Spectroscopic Monitoring of Local Conformational Changes during the Intramolecular Domain−Domain Interaction of the Ryanodine Receptor[†]

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ABSTRACT: The amino (N)-terminal and central regions of the ryanodine receptor (RyR) containing most mutation sites of malignant hyperthermia (MH) and central core disease (CCD) seem to be involved in the Ca^{2+} channel regulation. Our recent peptide probe study (Yamamoto, T., El-Hayek, R., and Ikemoto, N. (2000) *J. Biol. Chem.* 275, 11618−11625) suggested the hypothesis that a close contact between the N-terminal and central domains (zipping) stabilizes the closed-state of the channel, while removal of the contact (unzipping) deblocks the channel, causing channel-activation effects. We here report the results of our recent effort to monitor local conformational changes in the putative domain−domain interaction site to test this hypothesis. The conformation-sensitive fluorescence probe, methyl coumarin acetamide (MCA), was incorporated into RyR in a protein- and site-specific manner by using DP4 (the peptide corresponding to the Leu^{2442}-Pro^{2477} region of the central domain) as a site-directing carrier. The site of MCA labeling was localized in the 150 kDa N-terminal region of RyR, indicating that DP4 and its in vivo counterpart (a portion of the central domain) interact with the N-terminal region. RyR-activating domain peptides, DP4 and DP1 (corresponding to the Leu^{590}-Cys^{609} region of the N-terminal domain), and depolarization of the T-tubule moiety of the triad (physiologic stimulation) induced a rapid decrease in the fluorescence intensity of the protein-bound MCA and Ca^{2+} release at a somewhat slower rate. The accessibility of the protein-bound MCA to the fluorescence quencher was increased in the presence of DP4. These results are all consistent with the above hypothesis.

Skeletal muscle-type E−C[1] coupling is activated presumably by the voltage-mediated interaction of the DHP receptor α1 subunit II-III loop to the putative signal reception site of the ryanodine receptor (RyR) (*1*−*7*). The RyR Ca^{2+} channel is activated also by a variety of types of pharmacological and chemical agonists, which appear to bind to different regions of the bulky cytoplasmic domain of RyR (*8*−*10*). These signals open the Ca^{2+} channel located in the transmembrane region of the RyR. Therefore, there must be a "relay switch" mechanism built in RyR by which various activation signals are translated into the opening of the Ca^{2+} channel.

Our recent studies (*11*−*14*) have led us to the concept that the interaction among the putative regulatory domains within the RyR plays a key role in the above-mentioned coupling between the signal reception at the cytoplasmic domain and the opening of the channel. In searching for such regulatory domains, we have paid particular attention to the fact that, in some muscle diseases, such as malignant hyperthermia (MH) and central core disease (CCD), there is a close correlation between the mutation that has taken place within RyR (*15*−*32*) and the abnormal mode of Ca^{2+} channel regulation (*33*−*39*). We have examined several domain peptides corresponding to the regions where MH/CCD mutations were reported and found interesting effects of these peptides. Namely, DP4 (peptide corresponding to the Leu^{2442}-Pro^{2477} region) and DP1-2 (peptide corresponding to the Leu^{590}-Gly^{628} region) produced (i) significant activation of ryanodine binding and SR Ca^{2+} release from the SR (hyperactivation effect) and (ii) an increase in the sensitivity of the Ca^{2+} channel to various agonists of RyR (hypersensitization effect) (*11*−*14*). These effects were essentially the same effects as would have been produced by the MH/CCD mutations in these domains. Upon the reproduction of an Arg2458Cys human MH mutation (*32*) in DP4 (DP4-mut), the activation effect of DP4 was abolished (*12*). These findings led us to the following hypothesis. To facilitate discussion, the hypothesis is schematically illustrated in the model in Scheme 1. As shown in the model, a close contact ("zipping") of the interacting domains, domain *x* and domain *y*, stabilizes the closed state of the Ca^{2+} channel, while dissociation of such contact ("unzipping") destabilizes the

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[1] Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; CCD, central core disease; DHP, dihydropyridine; E−C coupling, excitation−contraction coupling; HEPES, [2-hydroxyethyl] piperazine [2-ethanesulfonic acid]; MCA, methyl coumarin acetamido; MES, 2-(*N*-morpholino)ethanesulfonic acid; MH, malignant hyperthermia; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PEP, phosphoenolpyruvic acid; PMSF, phenylmethanesulfonyl fluoride; RyR, ryanodine receptor; SAED, sulfosuccinimidyl-2-[7-azido-4-methylcoumarin-3-acetamido]ethyl-1,3′-dithiopropionate; SR, sarcoplasmic reticulum; UV, ultraviolet.

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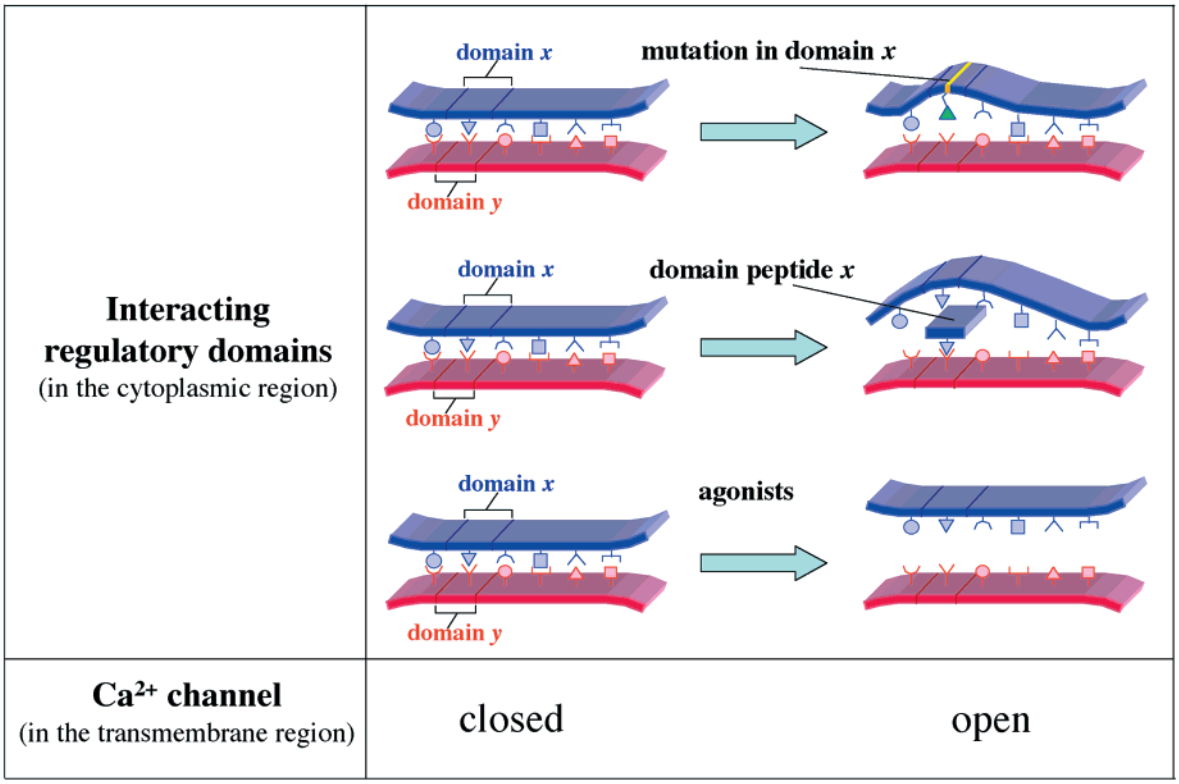
Scheme 1: Schematic Illustration of the Working Hypothesis That Zipping and Unzipping Actions of the Two Regulatory Domains within the Ryanodine Receptor Control the Close and Open States of the Ca^{2+} Channel[a]



[a] The model assumes that a close contact between domain *x* and domain *y* located in the cytoplasmic region of RyR (zipping) stabilizes the closed state of the channel located in the trans-membrane region. An MH/CCD mutation taken place in domain *x* weakens the *x*−*y* interdomain interaction. This causes unzipping and opens the channel (actually deblocking). The peptide corresponding to domain *x*, (i.e., domain peptide *x* (DP*x*)) binds to domain *y* in competition with domain *x*, which also causes unzipping and channel activation. This unzipping mechanism may be used as the channel activation mechanism in the E−C coupling process.

closed state of the channel, activating the channel. Mutations that have occurred in either domain *x* or *y* (or both) weaken the *x*−*y* interaction, or destabilize the closed state (the model shows an example when mutation has occurred in domain *x*). This results in the hyperactivation/hypersensitization effects seen in the MH/CCD channels. Similarly, domain peptide *x* (i.e., the peptide corresponding to domain *x*) interferes with *x*−*y* interaction because it binds to domain *y* in competition with domain *x*. This will produce unzipping and activation effects similar to those produced by mutations. The model (Scheme 1) also suggests that this zipping/unzipping mechanism may be involved in the channel activation process induced by various agonists of RyR.

The aims of the present study are to develop suitable techniques to monitor a local conformational change occurring in the interacting regulatory domains and to perform a stringent test of the previously mentioned hypothesis. To monitor the conformational change, we introduced the fluorescent conformational probe, methyl coumarin acetamide (MCA), to the DP4 binding site in a site-specific manner using DP4 as a site-directing carrier. According to the previously mentioned *x*−*y* interaction hypothesis, domain *y* would be labeled with MCA if the MCA labeling is mediated by a domain peptide *x* (DP*x*). As shown here, the activation of domain peptides DP4 and DP1, also T-tubule depolarization (physiological activation signal), produced a rapid decrease of the MCA fluorescence in a concentration-dependent manner and then induced SR Ca^{2+} release. The mutated DP4 mentioned previously (DP4-mut) and another nonactivating domain peptide, DP3 (corresponding to the Asp^{324}-Val^{351} region; ref *12*), produced neither MCA fluorescence change nor Ca^{2+} release. This indicates that the MCA fluorescence is reporting a local conformational change, which presumably represents a causative mechanism of channel activation. The direction of the fluorescence change (i.e., the decrease, which would normally represent a change from hydrophobic to hydrophilic environment of the site of probe attachment) supports the notion that these activating domain peptides unzip the interacting domains. This is further supported by the results of fluorescence quenching experiments showing that the rate of quenching by a large-size quencher (i.e., the accessibility of the quencher to the protein-attached MCA) increased in the presence of the domain peptide. Overall, the data presented here support the notion that unzipping between the Leu^{2442}-Pro^{2477} portion (the in vivo domain corresponding to DP4) of the central domain and the DP4 binding portion of the N-terminal region is involved in the process of Ca^{2+} channel activation.

EXPERIMENTAL PROCEDURES

Preparation. Triad-enriched microsomal fraction was prepared from the rabbit back paraspinous and hind leg skeletal muscles by differential centrifugation as described previously (*40*). The microsomes from the final centrifugation were homogenized in a sample solution containing 0.3 M sucrose, 0.15 M K gluconate, proteolytic enzyme inhibitors (0.1 mM PMSF, 1 μg/mL leupeptin, and 2.0 μg/mL soybean

trypsin inhibitor), and 20 mM MES (pH 6.8) to a final concentration of 20−30 mg/mL and were frozen immediately in liquid N_2 and stored at −78 °C.

Peptides Used and Peptide Synthesis. We used four domain peptides: DP1, DP4, DP4-mut, and DP3. DP1, DP4, and DP3 correspond to the Leu^{590}-Cys^{609}, the Leu^{2442}-Pro^{2477}, and the Asp^{324}-Val^{351} regions of RyR of the rabbit skeletal muscle, respectively (*11*, *12*). DP4-mut was identical with DP4 except that the Arg residue corresponding to Arg^{2458} was replaced with Cys, mimicking the reported human MH/CCD mutation (*12*, *32*).The peptides were synthesized on

domain peptide	sequence
DP1	^{590}LDKHGRNHKVLDVLCSLCVC^{609}
DP4	^{2442}LIQAGKGEALRIRAILRSLVPLDDLVGIISLPLQIP^{2477}
DP4-mut	^{2442}LIQAGKGEALRIRAILCSLVPLDDLVGIISLPLQIP^{2477}
DP3	^{324}DTAPKRDVEGMGPPEIKYGESLCFVQHV^{351}

an Applied Biosystems model 431A synthesizer employing *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) as the α-amino protecting group. The peptides were cleaved and deprotected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography.

Reagents Used. Anti-RyR polyclonal antibody (polyclonal Ab), anti residue-416 antibody (anti-416), and anti residue-5029 antibody (anti-5029) were kindly provided by Dr. Kevin P. Campbell, Dr. Susan L. Hamilton, and Dr. Andrew R. Marks, respectively. Recombinant calpain II was purchased from Calbiochem. SAED was purchased from Pierce, and fluo-3 and QSY 7 carboxylic acid were purchased from Molecular Probes Inc.

Site-Specific MCA Labeling of the DP4 Binding Site of RyR. Site-specific fluorescent labeling of the DP4 binding site of the RyR moiety of the triad was performed using the cleavable heterobifunctional cross-linking reagent, sulfosuccinimidyl-3-((2-(7-azido-4-methylcoumarin-3-acetamido)ethyl)dithio)propionate (SAED; ref *41*) in the following way. First, peptide−SAED conjugate was formed by incubating 0.5 mM peptide with 0.5 mM SAED in a 20 mM HEPES (pH 7.5) for 60 min at 22 °C in the dark. The reaction was quenched by 20 mM lysine. Free SAED was removed using Sephadex G15 gel filtration. DP4−SAED conjugate retained essentially the same activities as the unmodified peptide. The peptide−SAED conjugate (5 μM in a final concentration) was mixed with 2 mg/mL triad protein in the sample solution (see Preparation section) containing a 1 mM BAPTA/calcium buffer (1.0 μM free Ca^{2+}) in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 2 min. β-Mercaptoethanol was added (100 mM in a 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 resuspended 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 (Olympus C-2020) using a 440 nm interference filter with a 40 nm bandwidth.

Digestion with Calpain II. Fluorescently labeled microsomes (1 mg/mL) were mixed with recombinant calpain II at the ratio of 6 units of calpain to 1 mg of SR protein in a solution containing 150 mM NaCl and 20 mM MOPS (pH 7.2). Digestion was started by adding 3 mM $CaCl_2$. After the digestion for 6 min at 22 °C, the reaction was stopped by adding 3 mM BAPTA.

Assays of Ca^{2+} Release Induced by the Domain Peptides. To induce Ca^{2+} release triggered by domain peptides, the microsomes (0.4 mg/mL) were incubated in a solution containing 0.15 M K gluconate, 1 mM MgATP, 40−50 μM $CaCl_2$, and 20 mM MES (pH 6.8) for 5 min to active Ca^{2+} loading. Then, 1 volume of this solution was mixed with 1 volume of a release solution containing 0.15 M K gluconate, 5.0 μM fluo-3, and 20 mM MES (pH 6.8) and various concentrations of domain peptides. The time course of Ca^{2+} release was monitored in a stopped-flow apparatus (Bio-Logic SFM-4 with an MOS-200 optical system; excitation at 440 nm, emission at 510 nm using an interference filter with 40 nm bandwidth) using fluo-3 as a Ca^{2+} indicator, as described previously (*42*). From 6 to 10 traces (each representing 1000 data points) of the fluo-3 signal were averaged for each experiment.

Spectrometric Monitoring of Conformational Changes in the Domain-Interaction sites of RyR, Induced by the Activating Domain Peptides. The time courses of fluorescence change of the MCA attached to the DP4 binding site (see previous discussion) induced by domain peptides (activating peptides (DP4 and DP1) and control (DP3)) were monitored with the stopped-flow fluorometer system (Bio-Logic SFM-4 with an MOS-200 optical system; excitation at 368 nm, emission at 455 nm using an interference filter with 70 nm bandwidth) as described previously (*43*, *44*). From 10 to 15 traces (each representing 1000 data points) of the MCA signal were averaged for each experiment.

Fluorescence Quenching of the MCA Fluorescence Attached to the DP4 Binding Site. To make a large-size collisional quencher, the quenching reagent, QSY 7 carboxylic acid, was conjugated with BSA by incubating 5 mM QSY 7 carboxylic acid with 0.5 mM BSA in 20 mM HEPES (pH 7.5) for 60 min at 22 °C in the dark. Unreacted QSY 7 carboxylic acid was removed by means of Sephadex G50 gel filtration. Fluorescence quenching by both QSY 7 carboxylic acid−BSA conjugate (a large-size quencher) and acrylamide (a small-size quencher) were performed by measuring steady-state fluorescence of labeled MCA (excitation at 368 nm, emission at 455 nm) in the presence or in the absence of 50 μM DP4. The data were analyzed using the Stern−Volmer equation (*45*).

Assays of Depolarization-Induced Ca^{2+} Release. To induce Ca^{2+} release by T-tubule depolarization, we employed the K^+ to Na^+ replacement protocol, which was originally devised in the skinned fiber system by Lamb and Stephenson (*46*) and was adopted to our triad system (*47*). The T-tubule moiety (1.0 mg/mL) of the triad was first polarized by incubating in the base solution (150 mM K gluconate, 15 mM NaCl, and 20 mM imidazole (pH 6.8)), containing 5.0 mM Mg·TP, 100−150 μM $CaCl_2$, and an ATP-regenerating system (2.5 mM PEP and 10 units/mL pyruvate kinase) for 10 min. Then, the T-tubule moiety was depolarized by mixing, in a stopped-flow apparatus (Bio-Logic SFM-4), 30 μL of the solution (solution A) containing the polarized triads with 120 μL of depolarization solution (solution B: 150 mM Na gluconate, 15 mM NaCl, and 20 mM imidazole (pH 6.8)). The time course of SR Ca^{2+} release was monitored in a

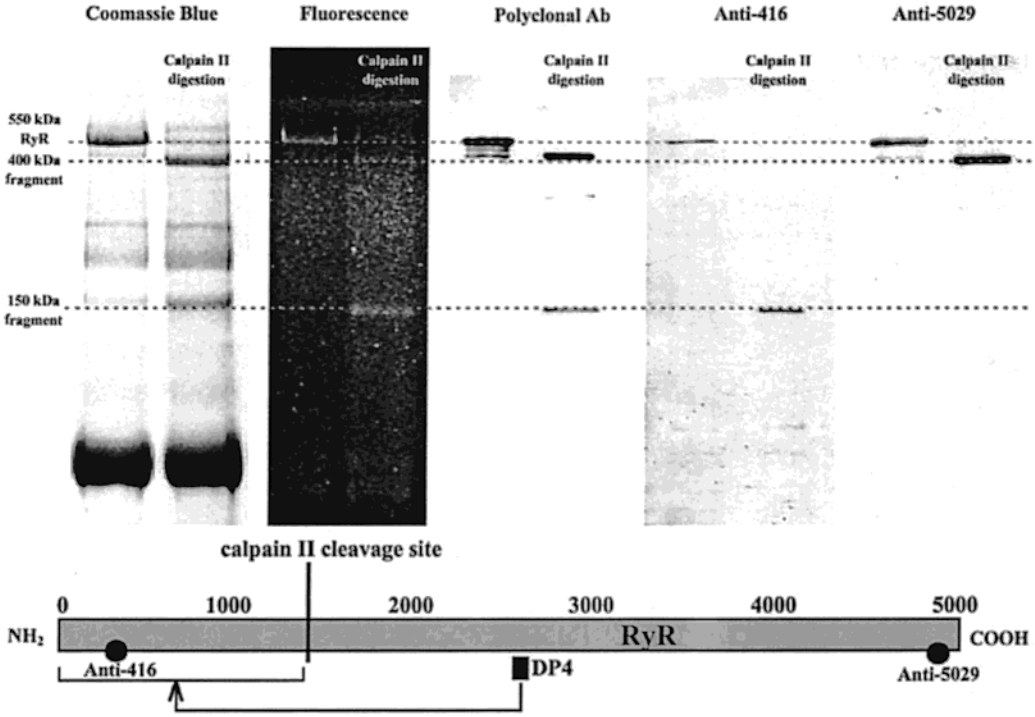


FIGURE 1: Site-directed MCA labeling of the DP4 binding domain of RyR. Note that the SAED−DP4 conjugate produced specific fluorescent labeling of the RyR moiety out of many proteins present in SR. The site of MCA labeling is localized in a 150 kDa calpain fragment of RyR. Because the 150 kDa calpain fragment was stained with anti-residue 416 antibody (anti-416), the MCA-labeling site (i.e., DP4 binding site) is within the 150 kDa N-terminal segment of RyR. Incidentally, a 400 kDa calpain fragment, which was not labeled with MCA, was stained with anti-residue 5029 antibody (anti-5029), indicating that this fragment represents the C-terminal segment of RyR.

stopped-flow apparatus using fluo-3 as a Ca^{2+} indicator, as described previously (*42*). From 6 to 10 traces (each representing 1000 data points) of the fluo-3 signal were averaged for each experiment.

Spectrometric Monitoring of Depolarization-Induced Conformational Change in the Domain Interaction Sites of RyR. The time courses of fluorescence change of the protein-bound MCA upon depolarization were monitored with the stopped-flow fluorometer (see previous discussion). From 10 to 15 traces (each representing 1000 data points) of the MCA signal were averaged for each experiment.

Statistics. Paired *T* test was employed to determine the statistical significance of the data.

RESULTS

Site-Directed MCA Labeling of the DP4 Binding Domain of RyR. The synthetic peptide corresponding to the Leu^{2442}-Pro^{2477} region of RyR (i.e., DP4) activates RyR and induces SR Ca^{2+} release (*12*−*14*). On the basis of the fact that most of the reported MH and CCD mutations are located in either the Cys^{35}-Arg^{614} region (N-terminal domain) or the Arg^{2168}-Arg^{2458} region (central domain) (*48*), we proposed the hypothesis that a close contact between these two domains of RyR produces the closed state of the Ca^{2+} channel, whereas the removal of such a contact by DP4 deblocks the channel, producing channel-activation effects (see introduction). According to this hypothesis, it was predicted that the central domain peptide DP4 would bind to the N-terminal domain. To test this prediction, also to establish the method that permits the labeling of RyR with an appropriate fluorescent conformational probe in a site-specific manner, we adopted the previously described site-directed MCA incorporation technique (*41*) with an important modification as follows. In the previous fluorescence conformational probe studies, we labeled the trans-membrane channel domain with MCA in a site-directed fashion by using neomycin as the site-direction marker and monitored local conformational changes occurring in the channel domain induced by T-tubule depolarization (*43*), polylysine (*42*), and peptide A (*44*). In more recent studies (*49*), we incorporated MCA into the peptide A binding site and monitored depolarization-induced conformational changes in the voltage-dependent signal reception site of RyR. In the present study, we made an attempt to introduce the MCA probe into the DP4 binding site, which is presumably located within the interacting regulatory domains. For this purpose, we followed the sequential procedures, namely, we (i) conjugated DP4 with SAED (azido-MCA-S−S-DP4) via the Lys^{2447} residue of DP4, (ii) incubated the triad with the SAED−DP4 conjugate to allow the conjugate to bind to the DP4 binding domain of RyR (i.e., the domain with which the Leu^{2442}-Pro^{2477} region interacts), (iii) cross-linked the SAED−DP4 conjugate to the designated site via the azido group by photolysis, and (iv) removed the DP4 moiety of the SAED−DP4 conjugate from the site by cleaving the S−S bond of SAED (for further detail, see Experimental Procedures).

Figure 1 shows five pairs of gels that were treated differently (left, undigested; right, after digestion with calpain II). The Coomassie blue gels show an overall view of the stained protein bands. Fluorescence gels show the fluorescence-labeling pattern of the protein bands. As seen, MCA fluorescence labeling took place almost exclusively at the 550-kDa component, which was identified as the RyR band by immunostaining with anti-RyR antibody (left, Polyclonal

Ab). This indicates that DP4 has been bound exclusively to the RyR moiety out of many proteins present in the triad/SR preparation. Brief digestion of RyR with calpain II cleaves the RyR polypeptide chain into two fragments of approximately 150 and 400 kDa. The 150 and 400 kDa fragments have originated from the N-terminal and the C-terminal segments of RyR, respectively, as evidenced by immunostaining with the site-specific antibodies. Interestingly, after calpain digestion, the MCA fluorescence that originally present in the 550 kDa RyR band was moved to the 150-kDa N-terminal fragment band, indicating that the specific MCA labeling site is localized in the 150 kDa N-terminal region. Namely, DP4 has bound to the N-terminal region. Because DP4 corresponds to the Leu^{2442}-Pro^{2477} segment of the central domain of RyR, these results indicate that at least a portion of the central domain including the Leu^{2442}-Pro^{2477} segment is interacting with a portion(s) of the N-terminal region. This is consistent with the hypothesis that the interdomain interaction between the N-terminal and central domains may play an important role in the regulation of the channel functions (*12*), although the exact location of the MCA binding site remains to be further investigated.

In this study, we carried out the same type of site-directed MCA labeling experiment with the use of the N-terminal domain peptide DP1 as a site-direction carrier, to examine the possibility that the MCA labeling might take place in the central domain region (viz., in the 400 kDa calpain fragment). However, we could not obtain appreciable MCA labeling of RyR in this case, probably because conjugation of DP1 with SAED altered the properties of DP1.

MCA Probe Attached to the DP4 Binding Site Reports a Local Conformational Change Occurring in the Domain−*Domain Interaction Region.* As described in our recent reports, several domain peptides, such as DP4 (*12*−*14*), activate RyR. According to the hypothesis, this is caused by unzipping of the regulatory domains that have been tightly bound with each other. Because MCA is attached to the DP4 binding domain (viz., one of such regulatory domains) as described previously, the unzipping that is produced by DP4 might be detected by monitoring the changes in the fluorescent intensity of the attached MCA. We examined this possibility in the experiment shown in Figure 2. In this experiment, the MCA-labeled SR was mixed, in a stopped-flow apparatus, with an equal volume of solution containing various concentrations of DP4. Then, we followed the time courses of the changes in the fluorescence intensity of MCA and induced Ca^{2+} release. As seen, DP4 produced a rapid decrease of the fluorescence intensity of the MCA attached to the DP4 binding domain (Figure 2A), and induced Ca^{2+} release (Figure 2B) in a concentration-dependent manner. The magnitude of the MCA fluorescence decrease and that of the amount of Ca^{2+} released show essentially identical DP4 concentration-dependence. Interestingly, the DP4 concentration-dependence of both MCA fluorescence change and Ca^{2+} release is also essentially identical to that of enhancement of ryanodine binding that was reported in our recent paper (*12*). These results suggest that the MCA fluorescence decrease observed here represents a mechanism that is tightly coupled with the activation of the Ca^{2+} channel. The DP4 concentration-dependence of the rate constants of the MCA fluorescence decrease and Ca^{2+} release are shown in Table 1. The rate constants of the MCA fluorescence change are

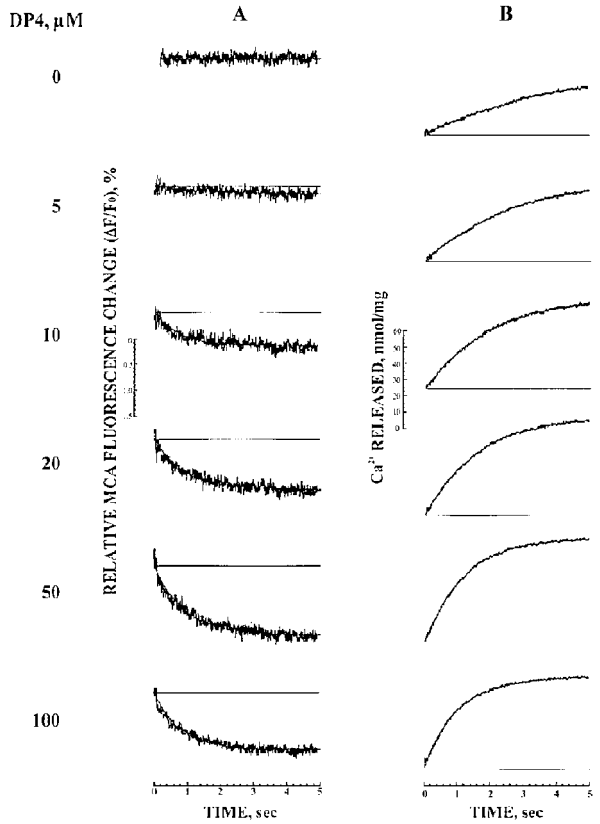


FIGURE 2: RyR-activating domain peptide, DP4, produces a rapid decrease of the fluorescence intensity of the RyR-bound MCA (A) and induces Ca^{2+} release (B) in a concentration-dependent manner. Upon increasing the concentration of DP4, both fluorescence change and Ca^{2+} release became faster and larger in parallel, suggesting that the MCA fluorescence change (a local conformational change in the DP4 binding region) and Ca^{2+} release are tightly coupled.

Table 1: Rate Constants (s^{-1}) of DP4-Induced MCA Fluorescence Decrease and Ca^{2+} Release[a]

	DP4, μM		
	20	50	100
MCA fluorescent change	0.763 ± 0.366	0.978 ± 0.221*	1.176 ± 0.094*
Ca^{2+} release	0.639 ± 0.286	0.833 ± 0.150	0.967 ± 0.213

[a] The rate constant of MCA fluorescence decrease is significantly larger than that of Ca^{2+} release at higher concentrations of DP4 examined (the statistical significance is indicated by the *P* value). This indicates that a local conformational change may be the causative mechanism for Ca^{2+} release. Data represent mean ± SD of four experiments performed in duplicate. Asterisk indicates $P < 0.05$.

significantly higher than those of Ca^{2+} release, as seen especially at higher peptide concentrations indicating that the MCA fluorescence change is reporting a causative mechanism for the channel activation. Importantly, the direction of the MCA fluorescence change (i.e., decrease) is consistent with the view that the microenvironment of the MCA attachment site is changing from a more hydrophobic environment to a less hydrophobic one. This is consistent with the concept that a close interaction between the portion of the central domain corresponding to DP4 and its counter domain in the N-terminal region (viz., the domain to which MCA has been bound) was interfered by the added DP4, causing unzipping of the interacting domain pair and activation of the Ca^{2+} channel (see hypothesis). In further support of this concept, the N-terminal domain peptide DP1

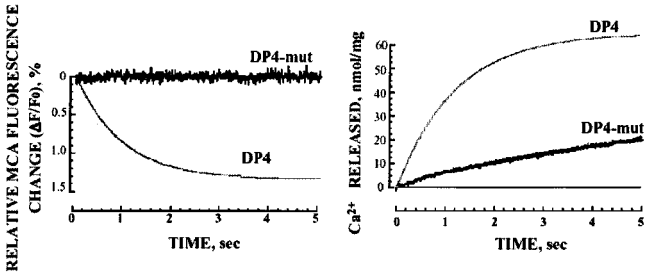


FIGURE 3: Mutant of DP4, DP4-mut, produces no MCA fluorescence change and virtually no Ca^{2+} release.

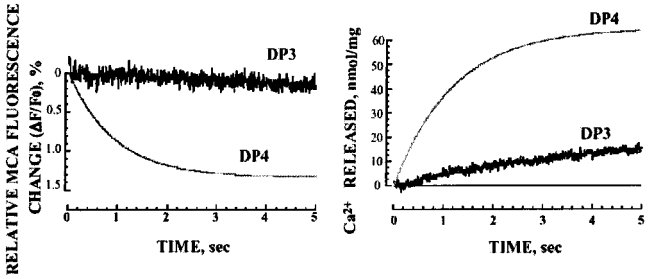


FIGURE 4: Another domain peptide DP3, which has virtually no effect on the ryanodine binding activity (ref *12*), produced no MCA fluorescence change and virtually no Ca^{2+} release.

produced a rapid fluorescence decrease of the MCA attached to the DP4 binding site (cf. Figure 6) and induced Ca^{2+} release (data not shown) in a concentration-dependent manner.

As shown in our previous studies with isolated triads (*12*) and saponin-permeabilized muscle fibers (*14*), single mutation that was produced in DP4 mimicking an Arg2458Cys mutation in an MH patient (producing DP4-mut) abolished the channel activating function that would have been present in DP4. This provides us with an excellent negative control to test the physiological significance of DP4 as a probe. Figure 3 shows that 50 μM DP4-mut, the concentration equivalent to the maximally activating concentration of DP4 (see Figure 2), produced virtually no effect on the MCA fluorescence and only negligible effect on Ca^{2+} release. As yet another negative control, we examined the peptide corresponding to the Asp^{324}-Val^{351} region of RyR (i.e., DP3) which according to our previous study (*12*) has no appreciable activation effect on RyR function. As shown in Figure 4, 50 μM DP3 (the concentration equivalent to the maximally activating concentration of DP4) induced virtually no MCA fluorescence change and a negligible effect on Ca^{2+} release.

Fluorescence Quenching Studies Further Support the Concept That Unzipping of the Interacting Domains Is Involved in the Activation Process. The direction of the MCA fluorescence change produced by domain peptides and T-tubule depolarization (i.e., the decrease) is consistent with the opening the gap between the interacting domains (i.e., unzipping). To further test this idea, we carried out fluorescence quenching experiments. As the fluorescence quencher, we conjugated QSY with bovine serum albumin (BSA) with a view to make a relatively bulky fluorescence quencher, which is accessible to the MCA fluorescence quenching only when the interacting domains are sufficiently unzipped. In the experiment shown in Figure 5A, the MCA-labeled SR was mixed, in a stopped-flow apparatus, with various concentrations of a large-size quencher BSA-QSY in the absence and in the presence of DP4, and the fluorescence intensity of the MCA before quenching (F_0) and that after quenching (F) were determined. The data were plotted according to the Stern−Volmer equation (*45*): $F_0/F = 1 + Ks[Q]$, where Ks is the quenching constant and [Q] is the quencher concentration. As seen in Figure 5A, the slope of the plot, which represents Ks, was significantly larger in the presence of DP4 compared with in its absence. As a control, we carried out the same experiment with a small-size quencher acrylamide (Figure 5B). The slope of the Stern−Volmer plot was essentially identical in the absence and the presence of DP4. These results suggest that the protein-bound MCA is accessible to the small-size quencher acrylamide, even in the zipped state, while it is less accessible to the large-size quencher BSA-QSY in the zipped state but becomes more accessible upon unzipping. Thus, these results provide further evidence that the interacting domains were, in fact, unzipped by DP4.

Competition Between Two Activating Domain Peptides Further Supports the Unzipping/Activation Hypothesis. Our previous ryanodine binding assay showed that when two activating domain peptides, such as DP4 and DP1, are added together, they do interfere with each other for the activating effect rather than producing additive activation effects (*12*). This was one of the pieces of evidence for the proposed hypothesis, because it excluded the possibility that the observed activation effects of these peptides might have been due to their binding to the activation sites. We reinvestigated this competitive phenomenon by utilizing the MCA technique devised in the present study, because the method offers a much higher temporal resolution. As shown in Figure 6 (top left), DP4 alone (50 μM) produces a rapid MCA fluorescence decrease, as described previously. Similarly, another activating peptide DP1 (*11*) alone (50 μM) produced similar MCA fluorescence change (Figure 6, bottom left). Interestingly, however, if the same concentration of DP1 was added after having been treated with 50 μM DP4, it produced virtually no further fluorescence change (Figure 6, top right). Similarly, the addition of 50 μM DP4 after preincubation with 50 μM DP1 produced virtually no fluorescence change. These results indicate that, after having already been unzipped by one peptide, the addition of the other peptide is virtually ineffective to produce further unzipping. This has quite important bearing for an analysis of the nature of the conformational change occurring in the regulatory domain of RyR, as elaborated in the Discussion.

Unzipping Mechanism Seems To Be Utilized for the T-Tubule Depolarization-Mediated Activation. The fact that an MH mutation causes hyperactivation and hypersensitization effects on depolarization-induced Ca^{2+} release (*37*) suggests that the same unzipping mechanism is used not only for the domain peptide-induced activation of the Ca^{2+} channel but also for the depolarization-induced activation. This was examined in the experiment shown in Figure 7 in which the T-tubule moiety of the triad was depolarized by the K^+-to-Na^+ replacement protocol and the changes in the fluorescence intensity of the MCA attached to the DP4 binding site and the time course of the induced Ca^{2+} release were followed. As seen, T-tubule depolarization produced a rapid decrease of the MCA fluorescence. Again, the rate constant of fluorescence decrease ($k = 1.75\ s^{-1}$) was

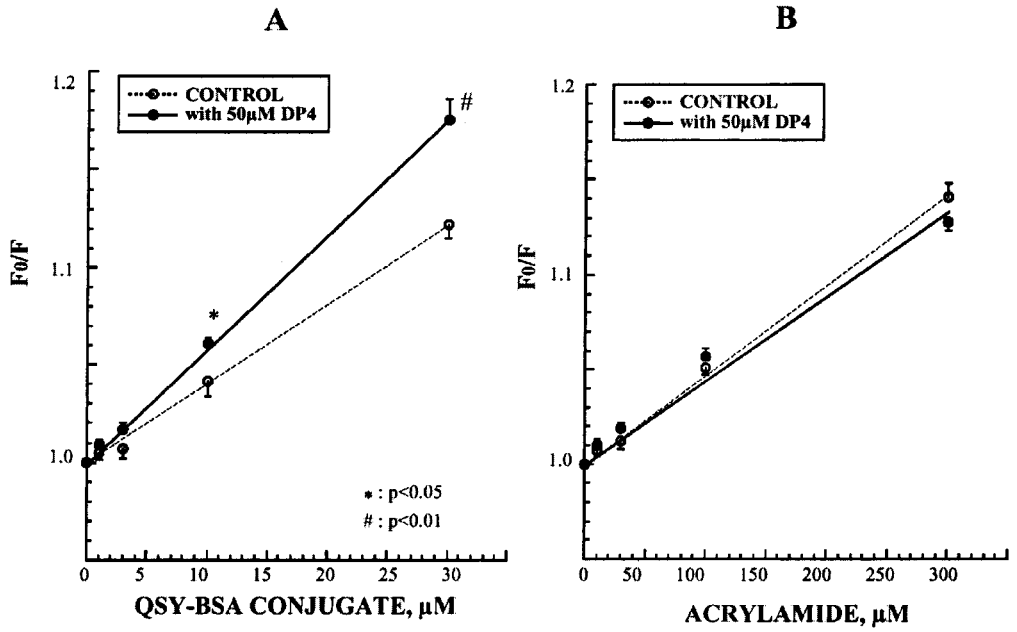


FIGURE 5: (A) Stern−Volmer plots of the fluorescence quenching data with QSY−BSA in the absence and the presence of added DP4. (B) Stern−Volmer plots of the fluorescence quenching data with acrylamide in the absence and the presence of added DP4. In the presence of the large-size quencher, the QSY−BSA conjugate, the efficiency of the MCA quenching (the slope of the plot) became significantly larger than that in its absence. However, the presence of DP4 had virtually no effect on the efficiency of the MCA fluorescence quenching by the small-size quencher, acrylamide.

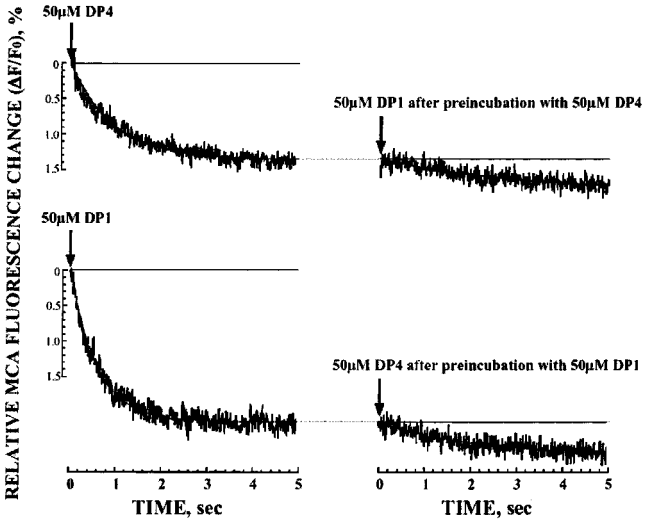


FIGURE 6: Two activating domain peptides (DP4 (central domain peptide) and DP1 (N-terminal domain peptide)) show competitive effects on the MCA fluorescence change. Note that preincubation with one of these domain peptides followed by the addition of the other negates the ability of causing the MCA fluorescence change by the latter.

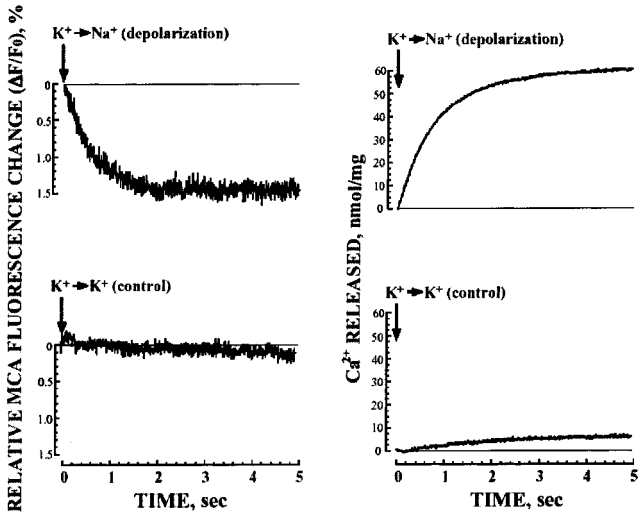


FIGURE 7: Depolarization of the T-tubule moiety of the triad produces a rapid decrease of the fluorescence intensity of the RyR-bound MCA (A) and induces Ca^{2+} release (B), indicating that the same unzipping mechanism is involved in the physiological activation of the Ca^{2+} channel. The T-tubule moiety of the triad was depolarized by using the K^+-to-Na^+ replacement protocol, as described in Experimental Procedures. As a control, the same stopped-flow mixing was carried out without ionic replacement (K^+-to-K^+), namely, with no depolarization. Simple mixing without T-tubule depolarization produced no MCA fluorescence change nor Ca^{2+} release.

significantly higher than that of Ca^{2+} release ($k = 1.58\ s^{-1}$), suggesting that the domain unzipping is used as the relay-switch mechanism in the normal operation of E−C coupling as well.

DISCUSSION

The Ca^{2+} channel seems to be regulated by mediation of a global conformational change of RyR, which is performed presumably by intricate interactions among many domains within RyR. A number of putative regulatory domains have been suggested in the literature, such as calmoduline binding sites (*50*−*55*), phosphorylation sites (*56*, *57*), Ca^{2+}- and nucleotide-regulatory domains (*57*−*59*), FKBP-binding region (*60*, *61*), putative regulatory domains where the known MH and CCD mutation sites are located (*62*), and so forth. The possibility that mutual interactions of these domains may be involved in the channel regulation was suggested in an early report of Zorzato et al. (*63*). Namely, an antibody raised against the segment containing the Gly^{341} human MH mutation site produced a considerable increase in the Ca^{2+}-induced Ca^{2+} release rate at pCa 8, and shifted the half-

maximal [Ca^{2+}] for activation to a lower value; the same type effects as seen in MH. According to ligand overlays, binding of the Gly^{341}-containing peptide took place to the regions encompassing residues 3010−3225 and 799−1172, suggesting that the interaction among two or three different domains of RyR may be involved in the mechanism of channel regulation. Recently, we initiated the similar type of approach by using a group of peptides corresponding to various regions of RyR containing the known MH/CCD mutation sites. Presumably these MH/CCD mutation domains represent the prime candidates for the domains involved in the regulation of the RyR Ca^{2+} channel, because the mutations that have occurred in these domains produce significant modifications of the channel functions (i.e., hyperactivation and hypersensitization effects). As described in the introduction, some of these domain peptides that we have tested (e.g., DP4 and DP1) were found to produce the same type effects as produced by MH/CCD mutations within the corresponding in situ domains. These findings suggested the model that a tighter interaction between the interacting domains stabilizes the closed state of the channel and that the competitive binding of the exogenously added domain peptides to the domain interaction site weakens the domain−domain interaction, causing the observed activation effects (cf. Scheme 1 and the introduction).

Some of the domain peptides of RyR serve as useful probes to investigate the events within RyR, as shown in our recent publications (*11*−*14*). Once the physiologic significance of the peptide function is established by stringent control experiments, such a peptide serves as a powerful tool for an analysis of the channel-regulation mechanism. One of the most important advantages with the peptide probe approach is that the peptide can serve as a delivery vehicle to introduce the fluorescent conformational probe MCA to the designated domain in a site-directed manner. Taking an example of interacting domains *x* and *y* described in the introduction, the domain peptide corresponding to domain *x* (i.e., DP*x*) would serve as a site-directing marker for specific MCA labeling of domain *y* (the counter-domain of domain *x*), because DP*x* binds to domain *y* as domain *x* binds to domain *y*. One of the most important aspects of the present study is the finding that the conjugate of DP4 with the heterobifunctional photoaffinity cross-linking reagent SAED {SAED−DP4 conjugate has the structure (azido photoaffinity group)-MCA-S−S-DP4} resulted in a specific MCA labeling of the 150 kDa N-terminal region of RyR. Because DP4 has the same sequence structure as the Leu^{2442}-Pro^{2477} region (a portion of the central domain of RyR), this would indicate that the counter domain, with which the Leu^{2442}-Pro^{2477} region of RyR interacts, is localized in the 150 kDa N-terminal region. In the other words, the central domain is interacting, at least partly, with the N-terminal segment. This is in a good agreement with our prediction that there may be some interactions between the N-terminal and central MH/CCD mutation domains (*12*, *48*). However, the exact location of the MCA-labeling site within the N-terminal mutation domain remains to be investigated by further studies.

An exciting new finding in the present study is that the MCA probe that had been covalently attached to the DP4-binding domain (viz., the putative counter domain of the Leu^{2442}-Pro^{2477} domain) could successfully report a rapid conformational change occurring in the vicinity of the probe attachment site. Thus, the addition of the activating domain peptide DP4 produced a rapid decrease in the fluorescence intensity of the attached MCA in a concentration-dependent manner. The change was sufficiently rapid to be a causative mechanism for Ca^{2+} release, and the concentration-dependence of the magnitude of MCA fluorescence change was essentially identical to that of Ca^{2+} release. Furthermore, DP4-mut, which was incapable of activating RyR (see previous discussion), was unable to produce the MCA fluorescence change. Similarly, another domain peptide DP3, which has no activation effect (*12*), was also unable to produce the MCA fluorescence change. Thus, there is a close correlation between the kinetics of the MCA fluorescence change and the channel activation pattern, indicating that the observed MCA fluorescence change represents the local conformational change, which has direct relevance to the mechanism of channel opening.

In our previous studies, we investigated a rapid conformational change of RyR induced by various stimuli, such as T-tubule depolarization (*43*), polylysine (*42*), and peptide A (*44*), by using the MCA probe that had been attached to the neomycin binding region (or the trans-membrane channel domain) (*41*). In those experiments, activation of RyR by these effectors always produced a rapid increase in the MCA fluorescence, which preceded Ca^{2+} release. This finding suggested that local conformational change occurring in the trans-membrane channel domain represents a mechanism prerequisite to the channel opening (*41*, *43*). In contrary to the signal of the MCA attached to the channel domain, which increased upon activation by these agonists described here previously (*42*−*44*), the MCA fluorescence attached to the DP4 binding domain decreased upon the activation via T-tubule depolarization and activating domain peptides DP4 and DP1, as shown in the present study. According to the preliminary data, the other agonists such as polylysine, which induced the MCA signal at the channel domain (*42*), did not induce any appreciable MCA fluorescence change at the DP4 binding domain (data, not shown). Such a sharp difference in the properties in the fluorescence signal depending upon the sites of the MCA attachment indicates that quite different types of conformational changes are occurring in the interacting regulatory domain pair and in the trans-membrane Ca^{2+} channel. These results further suggest that the conformational change detected by the MCA probe at the interacting domain represents a mechanism, which may be involved specifically in the voltage-mediated activation of the Ca^{2+} channel.

The decrease of the fluorescence intensity often (if not always) represents the change of the environment of the probe attachment site from a hydrophobic to a less hydrophobic nature. Therefore, the MCA fluorescence decrease that has been produced by domain peptides and T-tubule depolarization is consistent with the view that the gap between the interacting domains was opened under these activating conditions (namely, unzipped), exposing the site of MCA attachment (DP4 binding site within the N-terminal domain) to a more hydrophilic environment. The fluorescence quenching experiment presented in this study provided additional evidence that the MCA fluorescence decrease in fact represents the unzipping action of interacting domains. As shown here, the conjugate of QSY with BSA served as a suitable fluorescence quencher that presumably was less

accessible to the attached MCA in the zipped state and became more accessible to MCA only when the unzipping occurred. Thus, the extent of fluorescence quenching, or the accessibility of the quencher to the bound MCA, became much larger when RyR had been pretreated with domain peptides (i.e., after having been unzipped).

An interesting finding in the present study is that DP1 produced the same type of MCA fluorescence decrease-signal as produced by the central domain peptide DP4. Unlike DP4, DP1 corresponds to a portion of the N-terminal region, and the MCA probe is attached also to the N-terminal region. As a matter of fact, such a result was predicted from the hypothesis, because unzipping between the N-terminal and central domains would be produced by either DP4 or DP1. In further support of the hypothesis, pretreatment of RyR with DP1 prevented the MCA fluorescence change that might have been caused by the subsequent addition of DP4. Similarly, pretreatment with DP4 prevented fluorescence change by the subsequently added DP1. These results indicate that, after having produced the unzipping of the interacting domains by one domain peptide, further attempt to unzip by another domain peptide has become less effective. These results are also consistent with our previous ryanodine binding data showing that activation effects of RyR by DP4 and DP1 are not additive but that these peptides interfere with each other for their activation effects (*12*). These results are, again, consistent with the view that relatively large segments of the N-terminal domain and of the central domain of RyR are interacting to each other and that domain peptides corresponding to either of these segments produce the interference of interdomain interactions, resulting in unzipping of the domains and activation of the Ca^{2+} channel.

In conclusion, the present results show a general agreement with the hypothesis deduced from our recent studies that a close contact, or zipping, between two regulatory domains (specifically, the N-terminal and the central domains) of RyR stabilizes the closed state of the RyR Ca^{2+} channel, while unzipping such a contact is involved in the opening of the channel. Thus, the domain peptide corresponding to the Leu^{2442}-Pro^{2477} region of the central domain, DP4, mediated the incorporation of the fluorescent conformational probe MCA into the N-terminal domain in a site-directed manner, indicating that there is at least partial interaction between these two domains. The fluorescence intensity of the MCA probe that had been attached to the N-terminal domain showed a rapid decrease upon the addition of activating domain peptides (e.g., DP4 and DP1), consistent with the view that the MCA probe reported the change in the interacting domains from a zipped to an unzipped configuration. This view was further confirmed by the fluorescence quenching experiment. The present finding that T-tubule depolarization induced the MCA fluorescence decrease at the DP4-binding domain and then induced SR Ca^{2+} release suggests that the unzipping mechanism is used for the physiological activation process of E−C coupling.

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