for 3 h and stained with diaminobenzidine. Correlation of the digestion pattern with the fluorescence-labeling pattern permitted us to localize the MCA labeling sites in shorter peptides. In the case of peptide A-mediated MCA incorporation (upper panel), the shortest recognizable peptide showing the intense MCA fluorescence was a 160-kDa sub-fragment that reacted with Ab 1417 antibody but not with Ab 2727. Thus, the peptide A-mediated MCA incorporation site (i.e. peptide A-binding site) must be within the region encompassing residue 1400 (calpain cleavage site) and residue 2726 (see the diagram shown at the bottom). In the case of peptide C-mediated incorporation (lower panel), the shortest recognizable fluorescent band was a 100-kDa sub-fragment, which reacted with Ab 416 but not with Ab 1417. This indicates that the peptide C-binding site is in the 100-kDa segment located at the N-terminal side of the peptide A binding region, as shown in the diagram. The whole set of experiments shown in this figure was repeated four times for reproducible results.

Experimental Procedures

Preparation—Triad-enriched micromosal fractions were prepared from the rabbit back paraspinous and hind leg skeletal muscles by a method of differential centrifugation as described previously (22). Microsomes from the final centriufrication were homogenized in a sample solution containing 0.3 M sucrose, 0.15 M potassium gluconate, protolytic enzyme inhibitors (0.1 mM phenylmethanesulfonflouride, 1 μg/ml leupeptin, 2.0 μg/ml soybean trypsin inhibitor), 20 mM MES, pH 6.8, to a final concentration of 20–30 mg/ml, frozen immediately in liquid N2, and stored at −78 °C.

Peptides Used and Peptide Synthesis—We used two peptides, peptide A and peptide C, corresponding to the Thr671–Leu690 and Glu724–Pro750 regions of the II-III loop of the DHP receptor α1 subunit of the rabbit skeletal muscle, respectively (7). The peptides were synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc (N-(9-fluorenylmethoxycarbonyl)) as the α-amino-protecting group. The peptides were cleaved and de-protected with 85% trifluoroacetic acid and purified by reversed-phase high pressure liquid chromatography.

Reagents Used—Anti-RyR polyclonal antibody was kindly provided by Dr. Kevin P. Campbell. Anti-residue 416, anti-residue 1417, anti-residue 2727 antibodies were kindly provided by Dr. Susan L. Hamilton. Anti-residue 5029 antibody was kindly provided by Dr. Andrew R. Marks. [3H]Ryanodine was purchased from PerkinElmer Life Sciences. Recombinant calpain II was purchased from Calbiochem. Sulfosuccinimidyl 3-(2-(7-azido-4-methylcoumarin-3-acetamido)ethyl)dithio)propioniate (SAED) was from Pierce.

Site-specific MCA Labeling of the Peptide A- and Peptide C-Binding Site of the RyR—Site-specific fluorescent labeling of the peptide A- and peptide C-binding sites of the RyR moiety of the triad was performed using the cleavable hetero-bifunctional cross-linking reagent SAED (21) in the following way. First, peptide-SAED conjugates were formed by incubating 0.5 mM peptide with 0.5 mM SAED in 20 mM HEPES, pH 7.5, for 60 min at 22 °C in the dark. Both peptide A-SAED and peptide C-SAED conjugates retained essentially the same activities as those of the unmodified peptides. The reaction was quenched by 20 mM lysine. Free SAED was removed using Sephadex G15 gel filtration. The peptide-SAED conjugate (5 μM final concentration) was mixed with 2 mM triad protein in the sample solution (see “Preparation” under “Experimental Procedures”) containing 1 mM BAPTA/calcium buffer (1 mM free Ca2+) in the dark, and the mixture was incubated at 4 °C for about 5 min to ensure the access of the peptide-SAED conjugate to the peptide-binding sites. The incubation time of 5 min seemed to be sufficient to introduce the peptide-SAED conjugated to its target site located in the junctional triad, as judged from the fact that this incubation time was sufficient to produce a maximal MCA labeling and a maximal MCA fluorescence response upon T-tubule depolarization. Then, the mixture was photolysed with UV light in a Pyrex tube at 4 °C for 2 min. 8-Mercaptoethanol was added (100 mM final concentration) to cleave the disulfide bond of SAED. After incubation on ice for 1 h, the mixture was centrifuged at 100,000 × g for 15 min, and the sedimented vesicles were re-suspended in the sample solution to a final protein concentration of 20 mg/ml. Gels containing electrophoretically separated protein bands were illuminated with a 360-nm UV lamp through the UG-1 filter (Schott), and the fluorescence images were obtained with a digital camera.
Assays of Ca\(^{2+}\) Release Induced by the Voltage-independent Agonists—To induce Ca\(^{2+}\) release triggered by several voltage-independent agonists of the RyR as a control, the microsomes (0.4 mg/ml) were incubated in a solution containing 0.15 M potassium gluconate, 1 mM MgATP, 40–50 μM CaCl\(_2\), 20 mM MES, pH 6.8, for 5 min for active Ca\(^{2+}\) loading. Then one volume of the above solution was mixed with one volume of a release solution containing 0.15 M potassium gluconate, 5.0 μM fluo-3, 20 mM MES, pH 6.8, and various agonists (peptide A, polylsine, and caffeine). The time course of SR Ca\(^{2+}\) release was monitored in a stopped-flow apparatus using fluo-3 as a Ca\(^{2+}\) indicator as described previously (25). Six to 10 traces (each representing 1,000 data points) of the fluo-3 signal were averaged for each stopped-flow measurement. Several such measurements (n = 3–5) were repeated for each experiment shown in the figure.

Control Assays of the Effect of the Voltage-independent Agonists on the MCA Fluorescence—The time courses of fluorescence change of the protein-bound MCA upon mixing with various RyR agonists were monitored with the stopped-flow fluorometer as described previously (25). Ten to 15 traces (each representing 1,000 data points) of the MCA signal were averaged for each experiment.

Calculation of Kinetic Parameters and Statistics—The time courses of MCA fluorescence change and Ca\(^{2+}\) release were fitted by the equation: $y = A (1 - \exp(-kt))$, where A is the maximum amount of Ca\(^{2+}\) release, k is the rate constant of Ca\(^{2+}\) release, t is reaction time, and Ak is the initial rate of Ca\(^{2+}\) release since $(dy/dt)_{t=0} = Ak$. Unpaired t test was employed to determine the statistical significance.

RESULTS

Site-specific Fluorescence Labeling of the II-III Loop Peptide Binding Regions of the RyR—In our recent studies (12, 14, 20, 24), we incorporated the conformation-sensitive fluorescent probe MCA into the trans-membrane channel domain using the Ca\(^{2+}\) channel blocker neomycin as a site-directing carrier and monitored conformational changes in the channel domain induced by various types of RyR agonists. In the present study, we introduced the MCA probe into the putative II-III loop binding region of the RyR using peptide A and peptide C as site-directing carriers. For this purpose, the triad-enriched SR fraction was incubated with the SAFD-peptide A or the SAFD-peptide C conjugate (azido-MCA-S-S-peptide A or azido-MCA-S-S-peptide C) followed by photo-cross linking of the conjugate via the azido group. Then peptide A or peptide C (site-directing carrier) was removed from the cross-linking site by cleaving the S-S bond of SAFD, leaving the MCA that had been covalently attached to the cross-linked site (details are provided under “Experimental Procedures”).

Fig. 1A depicts Coomassie Blue staining, Western blot, and MCA fluorescence-labeling patterns of the electrophoretically separated protein bands of the triad-enriched SR preparation that has been subjected to the site-directed MCA labeling by mediation of peptide A or peptide C. Fig. 1A also contains the set of staining patterns of the sample that was labeled with MCA first and then digested with calpain II. The corresponding pic-
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### Table I

| Magnitude of depolarization | K⁺ | Na⁺ | Cl⁻ | Glutamate⁺ |
|-----------------------------|----|-----|-----|------------|
| A                          | 150| 15  | 15  | 150        |
| B                          | 150| 15  | 15  | 150        |
| After A + B                | 150| 15  | 15  | 150        |

### Table II

| Magnitude of depolarization | MCA fluorescence | Ca²⁺ release |
|-----------------------------|------------------|--------------|
|                            | A %             | k s⁻¹        | Ak nmol/mg/s | A %             | k s⁻¹        | Ak nmol/mg/s |
| G1                          | 0.71 ± 0.25     | 44.11 ± 8.34 | 31.2 ± 17.4  | 5.56 ± 1.46     | 0.39 ± 0.04  | 2.20 ± 0.73  |
|                            | (n = 5)         |              |              | (n = 4)         |              |              |
| G3                          | 1.94 ± 0.21     | 66.15 ± 19.20| 128.4 ± 50.8 | 14.91 ± 2.65    | 0.82 ± 0.08  | 12.13 ± 3.21 |
|                            | (n = 5)         |              |              | (n = 4)         |              |              |
| G6                          | 3.06 ± 0.41     | 87.56 ± 14.07| 267.5 ± 54.4 | 20.17 ± 3.55    | 1.12 ± 0.12  | 22.68 ± 6.19 |
|                            | (n = 5)         |              |              | (n = 4)         |              |              |
| G9                          | 5.03 ± 0.31     | 80.91 ± 20.98| 380.5 ± 91.6 | 49.03 ± 7.99    | 1.22 ± 0.14  | 60.01 ± 11.9 |
|                            | (n = 5)         |              |              | (n = 4)         |              |              |
| G9 + nimodipine             | 0.25 ± 0.08     | 5.74 ± 0.72   | 1.42 ± 0.81   | 8.85 ± 3.58     | 1.84 ± 0.85  | 16.25 ± 0.90 |
|                            | (n = 4)         |              |              | (n = 4)         |              |              |
| G9 + ionophores             | 0.84 ± 0.26     | 23.25 ± 4.80  | 19.45 ± 7.24  | 7.58 ± 0.90     | 2.50 ± 0.53  | 18.95 ± 1.41 |
|                            | (n = 3)         |              |              | (n = 3)         |              |              |

### Kinetic parameters of MCA fluorescence change

- **A**: the amount of fluorescence change
- **k**: the rate constant of fluorescence change
- **Ak**: the initial rate of fluorescence change

- **Ca²⁺ release**: the amount of calcium release
- **Ak**: the initial rate of calcium release

The data were obtained from the experiments shown in Figs. 3 and 5. Each datum represents the mean ± S.E.

### Notes

- *p < 0.01 vs. G9
- *p < 0.05 vs. G9

- Tryptic digestion cleaved the RyR more extensively, producing shorter fragments (Fig. 2). In this figure, MCA-labeling patterns were compared with Western blot patterns obtained with various site-specific anti-RyR monoclonal antibodies. In case of peptide A-mediated labeling (upper panel), after relatively extensive digestion, the major intensity of MCA fluorescence was localized in a 160-kDa tryptic subfragment. Because this subfragment was stained with anti-residue 1417 antibody (Ab 1417) but not with anti-residue 2727 antibody (Ab 2727), the peptide A-mediated MCA labeling site, i.e., peptide A-binding site, seemed to be localized in the region of the RyR encompassing residues 1400–2726, as illustrated at the bottom of Fig. 2. There is an additional lower molecular mass (145 kDa) tryptic sub-fragment labeled with MCA that matches approximately with the band intensely stained with anti-residue 416.

- Trypsin digestion cleaved the RyR more extensively, producing shorter fragments (Fig. 2). In this figure, MCA-labeling patterns were compared with Western blot patterns obtained with various site-specific anti-RyR monoclonal antibodies. In case of peptide A-mediated labeling (upper panel), after relatively extensive digestion, the major intensity of MCA fluorescence was localized in a 160-kDa tryptic subfragment. Because this subfragment was stained with anti-residue 1417 antibody (Ab 1417) but not with anti-residue 2727 antibody (Ab 2727), the peptide A-mediated MCA labeling site, i.e., peptide A-binding site, seemed to be localized in the region of the RyR encompassing residues 1400–2726, as illustrated at the bottom of Fig. 2. There is an additional lower molecular mass (145 kDa) tryptic sub-fragment labeled with MCA that matches approximately with the band intensely stained with anti-residue 416.
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Various agonists that activate the RyR by direct stimulation produce Ca\(^{2+}\) release without producing the fluorescence change of the MCA attached to the peptide A-binding site. The Ca\(^{2+}\)-loaded triad vesicles were mixed with various voltage-independent agonists such as 3 mM caffeine, 100 nM polylysine, and 50 \(\mu\)M peptide A. These agonists induce a large Ca\(^{2+}\) release but produce no appreciable change in the MCA fluorescence, indicating that the MCA labeling site (i.e., peptide A-binding site) is in the domain outside of the agonist binding-channel activation pathways. The values of kinetic parameters and the statistic variations calculated from the data of this figure are shown in Table III.

EVENTS IN THE COUPLED TRIAD

The MCA Probe Attached to the A-site, Not That Attached to the C Site, Reports T-tubule Depolarization-induced Local Events in the Coupled Triad—The MCA-labeled triad preparation by mediation of either peptide A or peptide C showed almost intact activity of depolarization-induced Ca\(^{2+}\) release as the unlabeled preparation, although there was a significant decrease in the rate of depolarization-induced Ca\(^{2+}\) release; at G9, 60.01 ± 11.9 nmol/mg/s (\(n = 4\)) in the MCA-labeled triads (see Table II) versus 435.8 ± 73.9 nmol/mg/s (\(n = 5\)) in the case of the unlabeled triads (cf. Ref. 14). Therefore, the labeled triads can serve as a useful in vitro model of E-C coupling. We examined whether any of these activators produce appreciable changes in the fluorescence intensity of the MCA that had been incorporated into either the A or C site on the RyR in a protein-specific manner. We found that depolarization of the T-tubule moiety of the triad induced a rapid increase in the fluorescence intensity of the MCA bound to the A-site but no appreciable change in the MCA bound to the C-site.

Fig. 3 depicts the time courses of the increase in the fluorescence intensity of the MCA bound to the A-site (left panel) and Ca\(^{2+}\) release (right panel) induced by various degrees of T-tubule depolarization after the K\(^{+}\)-to-Na\(^{+}\) replacement proto-
Fig. 5. There was no MCA fluorescence change if the depolarization procedure took place in the presence of nimodipine or in the presence of ionophores (monensin and valinomycin). 1 μM nimodipine (upper panel) or the mixture of 10 μM valinomycin and 10 μM monensin (lower panel) prevented the MCA fluorescence change from occurring even when a maximal degree of ionic replacement (G9) was applied. Consequently, the subsequent Ca\(^{2+}\) release was almost completely blocked. The values of kinetic parameters and the statistic variations calculated from the data of this figure are shown in the bottom part of Table II.

![Diagram of MCA fluorescence and Ca\(^{2+}\) release](image)

Fig. 6. T-tubule depolarization produced no appreciable change in the fluorescence intensity of the MCA incorporated to the peptide C-binding site of the RyR. The site-specific MCA incorporation into the RyR moiety of the triad was performed by mediation of peptide C as a site-directing carrier, and T-tubule depolarization (G9) was performed as described under “Experimental Procedures.” The values of kinetic parameters and the statistic variations calculated from the data of this figure are shown in Table IV.

![Diagram of MCA fluorescence and Ca\(^{2+}\) release](image)
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**TABLE IV**

| MCA fluorescence | Ca²⁺ release |
|------------------|--------------|
| **A** | **k** | **A₀** | **k** | **A₀** | **k** |
| % | s⁻¹ | %/s | nmol/mg | s⁻¹ | nmol/mg/s |
| G9 | 0.015 ± 0.005 | 0.252 ± 0.095 | 0.003 ± 0.000 | 43.50 ± 2.83 | 1.60 ± 0.31 | 69.67 ± 17.15 |

**TABLE V**

Comparison of the types of responses of the MCA attached to the peptide A binding site and the MCA attached to the neomycin binding site located at the trans-membrane channel domain

| Agonists | MCA/A-site | MCA/channel domain |
|----------|-------------|--------------------|
| T-tubule depolarization | + | + |
| Peptide A | – | + |
| Polylysine | – | – |
| Caffeine | – | + |

**DISCUSSION**

In this study, we addressed the two key questions as to (a) whether peptide A and peptide C bind to the RyR in a protein- and site-specific manner and (b) whether the fluorescent probe that is attached to the peptide-binding sites can report the local events that are relevant to the physiological coupling between the DHP receptor and the RyR. The site-directed fluorescence probe-labeling technique we used in this study (original description, Ref. 21) involves the use of a hetero-bifunctional cleavable photo-affinity cross-linking reagent, SAED, with such a structure, (azido group)-(fluorescent adduct MCA)-S-S-(succinimidyl). Upon forming the conjugate of a selected ligand via the reaction of its reactive amino group with succinimidyl of SAED, the ligand delivers the conjugate to its binding site in a site-directed fashion serving as a site-directing carrier. Photo-cross-linking of the conjugate via the azido group of SAED followed by removal of the ligand moiety by cleaving the S-S bond under reducing conditions permits site-specific covalent labeling of the ligand-binding site with MCA. In our recent studies, we used neomycin (a blocker of the RyR Ca²⁺ channel, which is known to bind to the trans-membrane channel domain, Ref. 28) as the site-directing carrier to introduce MCA to the channel domain and investigated conformational changes occurring in the channel domain upon activation of the channel by various types of agonists (14, 20, 25). In the case when the site of the ligand binding has not yet been characterized as in the present case with the II-III loop peptides, the site-directed labeling technique provides a powerful tool to identify and characterize the site of peptide binding. Furthermore, if the probe is introduced successfully in the specific site or the specific region as in the present case, the protein-bound probe can serve as a reporter of the local events occurring during E-C coupling. Thus, the application of this technique in the present study has permitted us to investigate both key questions a and b outlined above.

With regard to the specificity of peptide binding, the fact that specific fluorescence labeling of the RyR could be achieved by using peptide A or peptide C as a site-directing carrier clearly indicates that both peptide A and peptide C are the RyR-specific ligands. The present results also suggest that peptide A and peptide C bind to the specific domains; hence, their binding is not only protein-specific but also domain-specific. Thus, the chief fluorescence labeling of the peptide A-binding site (A-site) occurred in the 160-kDa region located at the C-terminal side of the primary calpain II cleavage site at residue 1400 (26), whereas the chief fluorescence labeling of the peptide C-binding site (C-site) occurred in the 100-kDa segment located at the opposite side (i.e., N-terminal side) of the primary calpain cleavage site. We propose that in the quaternary structure the A-site and the C-site are in a close apposition to each other for several reasons. First, according to our preliminary study (29), the fluorescence energy transfer could be detected between the donor and acceptor placed at the A- and C-sites, respectively. Second, several different regions of the RyR have been identified as the II-III loop binding domains in the literature. Using deletion strategy, Yamazawa et al. (30) identified the residue-1303–1406 (D2) region as a critical region. The chimera approach by Nakai et al. (31) suggest that the critical region is in a rather long 1635–2636 stretch. On the other hand, the II-III loop affinity column assay by Leong and MacLennan (32) suggests a short 1076–1112 segment. These findings are consistent with the view that the putative a II-III loop-binding core is constructed by multiple segments that are scattered in a relatively broad range of the primary structure (29). Third, we pay a particular attention to an interesting analogy of our present results to the structure of the so-called inositol 1,4,5-trisphosphate (IP₃) binding site located in the N-terminal region of the IP₃ receptor, where the basic residues critical for IP₃ binding are positioned at both sides of the site that is highly susceptible to proteolytic cleavage (33).

The most important aspect of this study is the finding that depolarization of the T-tubule moiety of the triad preparation produced a rapid increase of the fluorescence intensity of the MCA attached to the A-site. The chemical depolarization protocol with various degrees of K⁺–to–Na⁺ replacement permits generation of various degrees of depolarization in the T-tubule moiety of the triad as described previously (23, 25). As shown here, upon increasing the magnitude of T-tubule depolarization, the magnitude of the MCA fluorescence change increased, and the magnitude of the induced Ca²⁺ release increased in a proportionate fashion. The MCA fluorescence change was much faster than Ca²⁺ release, suggesting that the local conformational change in the peptide A-binding domain reported by the attached MCA probe represents a causative and prerequisite mechanism for the Ca²⁺ channel activation. The observed MCA fluorescence signal and the induced Ca²⁺ release are in fact mediated by the voltage change in the T-tubule moiety and by the DHP receptor voltage sensor, as evidenced by the facts that dissipation of the Na⁺/K⁺ gradient across the T-tubule membrane by the monensin/vanilomycin mixture and the antagonist of the DHP receptor nimodipine inhibited both MCA fluorescence change and subsequent Ca²⁺ release. In further support of this notion, the MCA signal was produced in a Ca²⁺-independent fashion (data not shown) like depolarization-induced contraction and Ca²⁺ release (21, 34, 35) but unlike other chemical and pharmacological agonists of the RyR, most of which have a stringent Ca²⁺ requirement.

In evaluation of the physiological significance of the above data, critically important is the fact that the MCA fluorescence...
exchange at the A-site can be seen in response solely to the one type of activation signal, i.e. T-tubule depolarization. Other voltage-independent agonists such as peptide A, polyleucine, and caffeine induced Ca\(^{2+}\) release but without producing the MCA fluorescence change. This is in a sharp contrast to the results of the experiments with the triad preparation in which MCA was attached to the trans-membrane channel domain by mediation of neomycin, where all types of agonists we tested produced the MCA fluorescence change (see Table V). Presumably, the conformational change that was reported by the MCA at the channel domain represents the gating behavior of the channel common to various types of agonists of the RyR. Furthermore, T-tubule depolarization produced the fluorescence change only when the MCA probe was placed at the A-site but did not if the MCA was at the C-site.

In attempts to account for the present results, we postulate two alternative models as shown in Fig. 7, models a and b. Model a assumes that upon T-tubule depolarization a portion of the DHP receptor comes very close to the RyR, forming a highly hydrophobic DHP receptor/RyR interface. If the A-site is located within such an interface region, the fluorescence intensity of the attached MCA will show a rapid increase upon forming such a hydrophobic DHP receptor/RyR interface. Although peptide A is capable of delivering the MCA probe to the A-site and the binding of peptide A to this site induces SR Ca\(^{2+}\) release, it is incapable of inducing the DHP receptor/RyR contact, which is a specific event produced by the DHP receptor voltage-sensing. Although we are inclined to model a, we cannot exclude an alternative model shown in model b. Namely, T-tubule depolarization produces characteristic conformational change (e.g. internalization of the attached MCA probe) in some region of the RyR, which is not necessarily in the DHP receptor/RyR interacting region. Because the blocking of the DHP receptor-to-RyR communication results in the inhibition of the T-tubule depolarization-induced characteristic events regardless of the location of the A-site (either the DHP receptor/RyR interface or in the cytoplasmic domain of the RyR), it is difficult to decide either model by kinetic experiments alone. We should probably wait for the information about the exact locations of the A-site and of the DHP receptor-interacting region within the three-dimensional image of the RyR. At any rate, the present finding that the T-tubule depolarization-induced characteristic events take place in the region where the A-site is located suggests that peptide A can serve as a useful tool at least for introducing the conformational probe to the physiologically important domain.

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