Defective domain–domain interactions within the ryanodine receptor as a critical cause of diastolic Ca^{2+} leak in failing hearts

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Aims A domain peptide (DP) matching the Gly²⁴⁶⁰–Pro²⁴⁹⁵ region of the cardiac type-2 ryanodine receptor (RyR2), DPc10, is known to mimic channel dysfunction associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), owing to its interference in a normal interaction of the N-terminal (1–600) and central (2000–2500) domains (viz. domain unzipping). Using DPc10 and two other DPs harboring different mutation sites, we investigated the underlying mechanism of abnormal Ca^{2+} cycling in failing hearts.

Methods and results Sarcoplasmic reticulum (SR) vesicles and cardiomyocytes were isolated from dog left ventricular muscles for Ca^{2+} leak and spark assays. The RyR2 moiety of the SR was fluorescently labelled with methylcoumarin acetate (MCA) using DPs corresponding to the 163–195 and 4090–4123 regions of RyR2 (DP163–195 and DP4090–4123, respectively) as site-directed carriers. Both DPs mediated a specific MCA fluorescence labelling of RyR2. Addition of either DP to the MCA-labelled SR induced domain unzipping, as evidenced by an increased accessibility of the bound MCA to a large-size fluorescence quencher. Both SR Ca^{2+} leak and Ca^{2+} spark frequency (SpF) were markedly increased in failing cardiomyocytes. Upon introduction of DP163–195 or DP4090–4123 into normal SR or cardiomyocytes, both Ca^{2+} leak and SpF increased to the levels comparable with those of failing myocytes. K201 (JTV519) suppressed all of the effects induced by DP163–195 (domain unzipping and increased Ca^{2+} leak and SpF) or those in failing cardiomyocytes, but did not suppress the effects induced by DP4090–4123.

Conclusion Defective inter-domain interaction between N-terminal and central domains induces diastolic Ca^{2+} leak, leading to heart failure and lethal arrhythmia. Mutation at the C-terminal region seen in CPVT does not seem to communicate with the aforementioned N-terminal and central inter-domain interaction, although spontaneous Ca^{2+} leak is similarly induced.

1. Introduction

A Ca^{2+} release channel protein, the ryanodine receptor (RyR2) in sarcoplasmic reticulum (SR), plays a key role in a transient increase of the intracellular Ca^{2+} concentration from nmol/L to $\mu\text{mol/L}$ during cardiac muscle contraction.¹ A considerable amount of evidence has been accumulated that diastolic Ca^{2+} leak through the RyR2 is one of the problems in failing hearts.¹ The Ca^{2+} leak reduces SR Ca^{2+} load, thereby decreasing the SR Ca^{2+} required for an efficient contraction, causing contractile

dysfunction. Moreover, the diastolic Ca^{2+} leak triggers delayed afterdepolarization (DAD) caused by the entry of Na^{+} via Na^{+} – Ca^{2+} exchanger, which occasionally results in lethal arrhythmia.² It has been suggested that dissociation of RyR2-bound FKBP12.6 due to PKA-mediated hyperphosphorylation may be the cause of diastolic Ca^{2+} leak seen in various types of heart failure.³ However, several reports suggest that PKA hyperphosphorylation and subsequent FKBP12.6 dissociation may not necessarily be the primary cause of Ca^{2+} leak and heart failure.^{4,5}

In our recent report we have shown that defective interactions between the N-terminal domain (1–600) and

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central domain (2000–2500) of RyR2, harboring many mutation sites of catecholaminergic polymorphic ventricular tachycardia (CPVT)^{6,7} or ARVC,⁸ cause Ca^{2+} leak in failing hearts and reduced SR Ca^{2+} load, leading to contractile dysfunction.⁹ The causative mechanism of such dysfunctions seems to be as follows. In normal hearts these domains are interacting with each other forming a zipped state, which stabilizes the closed state of the Ca^{2+} channel. However, in failing SR the interaction becomes loose (domain unzipping), which de-stabilizes the closed state making the channel leaky. Reversal of the unzipped state to a normal zipped state by K201 (JTV519) restores normal channel gating in otherwise leaky channels of failing SR.⁹

Almost 40% of the reported CPVT mutation sites are within C-terminal region of RyR2. Recently, Liu *et al.*¹⁰ reported that in the knock-in mouse model of human CPVT mutant of RyR2 (R4496C), sustained bi-directional ventricular tachycardia occurred, and that K201 was without effect on such lethal arrhythmia. These findings suggest that the mutation at C-terminal region may induce Ca^{2+} sparks or DAD, leading to the lethal arrhythmia, but the defectiveness of RyR2 due to the mutation within C-terminal domain may not be ascribable to the aforementioned defective inter-domain interaction between the N-terminal and central regions. George *et al.*¹¹ made an attempt to reconstitute the channel gating function by co-expressing a truncated cytoplasmic N-terminal segment (segment P) and C-terminal segment containing channel domain (segment C) of RyR2, and found that in order to confer the agonist-regulated gating function to the channel domain, both Segments P and C must contain a common overlapping segment designated as I-domain that corresponds to residues 3722–4610. This suggests that the I-domain transduces cytoplasmic events to the Ca^{2+} pore-forming domain. Taken together, inter-domain interaction among N-terminal, central, I-domain, and transmembrane channel domain appears to play a pivotal regulatory role in Ca^{2+} channel function, although the precise mode of conformational alterations arising from mutations in distinct RyR2 domains may be different.

In the present domain peptide (DP) probe study, we investigated the mode of inter-domain interactions among the three mutable domains (i.e. N-terminal, central, and I-domains) and their involvement in channel regulation. Here, we show that defective inter-domain interactions among these mutable domains indeed cause abnormal Ca^{2+} cycling in failing hearts.

2. Methods

2.1 Materials

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

2.2 Animal model

In beagle dogs weighing 10–13 kg, we induced heart failure by continuous application of rapid ventricular (RV) pacing at 250 b.p.m. using an externally programmed miniature pacemaker (Medtronic Inc., Minneapolis) for 28 days, as described elsewhere.¹² This study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH

Publication no. 85–23, revised 1996). 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.

2.3 Preparation of sarcoplasmic reticulum vesicles

We prepared SR vesicles from dog LV muscle as described elsewhere.¹² Left ventricles were homogenized in a solution containing 30 mM Tris-malate, 0.3 M sucrose, 5 mg/L leupeptin, and 0.1 mmol/L PMSF, pH 7.0 (solution I). The homogenate was centrifuged at 5500g for 10 min and the resultant supernatant was filtered through four layers of cheesecloth before centrifugation at 12 000g for 20 min. The supernatant was again filtered through cheesecloth and centrifuged at 143 000g for 30 min. The pellet was resuspended in a solution containing 0.6 mol/L KCl, 30 mmol/L Tris-malate, 0.3 mol/L sucrose, 5 mg/L leupeptin, 0.1 mmol/L PMSF, at pH 7.0 (solution II). This suspension was centrifuged at 143 000g for 45 min. The pellet was resuspended again in solution II, homogenized, and centrifuged at 143 000g as described earlier. The pellet was suspended in solution I and centrifuged at 143 000g. The resultant pellet represents the microsomal fraction that is enriched in SR vesicles, and it was homogenized in a solution containing 0.1 mol/L KCl, 20 mmol/L Tris-malate, 0.3 mol/L sucrose, 5 mg/L leupeptin, 0.1 mmol/L PMSF, at pH 7.0, to a final concentration of about 10–20 mg protein/mL. This fraction was rapidly frozen in liquid nitrogen and stored at -80°C . An aliquot was retained for determination of protein concentration by the method of Lowry *et al.*¹³

2.4 Peptides used and peptide synthesis

We used the following DPs: DPc10 (DP2460–2495), DPc10-mut, DP163–195, DP163–195-mut, DP4090–4123, and DP4090–4123-mut.

DPc 10 (DP2460–2495) 2460GFCPDHKAAMVFLDRVYGIEVQDFLLHLL
EVGFLP2495

DPc 10-mut (DP 2460–2495-mut R2474S) 2460GFCPDHKAAMVFLD
SVYGIEVQDFLLHLLVGVFLP2495

DP163–195 163HPASKQRSEGEKVRVGGDLLILSVSSERYLHLS195

DP163–195-mut (R176Q) 163HPASKQRSEGEKVRVGGDLLILSVSSER
YLHLS195

DP4090–4123 4090PAKDIGNVAVLLTNLSEHMPNDTRLQTFLELAE4123

DP4090–4123-mut (N4104K) 4090PAKDIGNVAVLLTKLSEHMPNDTRL
QTFLELAE4123

Peptides were synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc (N-(9-fluorenyl)methoxycarbonyl) as the alpha-amino protecting group, as described previously.¹⁴ The peptides were cleaved and de-protected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography.

2.5 Ca^{2+} -uptake and Ca^{2+} -leak assays

Ca^{2+} -uptake and the following Ca^{2+} leak assays were done as described previously.¹² SR vesicles (0.2 mg/mL) were incubated in 0.5 mL of solution containing 0.15 mmol/L potassium gluconate, 1 mmol/L MgCl_2 , 0.2 mmol/L EGTA-calcium buffer (free $[\text{Ca}^{2+}] = 0.3 \mu\text{mol/L}$), 10 mmol/L NaN_3 , and 20 mmol/L MOPS, pH 6.8. Ca^{2+} uptake was initiated by the addition of 0.5 mmol/L ATP into the reaction solution, and the time course of Ca^{2+} uptake was monitored spectrophotometrically with fluo 3 as a Ca^{2+} indicator (excitation at 480 nm, emission at 530 nm). After the Ca^{2+} uptake had reached a plateau, DP was added in the presence of $0.5 \mu\text{mol/L}$ thapsigargin to inhibit SR Ca^{2+} -ATPase activity, and the resultant Ca^{2+} leak was measured. The Ca^{2+} leak was expressed as the ratio of the amount of Ca^{2+} leaked out from the SR at 60 s after the addition of thapsigargin to the amount of total Ca^{2+} uptake.

2.6 Site-directed fluorescent labelling of the RyR

Specific fluorescent labelling of RyR2 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 (SAED) from PIERCE (IL), with various DPs (DPc10, DP163–195, or DP4090–4123), as site-specific carriers. This method for the site-directed fluorescent labelling of the RyR2 was basically the same as the method used for the DP4-mediated MCA (methylcoumarin acetate)-labelling of RyR1.¹⁵ Briefly, peptide-SAED conjugate was formed by incubating 0.5 mmol/L peptide with 0.5 mmol/L SAED in a 20 mmol/L HEPES (pH 7.5) solution for 60 min at 22°C in the dark. The reaction was quenched by 20 mmol/L lysine. Unreacted SAED was removed using ion exchange column (GE HiTrap Q XL). The peptide-SAED conjugate (5 µmol/L in a final concentration) was mixed with 2 mg/mL SR protein in the sample solution containing a 1 mmol/L EGTA/calcium buffer (1.0 µmol/L free Ca²⁺) in the dark and photolyzed with UV light in a Pyrex tube at 4°C for 2 min. Beta-mercaptoethanol was added (100 mmol/L in a final concentration) to cleave the disulfide bond of SAED. After incubation on ice for 1 h, the mixture was centrifuged at 100 000g for 15 min, and the sedimented vesicles were resuspended in the sample solution to a final protein concentration of 10 mg/mL.

2.7 Fluorescence quenching assay of the MCA probe attached to the binding sites of DPc10, DP163–195, or DP4090–4123

The zipping/unzipping mode of regulatory domains within RyR2 was evaluated as described previously.^{9,16} To make the large-size quencher, QSY[®] 7 carboxylic acid (Mr = 791.32) was conjugated with BSA by incubating 5 mmol/L QSY[®] 7 carboxylic acid with 0.5 mmol/L bovine serum albumin (BSA) in 20 mmol/L HEPES (pH 7.5) for 60 min at 22°C in the dark. Unreacted QSY 7 carboxylic acid was removed by means of Sephadex G50 gel filtration. Fluorescence quenching by both QSY[®] 7 carboxylic acid–BSA conjugate (a macromolecular quencher) and unconjugated QSY[®] (a small-size quencher) was performed by measuring steady-state fluorescence of the labelled MCA (excitation at 368 nm, emission at 455 nm) in the presence or absence of various peptides and/or compounds. The data were analysed using the Stern–Volmer equation:¹⁵ $F_0/F = 1 + K_Q [Q]$ where F and F_0 are fluorescence intensities in the presence and in the absence of added quencher; K_Q , quenching constant, which is the measure of the accessibility of the protein-bound probe to the quencher; $[Q]$, the concentration of QSY[®] 7 carboxylic acid–BSA conjugate.

The principle of the fluorescence quench assay of domain unzipping is that a large-size quencher QSY[®] 7 carboxylic acid–BSA conjugate is not accessible to the attached MCA in the zipped state, whereas it becomes accessible to the MCA in the unzipped state.

2.8 Immunoblot analysis

We performed immunoblot analyses of RyR2-bound FKBP12.6 as described previously.¹² Co-immunoprecipitation of FKBP12.6 with RyR2 from the solubilized SR was performed using anti-RyR antibody (Oncogene Research Products), followed by immunoblotting with anti-FKBP12 (C-19) antibody (Santa Cruz Biotechnology).

2.9 Preparation of isolated cardiomyocytes

Cardiomyocytes were isolated from the LV free wall as described previously.^{9,17} In brief, a wedge of LV free wall, perfused by a branch of the left circumflex coronary artery, was dissected free from 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 concentration of Ca²⁺ 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 h at 37°C in 5%CO₂/95%O₂ atmosphere.

2.10 Incorporation of domain peptides into cardiomyocytes

The DP was incorporated into the cells using a protein delivery reagent (Bioporter, Gene Therapy Systems, Inc., CA), as described previously.⁹ Successful incorporation of the DP into the cell was confirmed by detecting the fluorescence signal of the peptide pre-labelled with Alexa Fluor 488 (Molecular Probes, OR).

2.11 Analysis of Ca²⁺ sparks with laser scanning confocal microscopy

Ca²⁺ sparks were measured with 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), as previously described.¹⁸ Briefly, cardiomyocytes were loaded with fluo-4 AM (20 µmol/L; Molecular Probes) for 30 min at room temperature in the dark. To record Ca²⁺ sparks, line-scan mode was used, where a single cardiomyocyte was scanned repeatedly (325.7 Hz) along a line parallel to the longitudinal axis, avoiding nuclei. To monitor diastolic Ca²⁺ sparks, cardiomyocytes were stimulated until the Ca²⁺ transient reached steady state, then stimulation was stopped, and Ca²⁺ sparks recorded during the subsequent ~10 s rest. Data were analysed with SparkMaster, an automated analysis program which allows rapid and reliable spark analysis.¹⁹ The analysis involved general image parameters (number of detected sparks, spark frequency) as well as individual spark parameters (Amplitude; FWHM, full width at half maximum; FDHM, full duration at half maximum).

2.12 Statistics

Paired or unpaired *t*-test was used for statistical comparison of the data corresponding to the normal and defective inter-domain interactions. We also used ANOVA with a *post hoc* Scheffe's test for statistical comparison of concentration-dependent data. Data are expressed as means ± SD. We accepted a *P*-value less than 0.05 as statistically significant.

3. Results

3.1 Both DP163–195 and DP4090–4123 induce Ca²⁺ leak without dissociating FKBP12.6 from RyR2

Addition of 0.5 µmol/L thapsigargin to normal SR vesicles at the steady-state of ATP-dependent Ca²⁺ uptake produced little Ca²⁺ leak, while addition of 3–100 µmol/L DP163–195, or DP4090–4123, together with 0.5 µmol/L thapsigargin produced a pronounced leak (Figure 1A). However, neither DP163–195-mut nor DP4090–4123-mut induced Ca²⁺ leak (data not shown). Figure 1B shows the effect of DP2460–2495 (DPc10), DP163–195, or DP4090–4123 on dissociation of FKBP12.6 from RyR2 in normal SR. None of these peptides dissociated FKBP12.6 from RyR2. The Ca²⁺ leak induced by DP163–195 was almost completely inhibited by 0.3 µmol/L K201, whereas the Ca²⁺ leak induced by DP4090–4123 was not inhibited by K201 (Figure 1C). In failing SR, spontaneous Ca²⁺ leak took place without addition of the peptides

Figure 1 (A) Concentration-dependent effects of DP163-195 and DP4090-4123 on Ca^{2+} leak from normal sarcoplasmic reticulum. Representative time courses of Ca^{2+} leak following Ca^{2+} uptake (top figure) and summarized data (bottom figure). * $P < 0.01$ vs. baseline. (B) Effects of DPc10, DP163-195, and DP4090-4123 on dissociation of FKBP12.6 from RyR2. Before immunoprecipitation of RyR, sarcoplasmic reticulum vesicles were mixed with DP163-195 or DP4090-4123 for 30 min and then centrifuged, followed by western blotting. (C) Effect of K201 (0.3 $\mu\text{mol/L}$) on the DP163-195-induced or DP4090-4123-induced Ca^{2+} leak in normal sarcoplasmic reticulum. (D) Effect of K201 (0.3 $\mu\text{mol/L}$) on spontaneous Ca^{2+} leak seen in failing sarcoplasmic reticulum, in the presence of DP163-195 (30 $\mu\text{mol/L}$) or DP4090-4123 (30 $\mu\text{mol/L}$).

(DP163-195 and DP4090-4123), and the addition of these peptides produced little or no further increase in Ca^{2+} leak. The Ca^{2+} leak was inhibited by K201, although K201 was without effect in the presence of DP4090-4123 (Figure 1D).

3.2 Specific fluorescent labelling of the peptide binding domains of RyR2

Figure 2 shows the fluorescence gel pictures of the MCA-labelled RyR2. Each of these peptides used as a site direction carrier (DPc10, DP163-195, or DP4090-4123) mediated a specific MCA fluorescence labelling of RyR2. However, each of the human ARVC or CPVT mutants of these peptides {Arg-2474-Ser mutation (DPc10mut), Arg-176-Gln mutation (DP163-195-mut), or Asn-4104-Lys mutation (DP4090-4123-mut)} did not mediate fluorescence labelling (Figure 2, left). An excess concentration of each unlabelled peptide (10 mmol/L) also prevented DP(DP163-195 or DP4090-4123)-mediated MCA labelling (Figure 2, right). In our previous report,⁹ an excess concentration of unlabelled DPc10 also prevented the DPc10-mediated MCA labelling.

3.3 Spectroscopic monitoring of the changes in the mode of inter-domain interactions induced by domain peptide (DP163-195 or DP4090-4123)

In order to monitor the mode of the inter-domain interactions, we adopted the fluorescence quenching technique.¹⁶

The MCA probe that has been attached to the critical domain is expected to be inaccessible to a bulky fluorescence quencher (QSY-BSA conjugate) in a zipped configuration (because in this case the probe is occluded between the interacting domains), while it will become accessible to the quencher upon unzipping (see Methods). The accessibility of the protein-bound MCA to the QSY-BSA quencher, i.e. the extent of domain unzipping, can be quantitatively determined as the slope (=the quenching constant, K_Q) of the Stern-Volmer plot ($F_0/F = 1 + K_Q [Q]$).

The data of fluorescence quenching assay are shown in Figure 3. When MCA labelling was mediated by DP163-195 (Figure 3A), DP163-195 increased the slope (K_Q) in a concentration-dependent manner, indicating that this peptide interfered with the inter-domain interaction between the N-terminal and central domains, causing domain unzipping (Figure 5, a). In this MCA-labelled RyR2 using DP163-165 as a carrier, DP4090-4123 had no effect on the K_Q , indicating that DP4090-4123 has no effect on the inter-domain interaction between N-terminal and central domains (Figure 5, b). K201 inhibited the increase in K_Q induced by DP163-195 (cf: Scheme 1-f), but DP163-195mut had no effect. The data with the RyR2, MCA-labelled with DP4090-4123, are shown in Figure 3B. DP4090-4123 increased K_Q , indicating that the peptide produced domain unzipping between I-domain and the putative partner domain of I-domain (IP domain) (Figure 5, b). DP4090-4123-mut had no effect. Interestingly in this case when MCA was introduced via

Figure 2 Site-directed fluorescence labelling of RyR2 with methylcoumarin acetate. Site-specific methylcoumarin acetate fluorescence labelling was performed using either DPc10, DP163-195, or DP4090-4123 as site-directing carrier. No methylcoumarin acetate fluorescence was seen when corresponding DPMut was used (left figure). An excess concentration of each unlabelled peptide (10 mmol/L) also inhibited each domain peptide-mediated methylcoumarin acetate labelling (right figure).

Figure 3 Fluorescence quenching analysis of domain unzipping (upper figures). A fluorescent probe methylcoumarin acetate was attached to the RyR2 of normal and failing sarcoplasmic reticulum in a site-specific manner using either DP163-195 (A) or DP4090-4123 (B) as a carrier. Then, the accessibility of the RyR2-bound MCA to a macromolecular fluorescence quencher BSA-QSY conjugate, the Stern-Volmer fluorescence quenching constant (i.e. the slope of the F_0/F vs. [BSA-QSY] plot), was determined as a measure of the degree of domain unzipping. Statistical comparison of the slope of each plot, which is equivalent to the Stern-Volmer quenching constant (K_Q) (bottom figures). DP163-195 increased the accessibility of the protein-bound MCA (carrier: DP163-195) to the quencher, as shown by an increase of the slope of the plot. K201 reversed the effect of DP163-195 in normal SR and decreased an elevated level of quencher accessibility in failing SR. DP4090-4123 also increased the accessibility of the bound MCA (carrier: DP4090-4123) to the quencher in normal SR, but in this case K201 was without effect.

Figure 3 (right side) depicts the fluorescence quenching data obtained with the RyR2 of failing SR MCA-labelled with either DP163–195 (Figure 3A) or DP4090–4123 (Figure 3B) as well. The K_Q in the RyR2 of failing SR was significantly larger compared with the K_Q in the normal RyR2 without added peptide (Figure 5, d), but was comparable with the value of normal RyR2 that was reached after the addition of DP (4090–4123 or 163–195) (Figure 5, a or b). Addition of these DP to the failing RyR2 produced no further increase in the K_Q value, indicating that similar domain unzipping has taken place in the failing RyR2 even without addition of DPs. K201 decreased the K_Q of the failing RyR2 to a normal level (Figure 5, g).

In order to investigate how the domain-unzipping effect of DP163–195 or DP4090–4123 is reflected upon the cardiomyocyte function, we incorporated DP163–195 or DP4090–4123

We monitored spontaneous Ca^{2+} sparks in cardiomyocytes at two different extracellular $[\text{Ca}^{2+}]$: 2 and 5 mmol/L. As shown in *Figure 4B*, the occurrence of Ca^{2+} sparks was less frequent in normal cardiomyocytes. However, incorporation of DP163–195 or DP4090–4123 into normal cardiomyocytes resulted in a significant increase in the Ca^{2+} spark frequency particularly at $[\text{Ca}^{2+}]=5$ mmol/L. Failing cardiomyocytes showed high Ca^{2+} spark frequency similar to the peptide-treated normal myocytes, even without introduction of the peptides. By incorporation of the peptides, both FWHM and FDHM changed in the same direction as spark frequency, whereas only spark amplitude changed in an opposite direction. This indicates that as Ca^{2+} spark frequency increases, Ca^{2+} spark amplitude decreases, and a Ca^{2+} spark spreads more in time and spatial direction. Incorporation of either DP163–195-mut (ARVC Arg-176-Gln mutation) or DP4090–4123-mut (CPVT Asn-4104-Lys mutation) into normal cardiomyocytes produced no appreciable effect on the Ca^{2+} spark frequency (data not shown). Protein delivery reagent (Bioporter) alone also had no effect on the Ca^{2+} spark frequency (data not shown). In normal cardiomyocytes, K201 inhibited the DP163–195-induced Ca^{2+} sparks, but not those induced by DP4090–4123 (*Figure 4C*). Addition of K201 inhibited the Ca^{2+} spark in failing cardiomyocytes, even in the presence of DP163–195. However, when DP4090–4123 was

Figure 4 (A) Delivery of DP163-195 or DP4090-4123, fluorescently labelled with Alexa Fluor 488 (Molecular Probes, OR), into the isolated cardiomyocytes. Confocal microscopy clearly detects the fluorescence signal (shown as green) of DP163-195 or DP4090-4123 in the cardiomyocytes. Cell surface membrane was fluorescently labelled as red by wheat germ agglutinin-Alexa Fluor 633 conjugate (Molecular Probes, OR). (B) Spontaneous Ca^{2+} sparks in DP163-195- or DP4090-4123-incorporated normal and failing cardiomyocytes at either 2 or 5 mmol/L extra-cellular $[\text{Ca}^{2+}]_o$. (C) Effect of K201 on spontaneous Ca^{2+} sparks in DP163-195- or DP4090-4123-incorporated normal and failing cardiomyocytes at 5 mmol/L extra-cellular $[\text{Ca}^{2+}]_o$. Summarized data are shown at the right side. FWHM, full width at half maximum; FDHM, full duration at half maximum.

incorporated into the failing cardiomyocytes, K201 produced virtually no effect on the Ca^{2+} spark frequency. In response to K201, both FWHM and FDHM again changed in the same direction as Ca^{2+} spark frequency, whereas only Ca^{2+} spark amplitude changed in an opposite direction.

4. Discussion

We have previously reported that in failing hearts the inter-domain interaction between N-terminal (1-600) and central domain (2000-2500) of RyR2 becomes defective, resulting in abnormal intracellular Ca^{2+} handling, such as Ca^{2+} leak from RyR2 and prolonged Ca^{2+} transient.⁹ The present study has uncovered several new mechanisms of domain-mediated RyR2 channel regulation and its pathology. Thus, it was found that defective inter-domain interactions not only in the N-terminal domain/central domain but also in IP-domain (putative partner domain of I-domain, which remains to be identified)/I-domain causes various pro-arrhythmia states, such as increased frequency of spontaneous Ca^{2+} sparks and appearance of DAD.

There are several reports supporting the view that the I-domain (3722-4610) is involved in the regulation of channel gating. George *et al.*^{11,20} have shown that functional coupling between the cytoplasmic and transmembrane domains of RyR2 is mediated by the I-domain. Thus, sudden cardiac death (SCD)-linked mutations occurring in the I-domain (N4104K and R4496C) cause RyR2 channel instability via defective inter-domain interaction, resulting in Ca^{2+} release dysfunction. Other investigators also emphasized the important role of several sub-domains located within the I-domain in the regulation of channel gating. Xiong *et al.*²¹ found that the 4064-4210 residue segment of RyR1 shows a CaM-like sequence (CaMLD), and suggested that this region may be involved in the mechanism of Ca^{2+} -dependent channel activation. The fact that CPVT mutations are not randomly scattered over the I-domain, but rather clustered in a rather restricted region; namely in the 3778-4200 region, especially in the 4090-4123 region, suggests that the sub-domain corresponding to DP4090-4123 plays a critical role on normal channel gating.

We recently identified the K201-binding site at domain2114–2149 of the ryanodine receptor (RyR2), and the binding of K201 to this domain corrects the defective inter-domain interaction between N-terminal (1–600) and central regions (2000–2500) of RyR2 in pacing-induced failing hearts.²³ The effect of K201 on the mode of inter-domain interactions described here has provided a further, new insight into the underlying mechanism for diastolic spontaneous Ca^{2+} sparks and DAD seen in failing cardiomyocytes. As shown, the primary action of K201 on the channel function of RyR2 is the correction of unwanted domain unzipping between the N-terminal and central domains seen in failing SR or in DPc10-introduced normal SR.⁹ The increase in Ca^{2+} spark frequency seen in failing cardiomyocytes was inhibited by K201, but when DP4090–4123 was incorporated into the failing cardiomyocytes, Ca^{2+} sparks remained abnormal even in the presence of K201. These findings suggest that in the tachycardia-induced failing hearts Ca^{2+} sparks are indeed induced by defective inter-domain interaction between N-terminal and central domains. The fact that K201 is less effective in correcting the defective IP-domain/I-domain interaction suggests that

Figure 5 Model illustrating how defects in the inter-domain interactions within RyR2 cause channel dysfunctions (diastolic Ca^{2+} leak) in heart failure and ARVC2/CPVT. N, N-terminal domain (1–600); C, central domain (2000–2500); I, I-domain; IP, IP-domain (putative partner domain of I-domain; unidentified).

K201 may also be less effective in correcting the problem produced by CPVT mutations within the I-domain. The fact that K201 was not effective in the R4496C RyR2 mutant in knock-in mice, which showed bi-directional VT,¹⁰ seems to be consistent with the present data that neither Ca^{2+} leak nor Ca^{2+} sparks, induced by DP4090–4123, was inhibited by K201. The point mutation, such as R4496C or N4104K at I-domain, seen in CPVT may directly induce domain unzipping between IP-domain and I domain, independent of the mode of inter-domain interaction between N-terminal and central domains.

While elevation of SR Ca^{2+} content obviously increases the amount of releasable Ca^{2+} , it may become as a causative factor of spontaneous diastolic SR Ca^{2+} release.²⁴ In failing hearts, in which SR Ca^{2+} content is decreased,⁹ Ca^{2+} spark frequency was quite high even in baseline conditions, and increasing $[\text{Ca}^{2+}]$ further increased the Ca^{2+} spark frequency. This suggests that the threshold of SR Ca^{2+} load for the induction of spontaneous Ca^{2+} spark is made much

lower in failing hearts than in normal hearts. With regard to this, Jiang *et al.*^{25,26} recently proposed that in mutation-linked RyR2 disorder the threshold of SR Ca^{2+} content for spontaneous Ca^{2+} release, referred as store-overload-induced Ca^{2+} release (SOICR), was much lower than normal RyR2. The SOICR may also be involved in the defective channel gating in failing hearts, which may also be caused by defective inter-domain interaction in failing RyR2.

Although we have not identified IP-domain yet, the project for identification of IP-domain using LC/MS spectroscopy is now on-going.

In conclusion, three hot regions within RyR2 (N-terminal, central, and I- domains), harbouring many ARVC2 or CPVT mutations, may play a critical role in regulating channel function. Defective inter-domain interactions among these domains may lead to serious problems of channel gating, as seen in abnormal Ca^{2+} spark events and DAD, leading to heart failure and lethal arrhythmia.

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