Identification of Target Domains of the Cardiac Ryanodine Receptor to Correct Channel Disorder in Failing Hearts

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Background—We previously demonstrated that defective interdomain interaction between N-terminal (0 to 600) and central regions (2000 to 2500) of ryanodine receptor 2 (RyR2) induces Ca$^{2+}$ leak in failing hearts and that K201 (JTV519) inhibits the Ca$^{2+}$ leak by correcting the defective interdomain interaction. In the present report, we identified the K201-binding domain and characterized the role of this novel domain in the regulation of the RyR2 channel.

Methods and Results—An assay using a quartz-crystal microbalance technique (a very sensitive mass-measuring technique) revealed that K201 specifically bound to recombinant RyR2 fragments 1741 to 2270 and 1981 to 2520 but not to other RyR2 fragments from the 1 to 2750 region (1 to 610, 494 to 1000, 741 to 1503, 1245 to 1768, 2234 to 2750). By further analysis of the fragment [741–2270], K201 was found to specifically bind to its subfragment 2114–2149.

With the use of the peptide matching this subfragment (DP2114–2149) as a carrier, the RyR2 was fluorescently labeled with methylcoumarin acetate (MCA) in a site-directed manner. After tryptic digestion, the major MCA-labeled fragment of RyR2 (155 kDa) was detected by an antibody raised against the central region (Ab2112). Moreover, of several recombinant RyR2 fragments, only fragment 2234–2750 was specifically MCA labeled; this suggests that the K201-binding domain 2114–2149 binds with domain 2234–2750. Addition of DP2114–2149 to the MCA-labeled sarcoplasmic reticulum interfered with the interaction between domain 2114–2149 and domain 2234–2750, causing domain unzipping, as evidenced by an increased accessibility of the bound MCA to a large-size fluorescence quencher. In failing cardiomyocytes, the frequency of spontaneous Ca$^{2+}$ spark was markedly increased compared with normal cardiomyocytes, whereas incorporation of DP2114–2149 markedly decreased the frequency of spontaneous Ca$^{2+}$ spark.

Conclusions—We first identified the K201-binding site as domain 2114–2149 of RyR2. Interruption of the interdomain interaction between the domain 2114–2149 and central domain 2234–2750 seems to mediate stabilization of RyR2 in failing hearts, which may lead to a novel therapeutic strategy against heart failure and perhaps lethal arrhythmia. (Circulation. 2008;117:762-772.)

Key Words: calcium ▪ heart failure ▪ ion channels ▪ ryanodine receptor ▪ sarcoplasmic reticulum

The Ca$^{2+}$ release channel of the sarcoplasmic reticulum (SR), referred to as ryanodine receptor 2 (RyR2), plays a central role in cardiac excitation-contraction coupling.1 Several pieces of evidence show that abnormality within the RyR2 causes Ca$^{2+}$ leak, leading to contractile and relaxation dysfunction,2-3 and triggers lethal arrhythmia through induction of delayed afterdepolarization.4 The Ca$^{2+}$ leak may be induced by FKBP12.6 dissociation due to protein kinase A–mediated hyperphosphorylation of the RyR2 in several types of heart failure,2 but there are conflicting reports that protein kinase A hyperphosphorylation and subsequent FKBP12.6 dissociation are not necessarily the cause of heart failure.5,6 Prevention of Ca$^{2+}$ leak or hypersensitized channel gating can be achieved by β-blocker7,8 and angiotensin II receptor antagonist9 via restoration of FKBP12.6-mediated stabilization of RyR2. As described previously, we found that K201 (JTV519),10 the 1,4-benzothiazepine derivative, corrects the defective FKBP12.6-mediated control of the RyR2, improving cardiac function during the development of heart failure.11 Marks and colleagues12,13 also demonstrated that K201 increased the binding affinity of FKBP12.6 to the RyR2, which stabilized the closed state of RyR2 channels and...
prevented the abnormal Ca\textsuperscript{2+} leak that otherwise would have triggered ventricular arrhythmias and contractile dysfunction. These studies suggest that securing the stabilization of the RyR2 may be an attractive therapeutic strategy against heart failure.

To date, many RyR2 missense mutations have been found to be linked with the inherited form of sudden cardiac death: catecholaminergic polymorphic ventricular tachycardia.\textsuperscript{14,15} All RyR2 mutations cluster into 3 regions of the channel that correspond to the 3 malignant hyperthermia/central core disease mutation regions (designated as N-terminal domain, central domain, and C-terminal channel-forming domain), suggesting that these specific domains play a key role in channel regulation as a common mechanism underlying both RyR2 and RyR1 isoforms.

From the unique distribution of mutation sites in the RyR, a new concept that the interaction between the N-terminal domain and the central domain is involved in Ca\textsuperscript{2+} channel regulation has emerged from the recent domain peptide probe studies of Ikemoto and colleagues.\textsuperscript{16,17} 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 the 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, the peptide corresponding to the Leu\textsuperscript{2422}-Pro\textsuperscript{2477} region of the central domain (DPs4) was found to bind to the N-terminal region of the RyR1 and produce malignant hyperthermia (MH)–like hyperactivation/hypersensitization effects. Spectroscopic studies have been consistent with the hypothesis that DPs4 produces unzipping of interacting domains.\textsuperscript{16,17} Significantly, introduction of a single amino acid substitution corresponding to an MH mutation in DPs4 results in the peptide losing its channel-stabilizing capacity. These data presented strong evidence that mutation in DPs4 results in the peptide losing its channel-activating capacity. For instance, the peptide corresponding to the Gly\textsuperscript{2460}-Gly\textsuperscript{2495} region of the central domain (DPc10) caused a Ca\textsuperscript{2+} leak through the RyR2 channel and destabilized both cAMP-dependent hyperphosphorylation of, and FKBP12.6 dissociation from, the RyR2. Interestingly, the unzipped state has already taken place in failing hearts, accompanied with an abnormal Ca\textsuperscript{2+} leak.\textsuperscript{20} These findings suggest that destabilization of the zipped state of the interacting domains is a key mechanism for the development of RyR2 channel dysfunctions in the failing heart. We further demonstrated that both K201 and the antioxidant edaravone corrected the defective interdomain interaction in failing hearts.\textsuperscript{20,21}

The main aim of the present study is to provide detailed information to better understand the mechanism of the pharmacological action of K201. First, to identify the drug-binding site within the RyR2, we screened recombinant fragments and domain peptides corresponding to various regions of RyR2 using the quartz-crystal microbalance (QCM) technique (a highly sensitive mass-measuring technology). We then investigated the effect of K201 on the mode of interdomain interactions among key regulatory domains, including the newly identified drug-binding domain. As shown in our report, binding of K201 to domain 2114–2149 seems to play a critical role in correcting the defective interdomain interaction within RyR2 and hence preventing the Ca\textsuperscript{2+} leak seen in failing hearts.

## Methods

### Materials

FK506 and K201 were provided by Fujisawa Pharmaceutical Co Ltd (Osaka, Japan) and Aeta Co Ltd (Tokyo, Japan), respectively.

### Homology Alignment Analysis

A homology alignment analysis of RyR2 and annexin V was done with the use of MagAlign software (Lasergene Inc).

### Animal Preparation

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, Minneapolis, Minn) for 28 days, as described previously.\textsuperscript{3} 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 SR Vesicles

We prepared SR vesicles from dog left ventricular muscle as described previously.\textsuperscript{3}

### Peptides Used and Peptide Synthesis

We used the following domain peptides for the experiments: DPc10 (DP\textsuperscript{2480–2495}), DP\textsuperscript{2114–2149}, DP\textsuperscript{2460–2495}, DP\textsuperscript{2114–2149}, and DP\textsuperscript{2460–2495}.

### Construction and Expression of RyR2 Fragments

The peptides corresponding to various regions of RyR2 (1 to 610, 1981 to 2520, 2234 to 2750) were polymerase chain reaction amplified with oligonucleotide primers designated to contain 2 restriction enzyme sites. Detailed methods are described in the online-only Data Supplement.

### QCM Measurements

Binding of K201 to RyR2 fragments was detected by using a 27-MHz QCM (Nihon, Inc, Japan), which is a highly sensitive mass-measuring apparatus.\textsuperscript{23,24} The QCM Au electrode was coated...
with K201 and immersed in a solution (500 μL) containing (in mmol/L) 150 NaCl, 20 MOPS (pH 7.4). The amount of drug binding was determined from the frequency changes due to changes in mass on the electrode (with sensitivity on the order of subnano-gram) on injection of a small volume (2 to 5 μL) of solution containing RyR2 fragments (30 μmol/L). No significant nonspecific binding of RyR2 fragments to the Au electrode was detected.

Ca2+ Uptake and Ca2+ Leak Assays
Ca2+ uptake and the following Ca2+ leak assays were done as described previously. Detailed methods are described in the online-only Data Supplement.

Site-Directed Fluorescent Labeling of the RyR
Specific fluorescent labeling of RyR2 in SR vesicles was performed as described in the online-only Data Supplement in detail. To determine the location of the incorporated methylcoumarin acetate (MCA) within the primary structure of RyR2, fluorescently labeled microsomes (1 mg/mL) were digested with TPCK trypsin (Calbiochem), added at various amounts of TPCK trypsin to 1 μg of SR protein in a solution containing (in mmol/L) 150 NaCl and 20 MOPS (pH 7.2). Digestion was started by adding TPCK trypsin (25 to 400 ng/mL) to the SR solution; after incubation for 10 minutes at 22°C, the reaction was stopped by adding trypsin inhibitor (40 μg/mL).

Fluorescence Quench Assay of Interdomain Interactions Within the RyR2
The state of interactions (zipping/unzipping) of regulatory domains within the RyR2 was evaluated by the fluorescence quench technique described previously. In brief, 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 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, as a control) was performed by measuring steady state fluorescence of the labeled MCA (excitation at 368 nm, emission at 455 nm) in the presence or absence of effectors. The data were analyzed with the use of the Stern-Volmer equation.

Immunoblot Analysis
The amount of RyR2-bound FKBP12.6 was determined by immunoblot analysis as described previously, which involved coimmunoprecipitation of FKBP12.6/RyR2 with anti-RyR2 antibody (Oncogene Research Products) and immunoblotting with anti-FKBP12 (C-19) antibody (Santa Cruz Biotechnology, Santa Cruz, Calif).

Preparation of Isolated Cardiomyocytes
Cardiomyocytes were isolated from the left ventricular free wall as described previously. The method is described briefly in the online-only Data Supplement.

Cell Shortening and Ca2+ Transient Measurement
Measurements of myocyte cell shortening and intracellular calcium were performed with the use of fura 2-AM, as described previously. Measurements of myocyte cell shortening and intracellular calcium were performed with the use of fura 2-AM, as described previously.

Analysis of Ca2+ Sparks With Laser Scanning Confocal Microscopy
Ca2+ sparks were measured as previously described on a laser scanning confocal microscope (LSM-510, Carl Zeiss) equipped with an argon ion laser coupled to an inverted microscope (Axiovert 100, Carl Zeiss) with a Zeiss ×40 oil-immersion Plan-Neofluor objective (numerical aperture, 1.3; excitation at 488 nm; emission >505 nm). Briefly, cardiomyocytes were loaded with fluo-4-AM (20 μmol/L; Molecular Probes) for 30 minutes at room temperature. Line-scan mode was used, in which a single cardiomyocyte was scanned repeatedly (325.7 Hz) along a line parallel to the longitudinal axis, avoiding nuclei. To monitor Ca2+ sparks, cardiomyocytes were stimulated until the Ca2+ transient reached steady state, then stimulation was stopped, and Ca2+ sparks were recorded during the subsequent ~10-second rest. Data were analyzed with SparkMaster, an automated analysis program that allows rapid and reliable spark analysis. The analysis includes general image parameters (number of detected sparks, spark frequency) as well as individual spark parameters (amplitude, full width at half maximum, full duration at half maximum).

Statistical Analysis
Linear regression analysis was used for comparison of the fluorescence quench assay in Figure 5. Statistical analysis was performed by 2-way ANOVA with a post hoc Scheffé test for comparison of FKBP12.6 dissociation data (Figure 3C), cell shortening, and Ca2+ transient data (Figure 6C). For comparison of Ca2+ spark parameters, 1-way ANOVA with a post hoc Scheffé test or paired t test was used in normal or failing cardiomyocytes, respectively (Figure 7B). Data are expressed as mean ± SD. We accepted a probability value <0.05 as statistically significant.

Results
K201 Binds to Annexin V–like Domain Within RyR2 (Domain 2114–2149)
K201 was found to interact with annexin V, and the cocrystal structure of K201 bound to annexin V was reported by Kaneko et al. We investigated whether there is any amino-acid sequence homology to annexin V in particular regions of RyR2, using MagAlign, an alignment software by Lasergene. In the whole region of the RyR2, domain containing a striking similarity to the corresponding sequence of annexin V, as shown in Figure 1. This portion contains no arrhythmogenic right ventricular cardiomyopathy/catecholaminergic polymorphic ventricular tachycardia mutation sites, but the corresponding portion of RyR1 harbors several MH/CCD (central core disease) mutation sites, i.e. R2163C, R2163H, R2163P, V2168M, and I2182F. Importantly, the domain of RyR2 includes domain 2114–2149, whose sequence corresponds to DPs5, a peptide that was previously shown to have a potential to reverse DPs5-induced domain unzipping in RyR1.

We synthesized the cardiac counterpart of DPs5, namely, DP 2114–2149, and investigated its drug-binding properties and functional effects. As shown in Figure 2A, QCM recording shows that there is a rapid binding of K201 with DP 2114–2149. No appreciable binding was observed in the N-terminal (2086–2750 residue region contains the 2 well-characterized domains) that together work as a regulatory switch for channel gating. As shown in Figure 2B, K201 specifically bound to fragment 1741–2270 and fragment 1981–2520, which contain the DP 2114–2149 sequence. However, there was no binding to other fragments that contain no DP 2114–2149 sequence.
Figure 1. Sequence similarity of amino acid residues between RyR2 and annexin V. Identical amino acid is shown in dark gray, and similar amino acid is shown in light gray.

DP**2114–2149** Prevents the FK506-Induced Ca**2+** Leak Without Reassociation of FKBP12.6 to RyR2

To investigate the role of K201-binding domain**2114–2149** of RyR2 in Ca**2+** channel regulation, we examined the effect of synthetic peptide DP**2114–2149** on the time course of FK506-induced Ca**2+** leak in normal SR vesicles. As shown in Figure 3A, addition of 0.3 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 30 μmol/L FK506 together with 0.3 μmol/L thapsigargin produced a pronounced Ca**2+** leak. However, this FK506-induced Ca**2+** leak was almost completely inhibited by addition of DP**2114–2149** (IC**50** = 0.1 μmol/L), similar to the inhibitory effect of K201 (IC**50** = 0.1 μmol/L) (Figure 3B). There was no appreciable effect of DP**2114–2149** on SR Ca**2+** uptake (data not shown). Because FK506-induced Ca**2+** leak seems to be a result from the dissociation of RyR2-bound FKBP12.6, we examined the effect of DP**2114–2149** on FK506-induced dissociation of FKBP12.6 from RyR2 in normal SR vesicles. As shown in Figure 3C, the addition of FK506 (1 μmol/L) almost completely dissociated FKBP12.6 from RyR2, regardless of the presence of DP**2114–2149** (30 μmol/L). This indicates that the inhibitory effect of DP**2114–2149** on Ca**2+** leak is not due to reassociation of FKBP12.6 to RyR2.

Identification of the Site of DP**2114–2149** Interaction Within RyR2

It appears that K201 and the peptide corresponding to the drug-binding domain (DP**2114–2149**) exert the identical inhibitory effect on Ca**2+** leak. This suggests that besides the drug-binding domain**2114–2149**, there is another regulatory domain with which the in vivo domain**2114–2149** or its corresponding DP**2114–2149** interacts. Presumably, the interaction between the drug-binding domain and its partner domain plays a critical role in channel regulation. In an attempt to identify the putative partner domain, we performed site-directed MCA labeling of RyR2 and RyR2 fragments (1 to 610, 741 to 1260, 1245 to 1768, 1741 to 2270, 2234 to 2750) using DP**2114–2149** as a carrier (Figure 4; for the principle of site-specific fluorescence labeling and protocol, see the online-only Data Supplement). This resulted in a specific fluorescence labeling of RyR2 (Figure 4A). After trypic digestion of MCA-labeled RyR2, the MCA-labeled fragment of RyR2 (155 kDa) was identified as the fragment derived from the central region of RyR2, as evidenced by the fact that it was immunostained by an antibody (Ab) raised against central region (Ab**2132**) but not by C-terminal Ab**963** and N-terminal Ab**12** (Figure 4A). Furthermore, as shown in Figure 4B, of several recombinant RyR2 fragments that cover the region**2234–2750**, only fragment**2234–2750** was specifically MCA labeled. This suggests that the 2234 to 2750 region is the aforementioned partner domain of the K201-binding domain**2114–2149**.

The extent of DP**2114–2149**-mediated MCA labeling of the RyR2 or fragment**2234–2750**, namely, the extent of binding of DP**2114–2149** to the domain**2234–2750** of RyR2, was about the same in the presence and in the absence of K201 (Figure 4C).

Spectroscopic Monitoring of DP**2114–2149**-Induced Changes in the Mode of Interdomain Interactions

To study the role of interdomain interaction between the K201-binding domain**2114–2149** and its partner domain**2234–2750**, we performed the fluorescence quench assay. The RyR moiety of the SR was fluorescently labeled with MCA with the use of DPC10 or DP**2114–2149** as a site-directing carrier; the carrier was removed from the RyR after MCA labeling to allow re-unzipping (see Figure in the online-only Data Supplement). The MCA probe that was attached to the critical domain is inaccessible to a bulky fluorescence quencher (QSY-BSA conjugate) in the zipped state of the interacting domains, although it becomes accessible to the quencher on unzipping of these domains (see Methods). To monitor the zipped and unzipped states of interacting domains, therefore, we employed the fluorescence quenching technique using a large-molecular-weight fluorescence quencher QSY-BSA (Figure 5). In agreement with our previous report,30 the extent of fluorescence quenching (KQ; the Stern-Volmer quenching constant determined from the slope of the plot, which is a measure of the extent of domain unzipping) of the MCA bound with the N-terminal domain was large in failing heart SR as well as in the normal SR that was treated with DPC10 to unzip the N-terminal/central domain-domain interaction. Addition of DP**2114–2149** or K201 reduced the KQ in failing SR (Figure 5A).
indicating that defective interdomain interaction (unzipping) in the failing RyR2 or in the DPC10-treated RyR2 was restored to a normal zipped state; this is reminiscent of the effect of K201 described in our previous report (see Figure 5 in Reference 20).

However, addition of DP2114–2149 or K201 to normal SR produced no appreciable change in KQ. These data suggest that domain unzipping between the N-terminal and central domains had taken place in failing SR, causing Ca²⁺ leak, and that K201 and

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**Figure 2.** Characterization of the binding of K201 with synthetic peptides (A), recombinant RyR2 fragments (B), and the purified RyR2 and/or annexin V (C) with the use of QCM. K201 was immobilized on the sensor chip surface as described in Methods. The tracings represent the time course of the binding of K201 to various peptides or RyR2 fragments. Inset, Either SR or purified RyR2 by SDS gel elution (see online-only Data Supplement) was separated in 6% SDS-PAGE.
Figure 3. A, Effect of DP2114-2149 on the time courses of FK506-induced Ca\(^{2+}\)/H\(_{11001}\) leak in normal SR vesicles. Note that the Ca\(^{2+}\)/H\(_{11001}\) leak was inhibited by addition of DP2114-2149 or K201 but not by DP2086-2114 or DP2150-2185. B, Concentration-dependent effect of DP2114-2149 or K201 on FK506-induced Ca\(^{2+}\)/H\(_{11001}\) leak in normal SR vesicles. C, Effect of DP2114-2149 (30 \(\mu\)mol/L) on FK506-induced dissociation of FKBP12.6 from RyR2 in normal SR vesicles. Values are mean±SD. The number of each group is 4 to 6. Note that the addition of FK506 almost completely dissociated FKBP12.6 from RyR2, regardless of the presence of DP2114-2149.
DP<sup>2114–2149</sup> corrected the defective unzipped state to a normal zipped state.

Interestingly, the situation was completely in a mirror image to the aforementioned data when we labeled the partner domain of the drug-binding domain with MCA using DP<sup>2114–2149</sup> as a site-directing carrier, namely, the K<sub>Q</sub> in the failing RyR2 was significantly smaller than normal SR, indicative of the zipped state in diseased conditions and the unzipped state in normal conditions. Addition of DPc10 to normal SR reduced the K<sub>Q</sub>, mimicking the situation seen in failing SR. Addition of DP<sup>2114–2149</sup> or K201 increased the K<sub>Q</sub> in failing SR vesicles, whereas there was no effect in normal SR. These results suggest that domain interaction between N-terminal and central domains is linked with another set of domain-domain interactions between the drug-binding domain<sup>2114–2149</sup> and its partner domain<sup>2234–2750</sup> and that these 2 sets of interdomain interactions are counterbalanced with each other, or coupled in a reciprocal manner, to regulate the RyR2 channel. These findings are summarized diagrammatically in the lower panels of Figure 5.

**Effect of DP<sup>2114–2149</sup> on Ca<sup>2+</sup> Transient and Ca<sup>2+</sup> Sparks in Normal and Failing Cardiomyocytes**

To investigate the effect of DP<sup>2114–2149</sup> in the in vivo conditions, we introduced DP<sup>2114–2149</sup> into the cardiomyocytes and investigated both Ca<sup>2+</sup> transient and cell shortening simultaneously. Successful incorporation of DP<sup>2114–2149</sup> into the cell was confirmed by detecting the Alexa fluorescence signal of fluorescently labeled DP<sup>2114–2149</sup> in the cell (Figure 6A). Neither protein delivery reagent (Bioporator) nor Bioporator plus DP<sup>2114–2149</sup> had any detectable effect on cell shortening and Ca<sup>2+</sup> transient in normal cardiomyocytes. As shown in Figure 6B and summarized in Figure 6C, in response to FK506 the duration of Ca<sup>2+</sup> transient was prolonged, and its peak was decreased in the untreated normal cardiomyocytes. However, DP<sup>2114–2149</sup> incorporation into the FK506-treated cardiomyocytes improved Ca<sup>2+</sup> transient and cell shortening. The Ca<sup>2+</sup> transient and cell shortening in the cardiomyocytes isolated from pacing-induced failing dog hearts were deteriorated, as in the FK506-treated cardiomyocytes. However, both Ca<sup>2+</sup> transient and cell shortening were partially restored toward normal by DP<sup>2114–2149</sup> incorporation.

As shown in Figure 7, Ca<sup>2+</sup> spark frequency was significantly increased both in FK506-added normal cardiomyocytes and in failing cardiomyocytes. Again, DP<sup>2114–2149</sup> incorporation significantly decreased the Ca<sup>2+</sup> spark frequency in both cardiomyocytes.

**Discussion**

A considerable body of evidence has been accumulated in the literature that defective operation of the SR Ca<sup>2+</sup> release channel, the ryanodine receptor, is one of the major causative factors of heart failure. We previously demonstrated that the interdomain interaction between the N-terminal domain and the central domain of RyR2 stabilizes channel gating, and their interaction becomes defectively loose in failing hearts, resulting in Ca<sup>2+</sup> leak and contractile dysfunction of cardiomyocytes. It was also shown that K201 reversed the mode of interdomain interaction from a defective unzipped config-
FK506 (30 μM) facilitates rebinding of FKBP12.6 in some cases, as shown in our previous study \(^1\) and by Wehrens et al. \(^12\) These findings allowed us to establish the drug-binding region as domain2114–2149. Although we identified the drug-binding domain, using a highly sensitive QCM technique for the drug-binding assay, we screened an entire area from the N-terminus to the residue covering an entire area from the N-terminus to the residue 2750 and showed that drug binding takes place to the constructs corresponding to the 1741 to 2520 region. Importantly, a synthetic peptide corresponding to the 2114 to 2149 region (DP2114–2149), but not its neighboring peptides, showed drug-binding capability. These findings allowed us to establish the drug-binding region as domain DP2114–2149. Although we

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DP2114–2149, the domain peptide matching the drug-binding domain of K201, also binds to other regions of RyR2. The well-known interdomain interaction between N-terminal (0 to 600) and central domains (2000 to 2500) and the newly identified interdomain interaction between the drug-binding domain2114–2149 and its partner domain2234–2750 are counterbalanced with each other or coupled in a reciprocal manner to regulate RyR2 Ca2+ channels. Thus, domain unzipping between the N-terminal (0–600) and central domains (2000–2500) in DPC10-incorporated normal or failing SR induces domain zipping between DP2114–2149 domain and its partner domain2234–2750 to destabilize the closed state of the channel. Interruption of the latter domain-domain interaction by K201 or DP2114–2149 restores a normal zipped state of the former domain-domain interaction, in turn restabilizing the channel.

Figure 7. A hypothetical model showing how interdomain interactions within the RyR2 are involved in the mechanism of pharmacological action of K201. The well-known interdomain interaction between N-terminal (0 to 600) and central domains (2000 to 2500) and the newly identified interdomain interaction between the drug-binding domain2114–2149 and its partner domain2234–2750 are counterbalanced with each other or coupled in a reciprocal manner to regulate RyR2 Ca2+ channels. Thus, domain unzipping between the N-terminal (0–600) and central domains (2000–2500) in DPC10-incorporated normal or failing SR induces domain zipping between DP2114–2149 domain and its partner domain2234–2750 to destabilize the closed state of the channel. Interruption of the latter domain-domain interaction by K201 or DP2114–2149 restores a normal zipped state of the former domain-domain interaction, in turn restabilizing the channel.

have not screened the carboxyl half of the RyR2 polypeptide chain, we propose that the domain2114–2149 is the specific K201-binding region of the entire molecule because the 2054 to 2163 region showed the best alignment match with the sequence of annexin V, whose specific interaction with K201 is well established. Nevertheless, we cannot completely eliminate the possibility that K201 also binds to other regions of the RyR2 under some specific conditions.

Another important finding in the present study is that DP2114–2149, the domain peptide matching the drug-binding domain2114–2149 mentioned above, showed a K201-like channel-stabilizing effect, namely, it restored normal interdomain interaction between the N-terminal and central domains (ie, a zipped configuration) in failing SR and in turn prevented its Ca2+ leak. Furthermore, we found that DP2114–2149 binds to a particular region of RyR2. As shown by the peptide-mapping analysis of the MCA-labeled RyR2 (Figure 4A), DP2114–2149-mediated specific MCA labeling took place in the central region (155 kDa) of RyR2. Moreover, of several recombinant RyR2 fragments covering the 1 to 2750 region, only fragment2234–2750 was MCA labeled with the use of DP2114–2149 as a site-directing carrier. This indicates that the druglike effect of DP2114–2149 described above is produced by its binding to the domain2234–2750. Because DP2114–2149 corre-

Figure 8. A hypothetical model showing how interdomain interactions within the RyR2 are involved in the mechanism of pharmacological action of K201. The well-known interdomain interaction between N-terminal (0 to 600) and central domains (2000 to 2500) and the newly identified interdomain interaction between the drug-binding domain2114–2149 and its partner domain2234–2750 are counterbalanced with each other or coupled in a reciprocal manner to regulate RyR2 Ca2+ channels. Thus, domain unzipping between the N-terminal (0–600) and central domains (2000–2500) in DPC10-incorporated normal or failing SR induces domain zipping between DP2114–2149 domain and its partner domain2234–2750 to destabilize the closed state of the channel. Interruption of the latter domain-domain interaction by K201 or DP2114–2149 restores a normal zipped state of the former domain-domain interaction, in turn restabilizing the channel.

sponds with the drug-binding domain2114–2149, these findings suggest that the interdomain interaction between the drug-binding domain2114–2149 and its partner domain2234–2750 plays an important role in the mechanism of drug action, as elaborated below.

As deduced from the present fluorescence quenching experiments, (A) the well-known interdomain interaction between N-terminal (0 to 600) and central domains (2000 to 2500) and (B) the newly identified interdomain interaction between the drug-binding domain2114–2149 and its partner domain2234–2750 are counterbalanced with each other or coupled in a reciprocal manner. Our postulated model is illustrated in Figure 8. In the normal RyR2, A is in a zipped configuration, whereas B is in an unzipped configuration to stabilize the closed state of the channel. In the failing RyR2 or in the DPC10-treated RyR2, A becomes unzipped, which is coupled with zipping of B, resulting in the destabilized, or partially opened, state of the channel. Addition of DP2114–2149 or K201 to the destabilized RyR2 produces domain unzipping in B, which in turn brings A back to a normal zipped state.

The mechanism by which K201 and DP2114–2149 unzips the interacting K201-binding domain and its partner domain has not yet been fully resolved. However, the mechanism of DP2114–2149-induced domain unzipping seems to be similar to the previously postulated mechanism of DPC10-induced domain unzipping,16,17 namely, the binding of the peptide to its partner domain competes with the in vivo domain-domain interaction, resulting in a disruption of normal interdomain interaction. In the case of K201, however, its domain unzipping effect cannot be explained simply by the interference of in vivo domain-domain interaction by the drug because the binding of DP2114–2149 to its partner domain is not affected by
K201 (Figure 4C). We tentatively propose that the binding of K201 to the domain (14–2149) of RyR2 produces a conformational change in its neighborhood, weakening the interaction of the drug-binding domain with its partner domain. In short, channel dysfunctions in failing hearts reflected on Ca2+ leak and abnormal Ca2+ sparks are due to abnormal interaction between the drug-binding domain (14–2149) and its partner domain (234–2750), causing abnormal unzipping between the N-terminal and central domains. K201 brings the former interdomain interaction to a normal state (unzipped state), in turn restoring the latter interaction to a normal zipped state. The next important question regards whether FKBP12.6 is required for the channel-stabilizing effect of K201. The fact that 1 μmol/L FK506 almost completely dissociates FKBP12.6 from RyR2 even in the presence of DP (2114–2149) (Figure 3C in this study) or K201 (Figure 3B in Reference 11) indicates that the function of DP (2114–2149) and K201 to inhibit Ca2+ leak does not depend on the facilitated binding of FKBP12.6 to RyR2. The findings that both K201 (20) and its binding domain (2114–2149) prevented domain unzipping between N-terminal and central domains in failing SR and in turn effectively inhibited Ca2+ leak without FKBP12.6 rebinding support the idea that correction of defective interdomain interaction may be a primary event for RyR2 stabilization. Recent findings by Hunt et al. (31) that K201 suppressed spontaneous Ca2+ release induced by Ca2+ overload in rat ventricular myocytes and in HEK293 cells expressing RyR2, irrespective of the state of FKBP12.6, support the idea that FKBP12.6 is not primarily involved in the corrective action of K201 on spontaneous Ca2+ release events. Although DP (2114–2149) specifically binds to RyR2, restores the normal zipped state of regulatory domains within RyR2, and inhibits FK506-induced Ca2+ leak, we cannot completely eliminate the possibility that the functional effects of DP (2114–2149) on cell shortening, SR Ca2+ release, and Ca2+ sparks are not direct consequences of DP (2114–2149) binding to RyR2 but secondary effects via other protein–protein interactions in vivo. Clearly, more work is required to address this issue adequately. In conclusion, the specific binding site of K201 was found to reside in the central domain within RyR2. The binding of K201 to this domain interferes with the novel interdomain interaction between the drug-binding domain (2114–2149) and central domain (234–2750). The specific interruption of the novel interdomain interaction within RyR2 seems to play a critical role in stabilizing the channel gating.

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Disclosures
None.

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A considerable body of evidence shows that the defective operation of the sarcoplasmic reticulum Ca\(^{2+}\) release channel, also known as ryanodine receptor 2 (RyR2), is a major causative factor of heart failure. Previously, we demonstrated that an interdomain interaction within RyR2, which stabilizes the channel gating, is defective in failing hearts, resulting in Ca\(^{2+}\) leak and contractile dysfunction. We have also shown that K201 (also known as JTV519) reversed the mode of interdomain interaction from a defective unzipped configuration to a normal zipped configuration and stopped Ca\(^{2+}\) leak. In this article, the mechanism by which K201 corrects the channel disorder of RyR2 is defined. The specific binding site of K201 was found to reside in the central domain of RyR2. The binding of K201 to this domain interferes with an interdomain interaction between the RyR2 drug-binding domain (2114 to 2149) and its partner domain. The specific interruption of this interdomain interaction within RyR2 seems to play a critical role in stabilizing the channel gating. These results further support the notion that fixing the defective interdomain interaction within RyR2 is a promising therapeutic strategy for treatment of heart failure.
Identification of Target Domains of the Cardiac Ryanodine Receptor to Correct Channel Disorder in Failing Hearts

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