Heart Failure

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

Takeshi Yamamoto, MD, PhD; Masafumi Yano, MD, PhD; XiaoJuan Xu, BS; Hitoshi Uchinoumi, MD; Hiroki Tateishi, MD; Mamoru Mochizuki, MD, PhD; Tetsuro Oda, MD, PhD; Shigeki Kobayashi, MD, PhD; Noriaki Ikemoto, PhD; Masunori Matsuzaki, MD, PhD

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 1741–2270, K201 was found to specifically bind to its subfragment 2114–2149. With the use of the peptide matching this subfragment (DP\(^{2114–2149}\)) 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 (Ab\(^{2132}\)). 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 DP\(^{2114–2149}\) 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 DP\(^{2114–2149}\) 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-blocker\(^7,8\) and angiotensin II receptor antagonist\(^9\) 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 colleagues\(^12,13\) also demonstrated that K201 increased the binding affinity of FKBP12.6 to the RyR2, which stabilized the closed state of RyR2 channels and

Received June 1, 2007; accepted November 28, 2007.
From the Department of Medicine and Clinical Science, Division of Cardiology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan (T.Y., M.Y., X.X., H.U., H.T., M. Mochizuki, T.O., S.K., M. Matsuzaki); Boston Biomedical Research Institute, Watertown, Mass (N.I.); and Department of Neurology, Harvard Medical School, Boston, Mass (N.I.).
The online-only Data Supplement, consisting of text and a figure, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.718957/DCL.

Correspondence to Masafumi Yano, MD, PhD, Department of Medicine and Clinical Science, Division of Cardiology, Yamaguchi University Graduate School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi, 755-8505, Japan. E-mail yanoma@yamaguchi-u.ac.jp
© 2008 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.107.718957

762
prevented the abnormal Ca\(^{2+}\) 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.\(^{14,15}\) All RyR2 mutations cluster into 3 regions of the channel that correspond to the 3 malignant hyperthermia/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\(^{2+}\) channel regulation has emerged from the recent domain peptide probe studies of Ikemoto and colleagues.\(^{16,17}\) 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\(^{2+}\) 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\(^{2+}\) 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\(^{2422}\)-Pro\(^{2477}\) 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.\(^{16,17}\) Significantly, introduction of a single amino acid substitution corresponding to an MH mutation in DPs4 results in the peptide losing its channel-stabilizing activity. These data presented strong evidence that DPs4 produces unzipping of interacting domains, suggesting that these specific domains play a key role in channel regulation as a common mechanism underlying both RyR2 and RyR1 isoforms.

Recently, we demonstrated the defective interdomain interaction between the N-terminal and central domains in pacing-induced failing hearts.\(^{20}\) Domain unzipping induced by a cardiac domain peptide corresponding to the Gly\(^{2460}\)-Pro\(^{2495}\) region of the RyR2 (DPc10) caused a Ca\(^{2+}\) leak through the RyR2 channel and facilitated 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\(^{2+}\) leak.\(^{20}\) 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.\(^{20,21}\)

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\(^{2+}\) leak seen in failing hearts.

### Methods

**Materials**

FK506 and K201 were provided by Fujisawa Pharmaceutical Co Ltd (Osaka, Japan) and Aetas 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.\(^3\) 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.\(^3\)

**Peptides Used and Peptide Synthesis**

We used the following domain peptides for the experiments: DPs10 (DP\(^{2460–2495}\)),

\[2460\text{GFCPDHKAA} \text{MVLFLD} \text{R} \text{VYGIEVQDFL} \text{H} \text{LLEVQGFLP}^{2495}\]

\[\text{DP}^{2114–2149}\]

\[2114\text{EDTINLLASLQGRLSSRM} \text{VGKKEEKLMIRGLGD}^{2250}\]

Peptides were synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc [N-(9-fluorenyl)methoxycarbonyl] as the aminomethyl protecting group, as described previously.\(^{22}\) The peptides were cleaved and deprotected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography.

**Construction and Expression of RyR2 Fragments**

The peptides corresponding to various regions of RyR2 (1 to 610, 1981 to 2520, and 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 (Initium, Inc, Japan), which is a highly sensitive mass-measuring apparatus.\(^{23,24}\) 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 mmol/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 mg 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 fluo-2-AM, as described previously. The method is described briefly in the online-only Data Supplement.

**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 (Domain²¹¹⁴–²¹⁴⁹)

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, domain²⁰⁵⁹–²¹¹⁶, but not other regions, showed 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 MI/CCD (central core disease) mutation sites, i.e., R2163C, R2163H, R2163P, V2168M, and I2182F. Importantly, the domain²⁰⁵⁹–²¹¹⁶ of RyR2 includes domain²¹¹⁴–²¹⁴⁹, whose sequence corresponds to DPs5, a peptide that was previously shown to have a potential to reverse DPs4-induced domain unzipping in RyR1.

We synthesized the cardiac counterpart of DPs5, namely, DP²¹¹⁴–²¹⁴⁹, 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 DP²¹¹⁴–²¹⁴⁹. No appreciable binding was observed in the N-terminal (1–600) and central (2000–2500) digerated regions of RyR2, as indicated in Figure 2B. This 1 to 2750 residue region contains the 2 well-characterized domains, the N-terminal [1–600] and central [2000–2500] domains that together work as a regulatory switch for channel gating.
sequence. We further assessed whether K201 specifically binds to purified RyR2, whose purity was confirmed by SDS-PAGE (inset of Figure 2C). As shown in Figure 2C, the purified RyR2 bound to K201 immobilized to the sensor tip. However, the binding was abolished when the K201 on the sensor tip had been reacted with annexin V before the addition of RyR2. Taken together, we conclude that K201 specifically binds to RyR2 at its domain2114–2149.

**DP2114–2149 Prevents the FK506-Induced Ca2+ Leak Without Reassociation of FKBP12.6 to RyR2**

To investigate the role of K201-binding domain2114–2149 of RyR2 in Ca2+ channel regulation, we examined the effect of synthetic peptide DP2114–2149 on the time course of FK506-induced Ca2+ 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 Ca2+ uptake produced little Ca2+ leak, whereas addition of 30 µmol/L FK506 together with 0.3 µmol/L thapsigargin produced a pronounced Ca2+ leak. However, this FK506-induced Ca2+ leak was almost completely inhibited by addition of DP2114–2149 (IC50 = 0.1 µmol/L), similar to the inhibitory effect of K201 (IC50 = 0.1 µmol/L) (Figure 3B). There was no appreciable effect of DP2114–2149 on SR Ca2+ uptake (data not shown). Because FK506-induced Ca2+ leak seems to be a result from the dissociation of RyR2-bound FKBP12.6, we examined the effect of DP2114–2149 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 DP2114–2149 (30 µmol/L). This indicates that the inhibitory effect of DP2114–2149 on Ca2+ leak is not due to reassociation of FKBP12.6 to RyR2.

**Identification of the Site of DP2114–2149 Interaction Within RyR2**

It appears that K201 and the peptide corresponding to the drug-binding domain (DP2114–2149) exert the identical inhibitory effect on Ca2+ leak. This suggests that besides the drug-binding domain2114–2149 there is another regulatory domain with which the in vivo domain2114–2149 or its corresponding DP2114–2149 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 DP2114–2149 as a carrier (Figure 4; for the principle of site-specific fluorescent labeling and protocol, see the online-only Data Supplement). This resulted in a specific fluorescent labeling of RyR2 (Figure 4A). After tryptic 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 (Ab2234) but not by C-terminal Ab2750 and N-terminal Ab12 (Figure 4A). Furthermore, as shown in Figure 4B, of several recombinant RyR2 fragments that cover the region2114–2149, only fragments234 to 2750 was specifically MCA labeled. This suggests that the 2234 to 2750 region is the aforementioned partner domain of the K201-binding domain2114–2149.

The extent of DP2114–2149-mediated MCA labeling of the RyR2 or fragment234 to 2750, namely, the extent of binding of DP2114–2149 to the domain2234 to 2750 of RyR2, was about the same in the presence and in the absence of K201 (Figure 4C).

**Spectroscopic Monitoring of DP2114–2149-Induced Changes in the Mode of Interdomain Interactions**

To study the role of interdomain interaction between the K201-binding domain2114–2149 and its partner domain2234–2750, we performed the fluorescence quench assay. The RyR moiety of the SR was fluorescently labeled with MCA with the use of DPC10 or DP2114–2149 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,20 the extent of fluorescence quenching (KQ; the Stern-Volmer quench 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 DP2114–2149 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 $K_\text{Q}$. These data suggest that domain unzipping between the N-terminal and central domains had taken place in failing SR, causing $\text{Ca}^{2+}$ leak, and that K201 and
Figure 3. A, Effect of DP2114-2149 on the time courses of FK506-induced Ca\textsuperscript{2+} leak in normal SR vesicles. Note that the Ca\textsuperscript{2+} 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\textsuperscript{2+} leak in normal SR vesicles. C, Effect of DP2114-2149 (30 µmol/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\textsuperscript{2114–2149} 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\textsuperscript{2114–2149} as a site-directing carrier, namely, the K\textsubscript{0}, 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 DPC\textsubscript{10} to normal SR reduced the K\textsubscript{0}, mimicking the situation seen in failing SR. Addition of DP\textsuperscript{2114–2149} or K201 increased the K\textsubscript{0}, 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\textsuperscript{2114–2149} and its partner domain\textsuperscript{2234–2750} 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\textsuperscript{2114–2149} on Ca\textsuperscript{2+} Transient and Ca\textsuperscript{2+} Sparks in Normal and Failing Cardiomyocytes**

To investigate the effect of DP\textsuperscript{2114–2149} in the in vivo conditions, we introduced DP\textsuperscript{2114–2149} into the cardiomyocytes and investigated both Ca\textsuperscript{2+} transient and cell shortening simultaneously. Successful incorporation of DP\textsuperscript{2114–2149} into the cell was confirmed by detecting the Alexa fluorescence signal of fluorescently labeled DP\textsuperscript{2114–2149} in the cell (Figure 6A). Neither protein delivery reagent (Biporter) nor Bioporter plus DP\textsuperscript{2114–2149} had any detectable effect on cell shortening and Ca\textsuperscript{2+} transient in normal cardiomyocytes. As shown in Figure 6B and summarized in Figure 6C, in response to FK506 the duration of Ca\textsuperscript{2+} transient was prolonged, and its peak was decreased in the untreated normal cardiomyocytes. However, DP\textsuperscript{2114–2149} incorporation into the FK506-treated cardiomyocytes improved Ca\textsuperscript{2+} transient and cell shortening. The Ca\textsuperscript{2+} 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\textsuperscript{2+} transient and cell shortening were partially restored toward normal by DP\textsuperscript{2114–2149} incorporation.

As shown in Figure 7, Ca\textsuperscript{2+} spark frequency was significantly increased both in FK506-added normal cardiomyocytes and in failing cardiomyocytes. Again, DP\textsuperscript{2114–2149} incorporation significantly decreased the Ca\textsuperscript{2+} spark frequency in both cardiomyocytes.

**Discussion**

A considerable body of evidence has been accumulated in the literature that defective operation of the SR Ca\textsuperscript{2+} release channel, the ryanodine receptor, is one of the major causative factors of heart failure.\textsuperscript{30} 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\textsuperscript{2+} leak and contractile dysfunction of cardiomyocytes.\textsuperscript{30} It was also shown that K201 reversed the mode of interdomain interaction from a defective unzipped config-
shown in our previous study and by Wehrens et al. K201 facilitates rebinding of FKBP12.6 in some cases, as require rebinding of FKBP12.6 to RyR2 in failing SR, but leak. This drug-induced recovery process does not appear to uration to a normal zipped configuration and stopped Ca\(^{2+}\) leak. This drug-induced recovery process does not appear to require rebinding of FKBP12.6 to RyR2 in failing SR, but K201 facilitates rebinding of FKBP12.6 in some cases, as shown in our previous study and by Wehrens et al. These results suggest that K201 primarily modifies domain-domain interaction and in turn increases the affinity of FKBP12.6 to RyR2, presumably by establishing a stable conformation that favors FKBP12.6 binding to RyR2.

The present study has provided several pieces of important information required for a better understanding of the molecular mechanism of pharmacological action of K201. One of the most important aspects in this context is that we could identify the drug-binding domain. Using a highly sensitive QCM technique for the drug-binding assay, we screened recombinant constructs corresponding to various regions 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. Impor-tantly, a synthetic peptide corresponding to the 2114 to 2149 region (DP\(^{2114-2149}\)), but not its neighboring peptides, showed drug-binding capability. These findings allowed us to establish the drug-binding region as domain \(^{2114-2149}\). Although we

Figure 5. Stern-Volmer plots of the fluorescence quenching data with QSY-BSA. Effect of DPc10 or DP\(^{2114-2149}\) on the extent of MCA fluorescence in normal and failing SRs is shown; MCA labeling was done in a site-directed manner with the use of DPc10 (A) or DP\(^{2114-2149}\) (B) as a carrier. Values are mean±SD. The number of each group is 4 to 6. Diagrams for data interpretation are added at the bottom of each figure. The slope of the Stern-Volmer plot corresponds to the accessibility of the quencher.

Figure 6. A. Delivery of DP\(^{2114-2149}\) fluorescently labeled with Alexa Fluor 350 (Molecular Probes) into the isolated cardiomyocytes. Confocal microscopy clearly detected the fluorescence signal (green) of DP\(^{2114-2149}\) in the myocytes. Cell surface membrane was fluorescently labeled as red by wheat germ agglutinin-Alexa Fluor 633 conjugate (Molecular Probes). B. Effect of DP\(^{2114-2149}\) incorporation on Ca\(^{2+}\) transient and cell shortening in FK506 (30 μmol/L)-added normal cardiomyocytes and in failing cardiomyocytes. C. Summarized data of various parameters in Ca\(^{2+}\) transient and cell shortening in normal and failing cardiomyocytes. Values are mean±SD. The number of cells for each group is 10 to 15.
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 suggests 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 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.

The mechanism by which K201 and DP2114–2149 unzip 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 domain2114–2149 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 Ca\(^{2+}\) leak and abnormal Ca\(^{2+}\) sparks are due to abnormal interaction between the drug-binding domain2114–2149 and its partner domain2234–2750, 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 \(\mu\)mol/L FK506 almost completely dissociates FKBP12.6 from RyR2 even in the presence of DP2114–2149 (Figure 3C in this study) or K201 (Figure 3B in Reference 11) indicates that the function of DP2114–2149 and K201 to inhibit Ca\(^{2+}\) leak does not depend on the facilitated binding of FKBP12.6 to RyR2. The findings that both K20120 and its binding domain2114–2149 prevented domain unzipping between N-terminal and central domains in failing SR and in turn effectively inhibited Ca\(^{2+}\) 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 al31 that K201 suppressed spontaneous Ca\(^{2+}\) release induced by Ca\(^{2+}\) 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 Ca\(^{2+}\) release events. Although DP2114–2149 specifically binds to RyR2, restores the normal zipped state of regulatory domains within RyR2, and inhibits FK506-induced Ca\(^{2+}\) leak, we cannot completely eliminate the possibility that the functional effects of DP2114–2149 on cell shortening, SR Ca\(^{2+}\) release, and Ca\(^{2+}\) sparks are not direct consequences of DP2114–2149 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 domain2114–2149 and central domain2234–2750. The specific interruption of the novel interdomain interaction within RyR2 seems to play a critical role in stabilizing the channel gating.

Sources of Funding

This work was supported by grants-in-aid for scientific research from the Ministry of Education in Japan (grants 18390234 and 18659228 to Dr Yano, 18590777 to Dr Yamamoto, 18591706 to Dr Kobayashi, and 19209030 to Dr Matsuzaki) and a grant from the National Heart, Lung, and Blood Institute (HL072841 to Dr Ikemoto).

Disclosures

None.

References

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

Takeshi Yamamoto, Masafumi Yano, XiaoJuan Xu, Hitoshi Uchinoumi, Hiroki Tateishi, Mamoru Mochizuki, Tetsuro Oda, Shigeki Kobayashi, Noriaki Ikemoto and Masunori Matsuzaki

Circulation. 2008;117:762-772; originally published online January 28, 2008;
doi: 10.1161/CIRCULATIONAHA.107.718957

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/117/6/762

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2008/02/08/CIRCULATIONAHA.107.718957.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/