Defective Regulation of Interdomain Interactions Within the Ryanodine Receptor Plays a Key Role in the Pathogenesis of Heart Failure

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Background—According to our hypothesis, 2 domains within the ryanodine receptor (RyR) of sarcoplasmic reticulum (SR) (N-terminal [0 to 600] and central [2000 to 2500] domains), where many mutations have been found in patients with polymorphic ventricular tachycardia, interact with each other as a regulatory switch for channel gating. Here, we investigated whether the defective FKBP12.6-mediated stabilization of RyR in heart failure is produced by an abnormal interdomain interaction.

Methods and Results—SR vesicles were isolated from dog left ventricular muscles, and then the RyR moiety of the SR was fluorescently labeled with methylcoumarin acetate (MCA) using DPc10, a synthetic peptide corresponding to Gly2460-Pro2495 of RyR (one of the mutable domains in polymorphic ventricular tachycardia), as a site-directing carrier; the carrier was removed from the RyR after MCA labeling. Addition of DPc10 induced an unzipped state of the interacting N-terminal and central domains, as evidenced by an increase in the accessibility of the RyR-bound MCA fluorescence to a large fluorescence quencher. Domain unzipping resulted in Ca\(^{2+}\)/H\(^{1+}\) leak through the RyR and facilitated cAMP-dependent hyperphosphorylation of RyR and FKBP12.6 dissociation from RyR. When DPc10 was introduced into the isolated myocytes, the magnitude of intracellular Ca\(^{2+}\)/H\(^{1+}\) transient decreased, and its decay time was prolonged. In the SR isolated from pacing-induced dog failing hearts, the domain unzipping has already occurred, together with FKBP12.6 dissociation and Ca\(^{2+}\) leak.

Conclusions—The specific domain interaction within the RyR regulates the channel gating property, and the defectiveness in the mode of the interdomain interaction seems to be the initial critical step of the pathogenesis of heart failure. (Circulation. 2005;111:3400-3410.)

Key Words: calcium ■ heart failure ■ ion channels ■ sarcoplasmic reticulum

A considerable body of accumulated evidence suggests that disturbed calcium homeostasis is a key mechanism for the pathogenesis of human heart failure.1–3 Our recent studies with a canine model of pacing-induced heart failure have shown (1) that the Ca\(^{2+}\) release function of the ryanodine receptor (RyR) is defective in heart failure,4 presumably because of a partial loss of FKBP12.6 from RyR5 and resultant conformational changes in the RyR,6 and (2) that this defective regulation of the RyR causes an abnormal Ca\(^{2+}\) leak.6 Marx et al,7 who investigated a causative mechanism of the partial loss of FKBP12.6 from RyR, found that protein kinase A (PKA)–mediated hyper-phosphorylation of RyR causes dissociation of FKBP12.6 from RyR, which in turn increases the sensitivity of the channel to activating Ca\(^{2+}\). β-Adrenergic receptor blockade has been shown to correct the defective interaction of FKBP12.6 with RyR that is triggered by the PKA-mediated hyperphosphorylation of RyR.5–10 More recently, we found that a new chemical compound JTV519, the 1,4-benzothiazepine derivative, prevented dissociation of FKBP12.6 from RyR, hence preventing abnormal conformational change in RyR and the subsequent Ca\(^{2+}\) leak.11 Such preventive effects of JTV519 are manifested in an early developmental stage of heart failure, thereby preventing severe heart failure.11 Wehrens et al12 also
demonstrated that JTV519 increased the affinity of FKBP12.6 for RyR2, which stabilized the closed state of RyR2 channels and prevented abnormal Ca\textsuperscript{2+} leak that would otherwise have triggered ventricular arrhythmias.

To date, >20 RyR2 missense mutations have been found to be linked with 2 inherited forms of sudden cardiac death: catecholaminergic polymorphic or familial polymorphic ventricular tachycardia\textsuperscript{13,14} and arrhythmogenic right ventricular cardiomyopathy type 2.\textsuperscript{15} All RyR2 mutations cluster into 3 regions of the channel that correspond to 3 malignant hyperthermia-central core disease mutation regions (designated as N-terminal domain, central domain, and channel forming domain) of RyR1. In particular, some mutations of RyR2 reported in cardiac disease patients are located in the regions corresponding to the skeletal N-terminal and central domains harboring most of the malignant hyperthermia mutations that cause an increased Ca\textsuperscript{2+} leak. This suggests that RyR2 shares a common domain-mediated channel regulation mechanism with RyR1 and that the increased Ca\textsuperscript{2+} leak of diseased RyR2 channels may be explained by the altered mode of interdomain interactions as described below.

The concept that the interactions between the N-terminal domain and the central domain of RyR1 are involved in Ca\textsuperscript{2+} channel regulation has emerged from the recent domain peptide studies of Yamamoto et al\textsuperscript{16} and Yamamoto and Ikemoto.\textsuperscript{17} According to this concept, in the resting or nonactivated state, the N-terminal and central domains make close contact at several subdomains (domain zipping). The conformational constraints imparted by the zipped configuration of these 2 domains stabilize and maintain the closed state of Ca\textsuperscript{2+} channel. Stimulation via excitation-contraction coupling or pharmacological agents weakens these critical interdomain contacts, resulting in loss of conformational constraints (domain unzipping) and thus lowering of the energy barrier for Ca\textsuperscript{2+} channel opening. Weakening of these interdomain interactions may also occur via mutation or with the use of synthetic domain peptides. For instance, a cardiac domain peptide (DP10c) corresponding to the Gly\textsuperscript{2460}-Pro\textsuperscript{2495} region of the rabbit RyR2 (equivalent to the Gly\textsuperscript{2409}-Pro\textsuperscript{2404} region of the human RyR2) was found to produce significant activation of the RyR2 Ca\textsuperscript{2+} channel, especially at low Ca\textsuperscript{2+} concentrations (0.1 to 0.3 \(\mu\)mol/L).\textsuperscript{18} A single Arg-to-Ser mutation made in DP10c, mimicking the Arg\textsuperscript{2474}-to-Ser\textsuperscript{2474} human mutation, abolished all of the effects that would have been produced by DP10c.\textsuperscript{18} These data presented strong evidence that synthetic domain peptides corresponding to key subdomains of RyR2 are capable of mimicking diseased conditions of the RyR2 channel by interfering with the interdomain interaction.

Interestingly, the binding region of FKBP12.6 to RyR2, which seems to be in the 2361 to 2496 residues according to Marx et al,\textsuperscript{7} is included in the sequence of DPc10 (2460 to 2495). This suggests the possibility that there is a close mechanistic relationship between the PKA-mediated FKBP12.6 dissociation and abnormal domain-domain interactions as such that seen in the DPc10-mediated channel hypersensitization.

In the present study, using the well-characterized domain peptide DPc10 as a probe, we investigated whether FKBP12.6-mediated stabilization of RyR is linked to the interdomain interactions in cardiac muscle.

**Methods**

**Materials**

FK506 was provided by Fujisawa Pharmaceutical Co Ltd. JTV519 was kindly provided by Dr Noboru Kaneko (Dokkyo University).

**Animal Disease Model**

In beagle dogs weighing 10 to 13 kg, we induced heart failure by continuous application of rapid ventricular pacing at 250 bpm using an externally programmable miniature pacemaker (Medtronic Inc) for 28 days, as described elsewhere.\textsuperscript{4–6} The care of the animals and the protocols used were in accord with guidelines laid down by the Animal Ethics Committee of Yamaguchi University School of Medicine.

**Preparation of Sarcoplasmic Reticulum vesicles**

We prepared sarcoplasmic reticulum (SR) vesicles from dog left ventricle (LV), essentially following the method of Kranias et al,\textsuperscript{19} with the modifications described elsewhere.\textsuperscript{4–6}

**Peptides Used and Peptide Synthesis**

We used the 2 domain peptides, DP10c and DP10c-mut: DP10c (DPc10 (DPc2460-2495)), 460GFCPDHKAAVMVLFLDSYVGYEVQDFLLHLLEVGFLP2495; DP10c-mut (DPc2460-2495-mut R2474S), 460GFCPDHKAAMVLFLDSYVGYEVQDFLLHLLEVGFLP2495. Peptides were synthesized on an Applied Biosystems model 431A synthesizer with Fmoc [N-(9-fluorenylmethoxycarbonyl) as the \(\alpha\)-amino protecting group, as described previously.\textsuperscript{18} The peptides were cleaved and deprotected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography.

**Ca\textsuperscript{2+} Uptake and Leak Assays**

\(\text{Ca}^{2+}\) uptake and the following \(\text{Ca}^{2+}\) leak assays were done as described previously.\textsuperscript{5,8,11} The amount of \(\text{Ca}^{2+}\) uptake was measured using \(^{45}\text{Ca}\) in the presence of 10 \(\mu\)mol/L ruthenium red and 5 mmol/L oxalate as described previously.\textsuperscript{20}

**Site-Directed Fluorescent Labeling of the RyR**

Specific fluorescent labeling of RyR in SR vesicles was performed using the cleavable hetero-bifunctional cross-linking reagent sulfo-succinimidyl 3-[(2-[7-azido-4-methylcoumarin-3-acetamido] ethyl)dithio]propionate from PIERCE, with DPc10 as a site-specific carrier. This method for the site-directed fluorescent labeling of the RyR2 was basically the same as the method used for the DP4-mediated methylcoumarin acetate (MCA)–labeling of RyR1.\textsuperscript{17}

To determine the localization of the MCA binding site, fluorescently labeled microsomes (1 mg/mL) were digested with recombinant calpain II (Calbiochem) added at a ratio of 6 U calpain to 1 mg SR protein in a solution containing 130 mmol/L NaCl and 20 mmol/L MOPS (pH 7.2). Digestion was started by adding 3 mmol/L CaCl\(_2\). After digestion for 6 minutes at 22°C, the reaction was stopped by adding 5 mmol/L BAPTA.

**Fluorescence Quenching of the MCA Fluorescence Attached to the DPc10 Binding Site**

The zipped and unzipped states of RyR were evaluated as described previously.\textsuperscript{17} The principle of the fluorescence quench assay of domain unzipping is that a large quencher BSA-QSY is inaccessible to the attached MCA in the zipped state, whereas it becomes accessible to the MCA site in the unzipped state. To form the quencher, QSY 7 carboxylic acid was conjugated with BSA by incubating 5 mmol/L QSY 7 carboxylic acid with 0.5 mmol/L BSA in 20 mmol/L HEPES (pH 7.5) for 60 minutes at 22°C in the dark. Unreacted QSY 7 carboxylic acid was removed by Sephadex G50 gel filtration. Fluorescence quenching by both QSY 7 BSA conjugate (a large quencher) and acrylamide (a small quencher) was performed.
by measuring steady-state fluorescence of labeled MCA (excitation, 368 nm; emission, 455 nm) in the presence or absence of chemicals. The data were analyzed with the Stern-Volmer equation.

**Immunoblot Analysis**

We performed immunoblot analyses for FKBP12.6 as described elsewhere.6,8,11 Using the method by Marx et al.7 we achieved coimmunoprecipitation of FKBP12.6 from SR using anti-RyR antibody (Oncogene Research Products), followed by immunoblotting with anti-FKBP12 (C-19) antibody (Santa Cruz Biotechnology). Relative phosphorylation level of RyR was determined by immunoblotting with anti-phosphoRyR2 (P2809), which was kindly provided by Dr Andrew R. Marks (Columbia University).

**Isolation of Cardiac Myocytes**

Cardiac myocytes were isolated from the LV free wall as described previously in detail.21 In brief, a wedge of LV free wall perfused by a branch of the left circumflex coronary artery was dissected free of the heart and perfused with collagenase-containing buffer. LV myocardium was minced with scissors in fresh collagenase-containing buffer. Then, rod-shaped adult canine cardiomyocytes were prepared by retrograde perfusion of quickly excised hearts with 95%O2/5%CO2–bubbled minimal essential medium (Sigma) supplemented with 50 μmol/L Ca2+, 0.5 mg/mL collagenase B, 0.5 mg/mL collagenase D, and 0.02 mg/mL protease type XIV. The Ca2+ concentration was then gradually increased to a final concentration of 1 mmol/L by changing the incubation medium (50 μmol/L, 125 μmol/L, 300 μmol/L, and then 1 mmol/L). The isolated canine cardiomyocytes were transferred to laminin-coated glass culture dishes and incubated for 12 hours at 37°C in 5%CO2/95%O2 atmosphere.

**Cell Shortening and Ca2+ Transient Measurement**

Measurements of myocyte cell shortening and intracellular Ca2+ were performed using fura-2 AM, as described previously.22
were stimulated by a field electric stimulator (IonOptix) at a stimulation frequency of 0.5 Hz. After stimulation of the cells, the cell shortening and peak Ca\(^{2+}\)/H11001 transient gradually increased and reached steady state within 1 minute. At the steady state (\(\sim 2\) minutes after initiation of pacing), the intracellular calcium concentration was monitored by a dual-excitation spectrofluorometer as the ratio of the fluorescence emission intensities (at 505 nm) elicited by excitation at 340 and 380 nm. DPc10 was introduced into the cells with a protein delivery reagent (Bioporter, Gene Therapy Systems, Inc). The successful introduction of DPc10 into the cell was confirmed by detecting the intracellular fluorescence signal of the peptide prelabeled with Alexa Fluor 350 (Molecular Probe). Briefly, DPc10–Alexa Fluor conjugate was formed by incubating 1 mmol/L DPc10 with 1 mmol/L Alexa Fluor carboxylic acid and succinimidyl ester in a 20 mmol/L HEPES buffer (pH 7.6) for 60 minutes at 22°C in the dark. Free Alexa Fluor was removed by Sephadex G15 gel filtration. The final concentration of DPc10 conjugated with the Alexa Fluor was estimated by measuring the absorbance at 280 nm. After the introduction of DPc10-Alexa Fluor conjugate into the cell, the fluorescence intensity of Alexa Fluor was measured with confocal microscopy (LSM 510, Carl Zeiss). The intracellular peptide concentration was determined from the concentration-fluorescence intensity plot.

Statistical Analysis
Statistical analysis was performed by ANOVA with a post hoc Scheffé’s test. Data are expressed as mean ± SD. We accepted a value of \(P<0.05\) as statistically significant.

Results
DPc10 Induces Ca\(^{2+}\) Leak and Facilitates PKA-Mediated Hyperphosphorylation and FKBP12.6 Dissociation
Addition of 1 μmol/L thapsigargin to normal SR vesicles at the steady state of ATP-dependent Ca\(^{2+}\) uptake produced little Ca\(^{2+}\) leak, whereas addition of 10 to 100 μmol/L DPc10,
together with 1 μmol/L thapsigargin, produced a pronounced leak. However, DPC10-mut (10 to 100 μmol/L) did not induce Ca\(^{2+}\) leak (Figure 1A). Following ATP-dependent Ca\(^{2+}\) uptake after preincubation of the SR vesicles with 3 to 30 μmol/L cAMP plus 1 μmol/L okadaic acid, 1 μmol/L thapsigargin was added. Under the conditions, a pronounced leak was observed (Figure 1A). Both DPC10- and cAMP-induced Ca\(^{2+}\) leaks were almost completely inhibited by 0.3 μmol/L JTV519 (Figure 1B). Figure 1C shows the effect of DPC10 and cAMP on the spontaneous Ca\(^{2+}\) leak in failing SR.

In agreement with our previous reports, there was a prominent Ca\(^{2+}\) leak in the failing SR even in the absence of cAMP or DPC10. Therefore, both cAMP and DPC10 had no further effect on the Ca\(^{2+}\) leak. JTV519 (0.3 μmol/L) abolished the spontaneous Ca\(^{2+}\) leak that otherwise had been seen in the failing SR. On the other hand, there was no appreciable effect of DPC10 on SR Ca\(^{2+}\) uptake (with DPC10, 8.96±3.48 nmol · mg\(^{-1}\) · min\(^{-1}\); without DPC10, 8.73±1.71 nmol · mg\(^{-1}\) · min\(^{-1}\); P=0.77).

Figure 2A through 2C shows the concentration-dependent effects of DPC10 or cAMP on the level of phosphorylation of RyR2 and the extent of dissociation of FKBP12.6 from RyR2 in normal SR. In the absence of cAMP, DPC10 had no appreciable effect both on dissociation of FKBP12.6 from RyR2 and on the PKA phosphorylation level (Figure 2A). However, preincubation of the SR vesicles with cAMP for 30 minutes, as in Ca\(^{2+}\) leak experiment, led to a dissociation of FKBP12.6 in a concentration-dependent manner concurrently with an increase in the PKA phosphorylation level (Figure 2B).

As shown in Figure 2C, in the presence of 100 μmol/L DPC10, FKBP12.6 dissociation from RyR occurred at a lower concentration of cAMP than in its absence, in parallel with an increase in the PKA phosphorylation level of RyR. This suggests that domain unzipping induces a conformational change that facilitates PKA phosphorylation and FKBP12.6 dissociation. In further support of our previous reports, the amount of RyR2-bound FKBP12.6 was lower in failing SR than in normal SR, which was inversely proportional to the level of hyperphosphorylation of RyR2 (Figure 2D). The amount of FKBP12.6 was unchanged even in the presence of JTV519, suggesting that the inhibition of Ca\(^{2+}\) leak by JTV519 (Figure 1C) is not mediated by rebinding of (RyR-unbound) FKBP12.6 to SR vesicles.

### Spectroscopic Monitoring of DPC10- and cAMP-Induced Changes in the Mode of Interdomain Interactions

According to the domain-mediated channel regulation hypothesis (see the Introduction), the above findings suggest that DPC10 produced unzipping of the interacting N-terminal and central domains, which then caused Ca\(^{2+}\) leak. To investigate this possibility, we adopted the fluorescence quench technique. The method involves (1) incorporation of the fluorescent probe MCA into a key site of the interacting N-terminal and central domain pair in a site-specific manner by using DPC10 as a site-direction carrier, (2) localization of the site of MCA attachment within the polypeptide chain of RyR2, and (3) examination of the accessibility of the incorporated MCA probe to a large fluorescence quencher. Figure 3A shows that the carrier DPC10 mediated an intense fluorescence labeling of the RyR2 band (left lane), but DPC10-mut could not mediate MCA labeling (middle lane). As shown in the “cold-chase” experiment shown in the right lane, an excess concentration of unlabeled DPC10 (10 mmol/L) prevented DPC10-mediated MCA labeling. Figure 3B shows the results of our attempt to localize the site of MCA attachment within the RyR2 polypeptide chain. As shown in the Coomassie blue–stained gels, digestion of the SR with calpain II (6 U/mg protein) cleaved the RyR2 into several fragments. Of these fragments, 2 fragments, a 410-kDa fragment and a 120-kDa fragment, retained the MCA label (fluorescent gel). Importantly, polyclonal antibody raised against the peptide corresponding to the 24CTATIHKEQQKL35 region of RyR2 reacted specifically with the 116-kDa fragment of recombinant calpain II added at ratio of 6 U calpain to 1 mg SR protein. Note that site of MCA labeling is localized in exactly same fragments as stained by anti–N-terminal antibody. Anti–N-terminal antibody (rabbit serum) was raised against peptide 24CTATIHKEQQKL35.
The MCA probe attached to the critical domain would be inaccessible to a bulky fluorescence quencher (QSY-BSA conjugate) in the zipped configuration, but it would become accessible to the quencher on unzipping (see Methods). To monitor the zipped and unzipped states of RyR2, we used QSY-BSA as a large quencher (Figure 4). Similar to the case of the recent report with DP4,17 the slope of the Stern-Volmer plot \( K_Q \), which represents the accessibility to the quencher, namely the degree of domain unzipping, was considerably increased by DPc10, indicating that DPc10 induced domain unzipping (Figure 4A). However, when we used, acrylamide, the small quencher, DP10 had virtually no effect on the efficiency of the MCA fluorescence quenching (not shown). These results suggest that the domain unzipping may involve a sizable opening of the gap between the interacting domains. Like DPc10, 30 \( \mu \)mol/L cAMP increased the \( K_Q \), although the extent of the increase was about half of the DPc10 (100 \( \mu \)mol/L)–induced increase (Figure 4B). Collectively, DPc10 induces domain unzipping; however, domain unzipping does not induce FKBP dissociation by itself. Rather, it seems to facilitate FKBP dissociation in response to PKA-mediated hyperphosphorylation of RyR.

As shown in Figure 4C, both DPc10- and cAMP-induced increases in the extent of fluorescence quenching were almost completely reversed by JTV519. Addition of FK506 that dissociates FKBP12.6 from RyR in normal SR (D). Note that accessibility of MCA to quencher (ie, degree of domain unzipping) in failing SR is larger than that of normal SR, but in presence of JTV519, former became about same as latter (E). Effect of FKBP12.6 reconstitution into FKBP12.6-depleted RyR2 on accessibility of bound MCA to quencher (F). Normal SR vesicles were mixed with 30 \( \mu \)mol/L cAMP for 30 minutes and then centrifuged, followed by addition of 30 nmol/L recombinant FKBP12.6, together with alkaline phosphatase for dephosphorylation of RyR2 (1:100 enzyme to protein),7 in presence or absence of 100 \( \mu \)mol/L DPc10. Same procedure, except for addition of cAMP, was performed also in failing SR vesicles. Note that FKBP12.6 rebinding restored zipped state that could be unzipped again in both cAMP-treated normal and failing SR vesicles.

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Interestingly, the extent of fluorescence quenching \( (K_Q) \) in failing heart SR without the addition of domain-unzipping reagents (DPc10 or cAMP) was about the same as that in the
normal SR that had been treated with DPc10 (Figure 4E). However, JTV519 reduced the Kq to a normal level in failing SR. These findings suggest that domain unzipping had already occurred in failing SR, causing Ca\textsuperscript{2+} leak and FKBP dissociation, and that JTV519 restored the zipped state and then restored normal channel function and a normal state of FKBP association. To further elucidate whether domain unzipping can be reversed by FKBP12.6 rebinding, we performed an FKBP12.6 reconstitution experiment under dephosphorylation conditions of RyR2 using alkaline phosphatase.\textsuperscript{7} As shown in Figure 4F, phosphatase treatment and FKBP12.6 rebinding restored a zipped state that could again be unzipped by DPc10 in both cAMP-treated normal SR and failing SR. In the presence of phosphatase, the amount of the re-bound FK12.6 was about the same among the normal SR, cAMP-treated normal SR, and failing SR, presumably because the phosphatase inhibits phosphorylation of RyR2 and hence restores the FKBP12.6 rebinding.

To further characterize local conformation in the vicinity of the DPc10-mediated MCA attachment site in normal and diseased conditions, we investigated the effects of DPc10 or JTV519 on the fluorescence intensity of the attached MCA. As shown in Figure 5A, DPc10 induced a rapid decrease in the fluorescence signal in normal SR, which was inhibited by...
Cell Shortening, %

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Iso indicates isoproterenol; FSK, forskolin.

*P<0.05, †P<0.01, untreated vs DPC10(+) and JTV519(−); ‡P<0.05, §P<0.01, DPC10(+) and JTV519(−) vs DPC10(+) and JTV519(+) in normal myocytes, or JTV519(−) vs JTV519(+) in failing myocytes; ††P<0.05 vs Iso(−) and FSK(−).

JTV519. The fluorescence intensity of the attached MCA is expected to decrease when the environment of the MCA site changes from hydrophobic to hydrophilic on domain unzipping. Thus, these results provide further evidence that DPC10 produced domain unzipping and JTV519 reversed it. In failing SR, DPC10 produced virtually no effect on the MCA fluorescence, presumably because domain unzipping had already taken place. On addition of JTV519, however, the MCA fluorescence increased to a higher level, indicating restored zipped state. Figure 5B shows the concentration-dependent effects of DPC10 or JTV519 on the changes in MCA fluorescence in normal and failing SR vesicles. DPC10 decreased the level of MCA fluorescence in a concentration-dependent manner in normal SR (reflecting the change from the zipped to the unzipped state), whereas JTV519 increased the MCA fluorescence level in failing SR (reflecting the restoration of the zipped state).

**Effects of DPC10 on Ca²⁺ Transients and Cell Shortening**

To investigate the effect of DPC10-induced domain unzipping in vivo in vivo conditions, we introduced DPC10 into the cardiac myocytes and investigated both Ca²⁺ transient and cell shortening simultaneously (the Table and Figure 6). To evaluate the efficiency of DPC10 introduction into the cell by the protein delivery reagent, Alexa Fluor 350–DPc10 conjugate was mixed with the protein delivery reagent. The Alexa Fluor fluorescence signal was clearly observed in all myocytes, indicating successful DPC10 introduction into myocytes by this method (Figure 6A). There was no statistically significant difference in the estimated concentration of DPC10 (see Methods) between JTV-treated (2.92 ± 0.65 μmol/L) and untreated DPC10-introduced myocytes (2.91 ± 0.71 μmol/L; P=0.82). As shown in Figure 6B, neither protein delivery reagent (Biporter) alone nor DPC10-mut (R2474S) with Biporter had any significant effect on cell shortening and Ca²⁺ transient. This suggests that protein delivery reagent has no direct effect on myocyte function and that DPC10-mut does not interact with RyR and has no effect on myocyte functions. As shown in Figure 6C, in response to isoproterenol or forskolin, the duration of Ca²⁺ transient was prolonged, its peak was decreased, and the diastolic level of cytoplasmic [Ca²⁺] was increased. However, isoproterenol or forskolin produced no significant change in Ca²⁺ transient in these myocytes. Because DPC10 did not change SR Ca²⁺ uptake function, as mentioned earlier, these results suggest that, as in the aforementioned in vitro experiments, DPC10 produced domain unzipping and then Ca²⁺ leak. The increase in cell shortening in response to isoproterenol or forskolin was less in the DPC10-treated myocyte than the untreated myocyte (Table). Coincubation with JTV519 (0.3 μmol/L) restored normal activities of both Ca²⁺ transient and cell shortening in the DPC10-treated myocyte. These findings, consistent in both in vitro and in vivo studies, suggest that the conformational state of the interacting domains (zipped or unzipped) is a critical factor for tuning the channel gating and for Ca²⁺ homeostasis in normal and diseased myocytes.

As shown in Figure 6D, Ca²⁺ transient and cell shortening in the myocytes isolated from the pacing-induced failing dog hearts were deteriorated compared with those in normal myocytes. The diastolic level of the cytoplasmic [Ca²⁺] was higher in failing myocytes than in normal myocytes. In response to isoproterenol or forskolin, both Ca²⁺ transient and cell shortening were partially restored toward normal by JTV519 (0.3 μmol/L). There was no significant difference in the Ca²⁺ transient and cell shortening properties between DPC10-treated and untreated failing myocytes (data not shown).

Because intracellular peak Ca²⁺ transient strongly depends on the SR Ca²⁺ loading, we hypothesized that SR Ca²⁺ loading may be reduced as a result of Ca²⁺ leak in DPC10-
introduced myocytes and that it may be increased with JTV519 by preventing the Ca\textsuperscript{2+} leak. To test this hypothesis, we performed caffeine application experiments (Figure 7). In DPC10-introduced normal myocytes, peak Ca\textsuperscript{2+} transient after addition of caffeine was reduced, whereas it was restored toward normal in the presence of JTV519 (Figure 7, top). In failing myocytes in which the peak Ca\textsuperscript{2+} transient after addition of caffeine was lower than in normal myocytes, JTV519 increased it (Figure 7, bottom). These findings suggest that prevention of Ca\textsuperscript{2+} leak by JTV519 is effective in increasing myocyte contractility by increasing SR Ca\textsuperscript{2+} loading.

**Discussion**

The most important finding deduced from this study is that the defective interdomain interaction may play a key role in abnormal Ca\textsuperscript{2+} leak and subsequent contractile and relaxation dysfunctions seen in heart failure. This conclusion is based on the following findings. First, DPC10 (10 to 100 μmol/L) induced domain unzipping (Figure 4A) and the subsequent Ca\textsuperscript{2+} leak (Figure 2A). DPC10-induced domain unzipping did not induce FKBP12.6 dissociation by itself, but it facilitated FKBP dissociation in response to PKA-mediated hyperphosphorylation (Figure 2C). Alternately, FKBP dissociation either by PKA phosphorylation or FK506 induced domain unzipping (Figure 4B) and Ca\textsuperscript{2+} leak (Figure 2B). This domain unzipping was reversed by JTV519 (Figure 4C and 4D). Thus, domain unzipping and FKBP dissociation produce synergistic effects via conformational changes in the vicinity of interacting domains, and JTV519 may prevent the conformational change. Second, in failing hearts, the unzipped state
has already taken place, but JTV519 restored the zipped state (Figure 4E). Thus, JTV519 stopped Ca\(^{2+}\) leak completely (Figure 1C), even though much of the FKBP12.6 had already been dissociated from RyR by hyperphosphorylation of RyR (Figure 2D). Third, in the experiment with isolated myocytes, the DPc10-introduced myocytes showed a prolonged duration of Ca\(^{2+}\) transient and a decreased velocity in cell shortening (Figure 6C). These abnormalities were corrected by the addition of JTV519 (Figure 6C). The SR Ca\(^{2+}\) content, assessed by rapid application of caffeine, was reduced both in DPc10-introduced normal myocytes and in failing myocytes, but it was restored by JTV519. This suggests that the inhibition of SR Ca\(^{2+}\) leak by JTV519 increased SR Ca\(^{2+}\) content and thereby augmented cell shortening (Figure 7). Thus, both in vitro and in vivo experiments carried out in the present study consistently support the notion that the unzipped state of the interacting domains plays a key role in various episodes seen in failing heart such as FKBP dissociation, hyperphosphorylation, and SR Ca\(^{2+}\) leak. This notion is further supported by the fact that phophatase treatment and FKB12.6 rebinding restore the zipped state that can be unzipped again by DPc10. Thus, it seems that restoration of the domain interaction from the unzipped to the zipped states is a new strategy for the treatment of polymorphic ventricular tachycardia and posttranscriptionally developed heart failure as well. Reduction of the Ca\(^{2+}\) leak seems to be advantageous from an energy point of view because SR Ca\(^{2+}\) leak itself requires increased SERCA2a activity to maintain a physiological level of cytosolic Ca\(^{2+}\).

It was shown that mutations in RyR2 (S2246L, R2474S, R4497C) linked to exercise-induced arrhythmias (in patients with catecholaminergic polymorphic ventricular tachycardia) reduced the affinity of FKB12.6 for RyR2 and increased single-channel activity after PKA phosphorylation.\(^{23}\) It should be noted that one of these mutations, R2474S, is the same mutation we have made in DPc10-mut. The present finding that DPc10 produced diseaselike effects but DPc10-mut did not is consistent with our prediction that DPc10, but not DPc10-mut, is capable of interfering with the domain interaction and producing a domain unzipping effect (see hypothesis in the Introduction). These results present valuable evidence that synthetic domain peptide was capable of mimicking native conformation of the corresponding in vivo domain, and the data obtained with the peptide are physiologically relevant. Indeed, the fact that DPc10 increased the sensitivity of PKA phosphorylation–induced FKB12.6 dissociation to cAMP in the SR (Figure 2C) is consistent with the recent finding that catecholaminergic polymorphic ventricular tachycardia–linked mutant RyR2 channels have a decreased affinity for FKB12.6 and display abnormal single-channel function after PKA phosphorylation.\(^{23}\)

Although RyR2 has been shown to be hyperphosphorylated, correlating with FKB12.6 dissociation both in various experimental models of heart failure\(^{6,7,24}\) and in human heart failure,\(^{7,10}\) the functional role of RyR phosphorylation by PKA remains to be elucidated. Li et al\(^{25}\) found that PKA phosphorylation of RyR did not increase calcium sparks in permeabilized myocytes when the cytosolic Ca\(^{2+}\) was clamped at 50 or 10 nmol/L, lower than diastolic Ca\(^{2+}\) concentrations. They also recently showed that PKA phosphorylation induced by isoproterenol significantly increased the rate of Ca\(^{2+}\) release without changing the amplitude of Ca\(^{2+}\) release when both SR Ca\(^{2+}\) load and Ca\(^{2+}\) current were held constant.\(^{26}\) This suggests that a major outcome of PKA-dependent phosphorylation of RyR2 is an increase in the excitation-contraction coupling gain during exercise and stress.

The data shown in this study suggest that abnormal RyR2 channel functions in failing hearts are caused by 3 major factors: domain unzipping, an increased level of PKA phosphorylation, and dissociation of the receptor-bound FKB12.6. As shown here, domain unzipping facilitated PKA phosphorylation and the resultant FKB12.6 dissociation (Figure 2C) in normal SR. In the absence of cAMP, DPc10 caused domain unzipping and Ca\(^{2+}\) leak, without causing any changes in the level of phosphorylation and in the amount of the RyR2-bound FKB12.6. These results

![Figure 7. Representative time courses of Ca\(^{2+}\) transients before and after addition of caffeine in normal and failing myocytes.](http://circ.ahajournals.org/)

Caffeine-induced increase in Ca\(^{2+}\) transients reflecting SR Ca\(^{2+}\) load was measured after stimulation train at 0.5 Hz by rapidly switching superfusing solution to one containing 20 mmol/L caffeine for 5 to 6 seconds. Note that peak Ca\(^{2+}\) transient after addition of caffeine (measure of SR Ca\(^{2+}\) load) was higher in normal myocytes than in failing myocytes and that JTV treatment (0.3 μmol/L) ameliorated SR load by inhibiting SR Ca\(^{2+}\) leak.
suggest that the increased tendency of domain unzipping may be the primary cause of the increased Ca\(^{2+}\) leak. However, the present data also show that the increased phosphorylation and FKBP12.6 dissociation produce SR Ca\(^{2+}\) leak directly or by facilitating domain unzipping. Thus, a reasonable conclusion of the present study seems to be that these 3 factors—domain unzipping, hyperphosphorylation, and FKBP12.6 dissociation—produce synergetic effects on Ca\(^{2+}\) leak in a cooperative manner.

As shown here, JTV519 inhibits DPc10-induced domain unzipping and prevents Ca\(^{2+}\) leak, and again, in the absence of cAMP, JTV519 has no effect on phosphorylation and the receptor-bound FKBP12.6 (data not shown). This suggests that the mechanism of pharmacological action of JTV519 is to stabilize the interdomain interaction and in turn to prevent FKBP12.6 dissociation and Ca\(^{2+}\) leak. In further support of this notion, we found that the amount of RyR2-bound FKBP12.6 in failing heart remained identical in the absence or presence of JTV519. However, we cannot analyze the possibility that the improvement in myocyte function by JTV519 has no effect on phosphorylation and the FKBP12.6 dissociation and Ca\(^{2+}\) leak. In conclusion, defective interactions of the regulatory domains in RyR2 seem to play a key role in the abnormal Ca\(^{2+}\) channel functions of RyR2 seen in the SR of failing hearts such as increased Ca\(^{2+}\) leak and dissociation of FKBP12.6. The present finding that the defective domain interaction is corrected by JTV519 provides one with a new clue for the development of therapeutic strategy against heart failure and possibly cardiac arrhythmia.

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**References**

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Defective Regulation of Interdomain Interactions Within the Ryanodine Receptor Plays a Key Role in the Pathogenesis of Heart Failure

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Correction

In the article, “Defective Regulation of Interdomain Interactions Within the Ryanodine Receptor Plays a Key Role in the Pathogenesis of Heart Failure,” by Oda et al, which appeared in the June 28, 2005, issue of the journal (Circulation. 2005;111:3400–3410), the authors would like to note the following error:

In the legend to Figure 3, anti-N-terminal antibody (rabbit serum) was raised against peptide “6EGEDIEQFLRTDDE19,” not “24CTATIHKEQQKL35.”

The authors regret this error.

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