Journal of the American College of Cardiology
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Published by Elsevier Inc.

Vol. 49, No. 16, 2007
ISSN 0735-1097/07/$32.00
doi:10.1016/j.jacc.2007.01.064

PRECLINICAL STUDY

Scavenging Free Radicals by Low-Dose Carvedilol Prevents Redox-Dependent Ca^{2+} Leak Via Stabilization of Ryanodine Receptor in Heart Failure

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Objectives We investigated whether defective intracellular Ca^{2+} handling is corrected by carvedilol in heart failure.

Background In heart failure, the interaction between the N-terminal and central domains of the ryanodine receptor (RyR), the domains where many mutations have been found in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT), is defective, as shown in our recent report.

Methods Sarcoplasmic reticulum vesicles were isolated from canine left ventricular muscle (normal or 4-weeks rapid ventricular pacing). The RyR was labeled with the fluorescent conformational probe methylcoumarin acetate (MCA) with DPc10 (a synthetic peptide corresponding to Gly^{2460}-Pro^{2495} of RyR, one of the mutable domains in CPVT) as a site-direction carrier.

Results Normal cardiac function was well preserved in carvedilol-treated/paced dogs (CV+) but not in the untreated/paced dogs (CV−). In CV−, the interdomain interaction within RyR was defective (i.e., in an unzipped state), as determined by the fluorescence quenching technique. However, in CV+, the domain interaction remained normal (i.e., in a zipped state). In CV−, oxidative stress of RyR (reduction in the number of free thiols) was severe, but it was negligible in CV+. In (CV−) failing cardiomyocytes, incubation with low-dose CV (30 nmol/l), which eliminated intracellular reactive oxygen species with no acute effect on cell shortening, markedly improved the contractile function and Ca^{2+} transient. However, after domain unzipping by DPc10, CV was without effect.

Conclusions Carvedilol, at a concentration that is sufficient to produce antioxidant effect, improves the intracellular Ca^{2+} handling and contractile dysfunction by correcting defective interdomain interaction within the RyR in the failing heart. (J Am Coll Cardiol 2007;49:1722–32) © 2007 by the American College of Cardiology Foundation

The interaction between 2 specific domains (N-terminal: residue 1-600 and central: 2000-2500 domains), where many reported malignant hyperthermia (MH) and central core disease (CCD) mutations are located, plays a critical role in Ca^{2+} channel regulation (1). In the resting or nonactivated state of ryanodine receptor (RyR), the 2 domains make close contact at several sub-domains (domain zipping). The conformational constraints imparted by the "zipped" configuration stabilize the closed state of the channel. Mutation in either of these domains causes weakening of the interdomain interaction and partial domain unzipping, even in resting or nonactivating conditions. Thus, diseased channels remain partially open (1).

The domain unzipping concept can also account for the pathogenesis of mutation-caused cardiomyopathies of RyR2 (e.g., arrhythmogenic right ventricular cardiomyopathy type 2 [ARVC2] and catecholaminergic polymorphic ventricular tachycardia [CPVT]), in which more than 40 mutations have been identified in the corresponding regions seen in MH and CCD (2). In our recent study of the pacing-induced heart failure model (3), we have shown that defective interdomain interaction destabilizes the channel

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Manuscript received June 12, 2006; revised manuscript received December 11, 2006, accepted January 1, 2007.

gating of the RyR2, causing serious consequences, such as Ca^{2+} leak, cyclic adenosine monophosphate (cAMP)-dependent hyperphosphorylation of RyR2, and FKBP12.6 dissociation from RyR2. This suggests that the weakened interdomain interaction is 1 of the key pathogenic mechanisms not only in mutation-caused cardiomyopathy (ARVC2 and CPVT) but also in heart failure. In further support of this view, we have shown that a benzothiazepine derivative JTV519 (K201) corrects domain unzipping in the diseased RyR2 and prevents the development of heart failure (3,4).

Calcium sulfate hemihydrate channels of RyR2 as well as RyR1 are known to be regulated by the redox state. For instance, oxidation or nitroxylation of the cysteine residues in the RyR produces considerable changes in the channel function (5). We recently demonstrated that oxidative stress-induced domain unzipping of these regulatory domains, leading to Ca^{2+} leak and that the antioxidant edaravone corrected the defectiveness of the interdomain interaction and thereby improved cardiac function against the development of heart failure (6). The oxidative stress-induced domain unzipping and subsequent Ca^{2+} leak were again corrected by JTV519 (K201) (6). These findings further support the notion that the defective interdomain interaction within RyR2 is a common source mechanism of Ca^{2+} leak leading to heart failure and fatal arrhythmia and that the defectiveness can be induced either by FKBP12.6 dissociation from RyR2 and oxidative stress or inherently by single point mutation within RyR2.

Carvedilol (CV) is a nonselective beta-blocker that also has an antioxidant effect. Recent clinical trials have clearly demonstrated that CV significantly improves cardiac function as well as the prognosis of patients with chronic heart failure (7). However, the underlying mechanism by which CV improves cardiac function remains to be elucidated. In this study, we investigated the possibility that the defective interdomain interaction and resultant Ca^{2+} leak seen in failing hearts can be corrected by CV-treatment, and we have found that this is in fact the case.

Methods

Materials. The FK506 and CV were provided by Astellas Pharma Inc. (Tokyo, Japan) and Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively.

Animal preparation. In beagle dogs weighing 10 to 13 kg, heart failure was induced by 28 days of rapid ventricular (RV) pacing at 250 beats/min (referred to as 4W-pacing) with an externally programmable miniature pacemaker (Medtronic Inc., Minneapolis, Minnesota), and both left ventricular (LV) pressure and 2-dimensional echocardiograms were measured in the conscious state, as previously described (8). Carvedilol was chronically administered immediately followed by the initiation of RV pacing. The care of the animals and the protocols used were in accord with guidelines laid down by the Animal Ethics Committee of Yamaguchi University School of Medicine.

Preparation of sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum (SR) vesicles were prepared from dog LV, as described elsewhere (8).

Ca^{2+}-uptake and -leak assays. Ca^{2+}-uptake and Ca^{2+}-leak assays were performed as described previously (8).

Peptides used and peptide synthesis. We used a synthetic peptide corresponding to residues 2460-2495 of the RyR2, ^{2460}GFCPDHKAAMVLFLDRVYGIEVQDFLLHLLEVGFLP^{2495} (DPc10), that includes 1 residue mutable in CPVT, as described previously (3,9).

Site-directed fluorescence labeling of the RyR2. Specific fluorescence labeling of the RyR2 moiety of the SR was performed with the cleavable hetero-bifunctional cross-linking reagent sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido) ethyl) dithio)propionate (SAED) from PIERCE (Rockford, Illinois), with DPc10 as a site-specific carrier, as described previously (3).

Fluorescence quenching of the MCA probe attached to the DPc10 binding site. The zipped and unzipped states of the interacting domains of the RyR2 were assessed by the fluorescence quench technique described previously (3,9). The principle of the fluorescence quench assay of domain unzipping is that a large-size quencher QSY (QSY-7, Molecular Probes, Eugene, Oregon)–bovine serum albumin (BSA) is inaccessible to the attached MCA in the zipped state, whereas it becomes accessible to the MCA site in the unzipped state. The MCA fluorescence data (excitation at 368 nm, emission at 455 nm) were analyzed with the Stern-Volmer equation.

Immunoblot analysis. Immunoblot analyses for FKBP12.6, SR Ca^{2+}-adenosine triphosphate (ATP)ase, and phospholamban (PLB) were carried out as described elsewhere (4,8). By employing the method of Marx et al. (10), we achieved co-immunoprecipitation of FKBP12.6 from SR with anti-RyR2 antibody (Oncogene Research Products, San Diego, California) followed by immunoblotting with anti-FKBP12 (C-19) antibody (Santa Cruz Biotechnology, Santa Cruz, California). The relative phosphorylation level of RyR was determined by immunoblotting with anti-phosphoRyR2 (RyR2-pSer^{2808}) that was kindly provided by Dr. Andrew R. Marks (Columbia University). Specific antibodies against pSer^{16}-PLB (Upstate Biotechnology, Lake Placid, New York) and an epitope common to all PLB forms (PLB; Upstate Biotechnology) were also used.

Abbreviations and Acronyms

CPVT = catecholaminergic polymorphic ventricular tachycardia
CV = carvedilol
DCFH-DA = 2′,7′-dichlorofluorescin diacetate
DPc10 = a synthetic peptide corresponding to Gly^{2460}-Pro^{2495} of ryanodine receptor
LV = left ventricle/ventricular
mBB = monobromobimane
MCA = methylcoumarin acetate
PKA = protein kinase A
PLB = phospholamban
ROS = reactive oxygen species
RV = rapid ventricular
RyR = ryanodine receptor
SIN-1 = 3-morpholinosydnonimine
SR = sarcoplasmic reticulum

Oxidative stress level in RyR2. The content of free thiols (the number of reduced cysteines) in the canine RyR2 was determined with the monobromobimane (mBB, Calbiochem, San Diego, California) fluorescence technique (6,11,12). The mBB fluorescence in the RyR2 (excitation at 382 nm, emission at 482 nm), normalized by protein abundance of RyR2, was defined as the relative content of free thiols in the RyR2.

In canine cardiomyocytes, a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes), was used for the assessment of intracellular reactive oxygen species (ROS) formation, as described previously (6,13). Fluorescence images (excitation at 490 nm and emission at 530 nm) were acquired with a microscope (LSM 510, Carl Zeiss, Oberkochen, Germany).

Ca^{2+} transient and cell shortening in canine cardiomyocytes. Cell shortening and intracellular calcium were measured as described previously (3). In brief, cardiomyocytes were incubated with 1 μmol/l fura-2 AM, 0.0045% pluronic F-127 (Sigma, St. Louis, Missouri) and 0.1% dimethyl sulfoxide for 30 min, then washed twice with N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES buffer) containing (in mmol/l) sodium chloride 126, potassium chloride 4.4, magnesium chloride 1.0, calcium chloride 1.08, HEPES 24, glucose 11, sodium hydroxide 11, sodium dihydrogen phosphate 10, and probenecid 0.5 (pH 7.4). Cells were stimulated by a field electric stimulator (IonOptix, Milton, Massachusetts) at 0.5 Hz. A dual excitation spectrofluorometer was used to record fluorescence emissions (505 nm) elicited from exciting wavelengths at 340 and 380 nm. The intracellular calcium was monitored as the ratio of fluorescence of the cell at 340 and 380 nm excitation.

Statistics. Paired *t* tests were performed for pre-post comparisons of hemodynamic data. Analysis of variance was used to compare data groups. When we identified a significant trend by the F test, we used Scheffé's post hoc test to compare the data. For comparison of multiple measurements, upon dose-dependent effect of CV on hemodynamic parameters, repeated measures analysis was used. Data are expressed as means ± SD. We accepted a p value <0.05 as statistically significant.

Results

Determination of an appropriate concentration of CV for chronic administration. To determine the dose of CV to be used for chronic administration, we evaluated the concentration-dependent effect of CV on hemodynamic parameters in normal conscious dogs (Fig. 1A). Carvedilol was orally administered for 3 days/each dose, starting at a dose of 0.01 $mg \cdot kg^{-1} \cdot day^{-1}$ with various increments to 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$. At a dose of 0.02 $mg \cdot kg^{-1} \cdot day^{-1}$, neither the baseline peak +dP/dt nor the isoproterenol-induced inotropic response was decreased, although the baseline heart rate decreased significantly (by approximately 15%) in normal conscious dogs. We used this low-dose of CV (0.02 $mg \cdot kg^{-1} \cdot day^{-1}$) for treatment of pacing-induced heart failure.

Hemodynamic data. In the CV-treated dogs with chronic RV pacing, normal systolic and diastolic functions were well preserved, and none of these dogs developed heart failure (Table 1, Fig. 1B). These data indicate that in the CV-treated dogs, there was no sign of heart failure after chronic RV pacing.

Levels of oxidative stress are elevated in failing hearts. The relative content of free thiols in the RyR2 (for definition, see Methods section) was considerably reduced in the untreated failing hearts but was normal in CV-treated hearts (Fig. 2A), indicating that the oxidation of the RyR2 is in fact involved in the oxidative stress in failing hearts and the normal level is restored by CV-treatment. To examine whether the preventive effect of CV is in fact due to the inhibition of the oxidation of the RyR2, we assessed the acute effect of CV on the oxidation of the RyR2 induced by 3-morpholinosydnonimine (SIN-1), which generates the nitric oxide-related species peroxynitrite ($OONO^-$). In agreement with our previous report (6), SIN-1 decreased the number of free thiols of the RyR2 (i.e., oxidation) (Fig. 2B). When SIN-1–was added together with CV, the number of free thiols was significantly higher than that without CV. This indicates that the inhibition of Ca^{2+} leak by CV is mediated in fact by the prevention of RyR2 oxidation by CV. The DPc10 (30 μmol/l), which was found to induce defective interdomain interaction and subsequent Ca^{2+} leak (3), did not change the relative content of free thiols. This indicates that CV reverses SIN-1–induced RyR oxidation and that defective interdomain interaction has little effect on the RyR oxidation. In contrast, when CV was added after washout of SIN-1, CV did not increase the number of free thiols (data not shown). This suggests that the actual effect of CV is scavenging of the oxidant $OONO^-$ rather than serving as a thiol-reducing agent.

Effects of CV on SR Ca^{2+}-leak and defective FKBP12.6-RyR2 interaction in failing hearts. Addition of 0.3 μmol/l thapsigargin to normal SR vesicles produced no detectable spontaneous Ca^{2+} leak, whereas addition of 30 μmol/l FK506 together with 0.3 μmol/l thapsigargin produced a pronounced leak (Fig. 3A). In contrast, in the failing SR vesicles from 4W-paced/CV-untreated hearts, the addition of thapsigargin alone produced a prominent Ca^{2+} leak, but the addition of FK506 produced no further increase (Fig. 3A). Importantly, neither the FK506-induced Ca^{2+} leak in normal SR nor Ca^{2+} leak in failing SR was inhibited by acute addition of CV, suggesting that CV has no direct effect on channel stabilization. In the SR vesicles from 4W-paced/CV-treated hearts, spontaneous Ca^{2+} leak was not observed, and FK506 increased Ca^{2+} leak as in the normal SR (Fig. 3A).

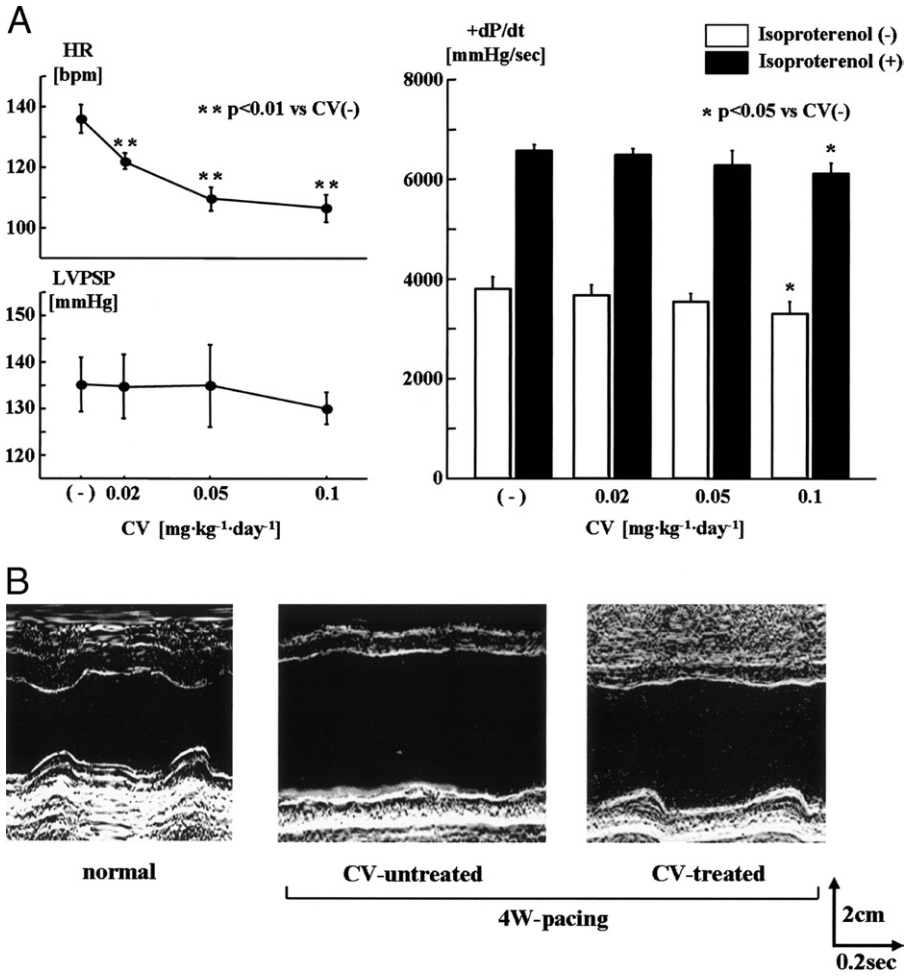

Figure 1 Hemodynamics and Echocardiograms

(A) Acute concentration-dependent effect of carvedilol (CV) on resting and isoproterenol-stimulated hemodynamic in normal conscious dogs. Isoproterenol (0.80 $\mu g \cdot kg^{-1} \cdot min^{-1}$ intravenously) was used to produce inotropic response. Data represent mean ± SD obtained from 4 dogs. **(B)** Representative M-mode echocardiograms. Note that left ventricular (LV) end-diastolic and -systolic diameters were each smaller in the CV-treated dog than in the CV-untreated (–) dog (both 4-week paced). HR = heart rate; LVPSP = left ventricular peak-systolic pressure; +dP/dt = peak (+) dP/dt of LV pressure. *p < 0.05; **p < 0.01 versus CV (−).

In the failing SR vesicles, the RyR2 was protein kinase A(PKA)-hyperphosphorylated, but CV treatment reduced the RyR2 phosphorylation to a level seen in the normal hearts (Fig. 3B). The amount of RyR2-associated FKBP12.6 was decreased by 4W-pacing, but FKBP12.6 dissociation was prevented by CV treatment (Fig. 3C). Acute addition of CV (100 μmol/l) to failing SR did not change the level of phosphorylation at RyR2-pSer^{2808}

Table 1 Hemodynamic Data

	HR (beats/min)	LVPSP (mm Hg)	LVEDP (mm Hg)	(+)dP/dt (mm Hg/s)	Tau (ms)	LVEDD (mm)	LVESD (mm)	LVFS (%)
Carvedilol-untreated (n = 7)								
Prepacing	134 ± 16	125 ± 10	7.7 ± 2.1	3,148 ± 646	19.3 ± 2.0	32.0 ± 1.5	22.2 ± 3.0	33.4 ± 8.1
4W-pacing	126 ± 13	119 ± 15*	31.7 ± 3.8*	1,378 ± 220*	37.5 ± 7.0*	39.8 ± 1.5*	34.8 ± 1.9*	12.7 ± 2.7*
Carvedilol-treated (n = 7)								
Prepacing	129 ± 24	129 ± 12	9.2 ± 4.3	2,988 ± 876	18.8 ± 4.9	32.0 ± 2.5	21.7 ± 1.9	31.7 ± 5.9
4W-pacing	123 ± 12	119 ± 16	16.3 ± 4.1*†	1,643 ± 138*†‡	27.0 ± 1.5*†	36.0 ± 1.7*†	28.4 ± 2.6*†	20.0 ± 4.0*†

Data represent mean ± SD. *p < 0.01 versus prepacing; †p < 0.01 versus carvedilol-untreated. ‡p < 0.05.

(+)dP/dt = peak +dP/dt of left ventricular pressure; HR = heart rate; LVEDD = left ventricular end-diastolic diameter; LVEDP = left-ventricular end-diastolic pressure; LVESD = left ventricular end-systolic diameter; LVFS = left ventricular fractional shortening: (LVEDD−LVESD)/LVEDD × 100; LVPSP = left ventricular peak-systolic pressure; Tau = time constant of left ventricular pressure decay during isovolumic relaxation period; W = week.

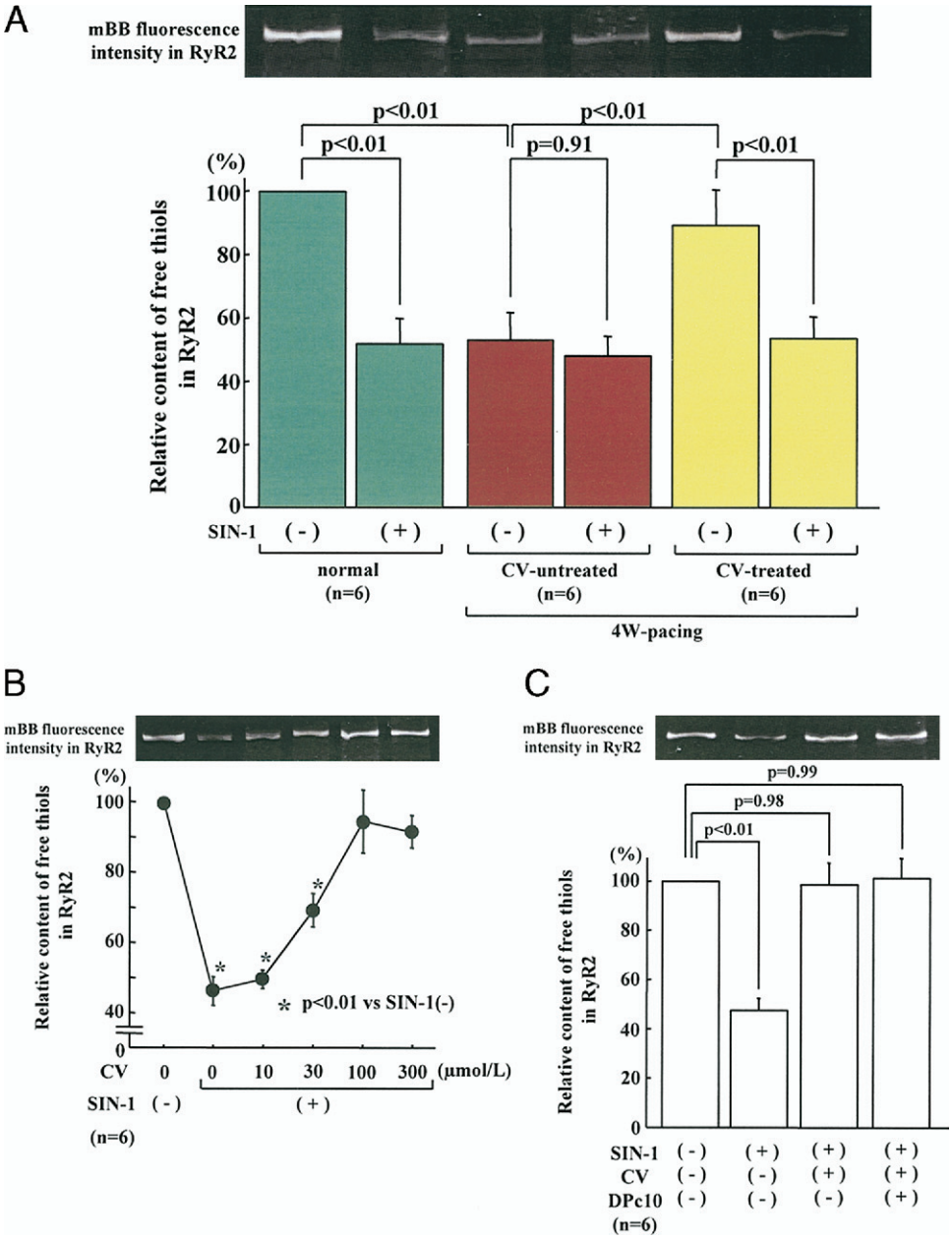

Figure 2 **Redox State in RyR2**

(A) Monobromobimane (mBB) fluorescence intensity of the ryanodine receptor (RyR)2 (corresponding to the content of free thiols) in the presence or absence of 100 μmol/l 3-morpholinosydnonimine (SIN-1). The relative content of free thiols in the RyR2 was obtained as the ratio of fluorescence intensity of the mBB to protein abundance of RyR2 and then expressed as a percentage of that in normal sarcoplasmic reticulum. **(B)** Acute concentration effect of carvedilol (CV) on SIN-1 (100 μmol/l)–induced decrease in mBB fluorescence intensity of the RyR2. **(C)** Acute effect of CV (100 μmol/l) on the mBB fluorescence intensity of the RyR2 in the presence or absence of SIN-1 (100 μmol/l) and/or DPc10 (a synthetic peptide corresponding to Gly^{2460}-Pro^{2495} of RyR) (30 μmol/l).

and the amount of RyR2-bound FKBP12.6 (Fig. 3C). These results indicate that CV prevents PKA-hyperphosporylation of RyR2 and FKBP 12.6 dissociation during the 4W-pacing but it has no acute and direct effect on the intrinsic activities of PKA-phosphorylation and FKBP12.6 binding of RyR2.

Effects of oxidative stress on Ca^{2+} leak, interdomain interaction within RyR2, and FKBP12.6-RyR2 interaction. To assess the direct antioxidant effect of CV on Ca^{2+} leak, we investigated the inhibitory effect of CV on SIN-1-induced Ca^{2+} leak in normal SR. As shown in Figure 4A, 100 μmol/l SIN-1 increased the Ca^{2+} leak, and this Ca^{2+}

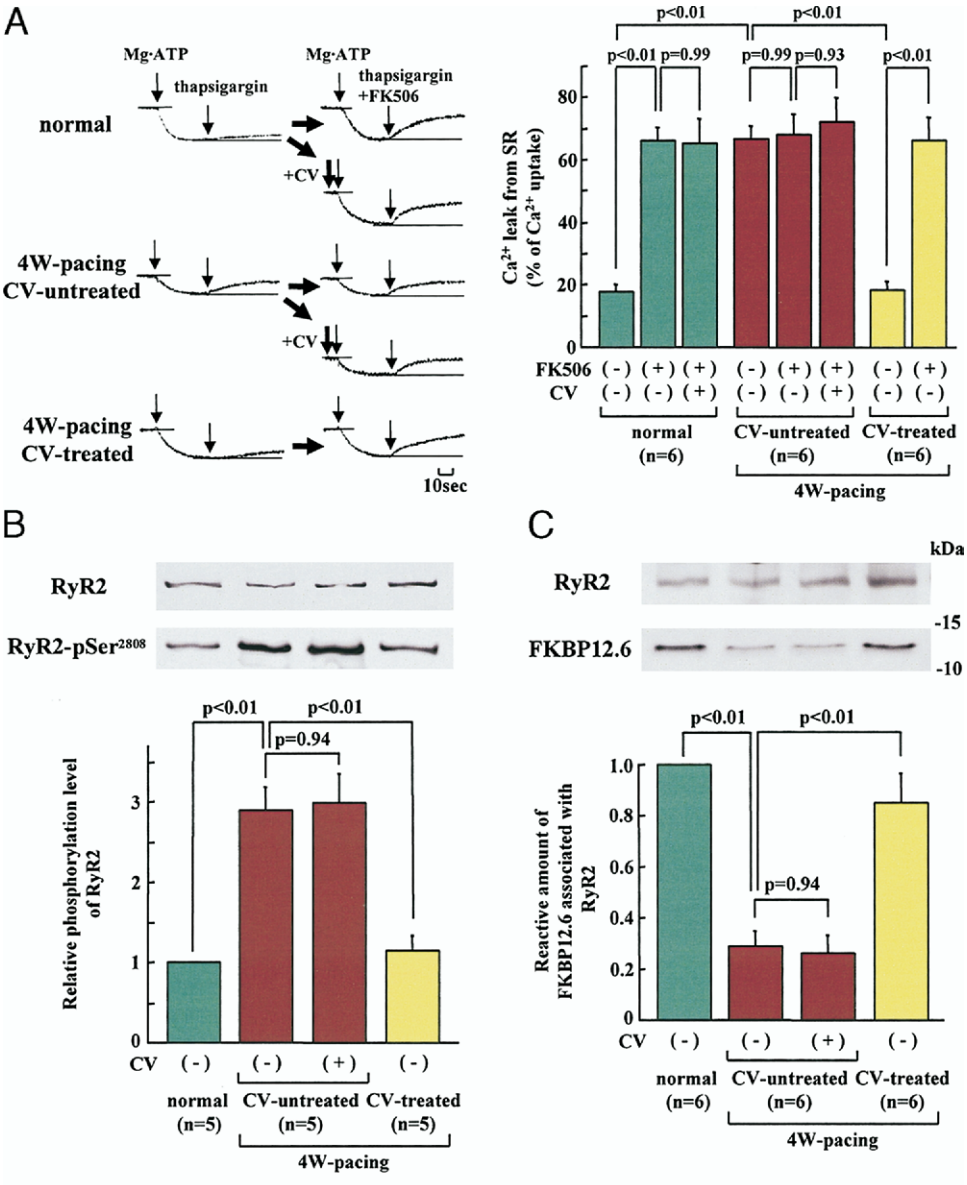

Figure 3 Ca^{2+} Leak, Ser^{2808}-Phosphorylation Level in RyR2, and FKBP12.6

(A) Representative time courses of Ca^{2+} uptake and the ensuing Ca^{2+} leak from SR vesicles in the presence or absence of 30 μmol/l FK506 and/or 100 μmol/l carvedilol (CV) **(left)**. Summarized data of Ca^{2+} leak in normal, CV-untreated, and CV-treated sarcoplasmic reticulum (SR) vesicles **(right)**. **(B)** Protein kinase A phosphorylation level of RyR2. **(C)** The amount of RyR2-bound FKBP12.6. To see the direct acute effect of CV on phosphorylation level of RyR2-$pSer^{2808}$ or amount of RyR2-bound FKBP 12.6 in failing SR vesicles, the SR vesicles were mixed with CV (100 μmol/l) for 30 min and then centrifuged, followed by immunoprecipitation and Western blotting. 4W-pacing = pacing at 250 beats/min for 4 weeks; ATP = adenosine triphosphate; other abbreviations as in Figure 2.

leak was completely inhibited by 100 μmol/l CV. However, this effect was completely abolished when 30 μmol/l DPc10 was added to induce Ca^{2+} leak through defective interdomain interaction within the RyR2 (3).

To monitor the zipped and unzipped states of the interacting domains of the RyR2, we used QSY-BSA as a macromolecular quencher (Figure 4B). As in the case of our recent study with DPc10 (3), the slope of the Stern-Volmer plot, which represents K_Q (the Stern-Volmer quenching constant: a measure of the extent of domain unzipping) was considerably increased by SIN-1 (100 μmol/l), indicating that SIN-1 in fact induced a sizable opening between the interacting domains (Fig. 4B, top panel). The SIN-1–induced increase in the extent of fluorescence quenching was almost completely reversed by 100 μmol/l CV. However, this inhibitory effect of CV was again completely abolished in the presence of 30 μmol/l DPc10 (Fig. 4B, top panel). These results suggest that the inhibition of Ca^{2+} leak

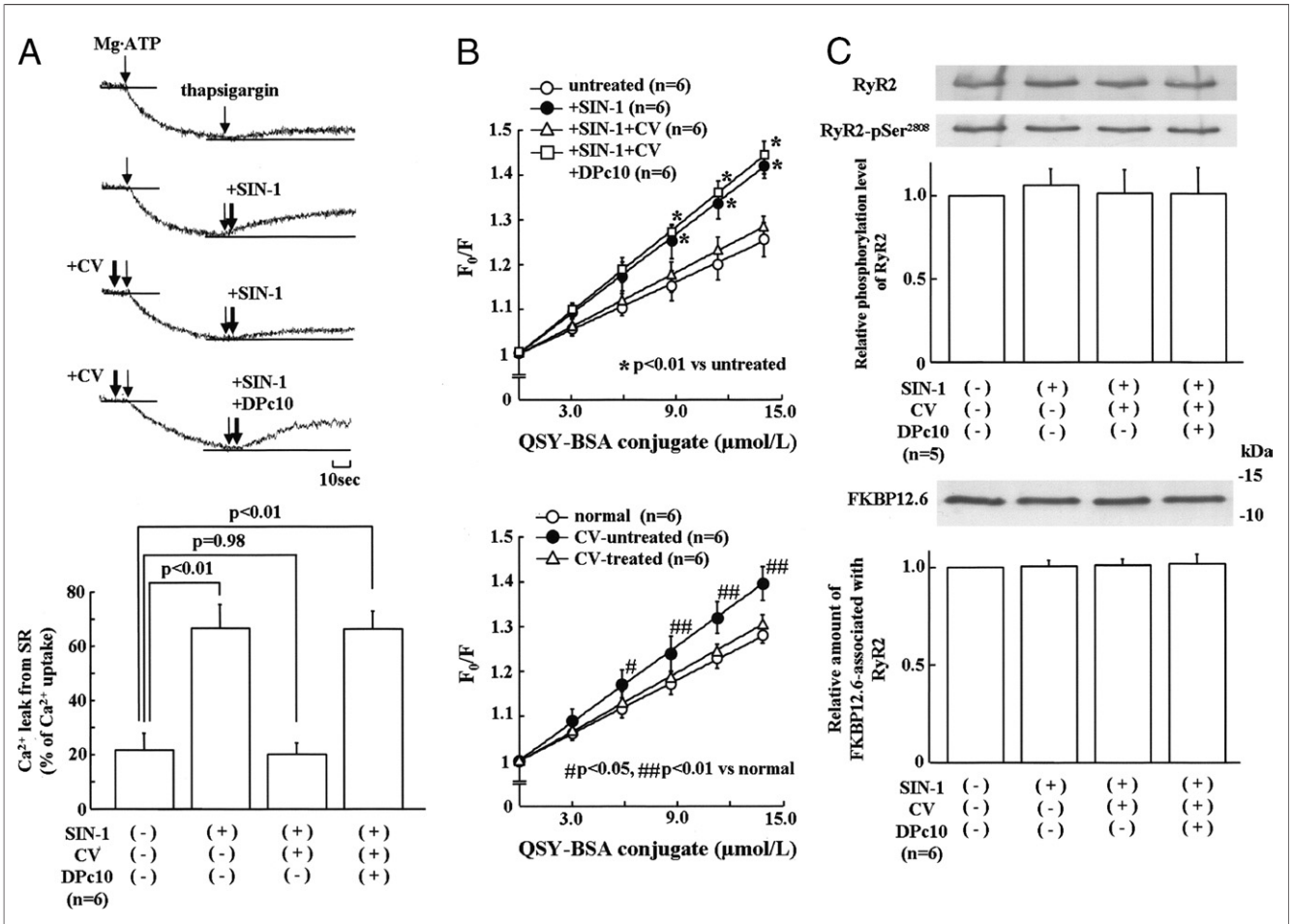

Figure 4 Ca^{2+} Leak and Interdomain Interaction Within RyR2

(A, top) Representative time courses of Ca^{2+} uptake and Ca^{2+} leak from SR vesicles in presence of SIN-1 (100 μmol/l), CV (100 μmol/l), or DPc10 (30 μmol/l). Summarized data of Ca^{2+} leak **(bottom)**. **(B)** Stern-Volmer plots of the fluorescence quenching data with QSY-bovine serum albumin (BSA). The **top panel** shows the effect of SIN-1 (100 μmol/l) in the presence or absence of CV (100 μmol/l) or CV (100 μmol/l) plus DPc10 (30 μmol/l) in normal SR. **Bottom panel** shows Stern-Volmer plots of QSY-BSA fluorescence quenching data of normal, failing (CV-untreated/paced), and CV-treated/paced groups. **(C)** Effect of SIN-1 (100 μmol/l) on phosphorylation level of RyR2-pSer^{2808} **(top)** or amount of RyR2-bound FKBP12.6 **(bottom)**, in the presence of CV (100 μmol/l) or DPc10 (30 μmol/l). Before immunoprecipitation of RyR, SR vesicles were mixed with CV or DPc10 for 30 min and then centrifuged, followed by Western blotting. Abbreviations as in Figures 2 and 3.

by CV is mediated by its reversal of the domain unzipping that has been produced by the SIN-1 oxidation. The extent of fluorescence quenching (K_Q) in failing SR vesicles was larger than for the normal SR. However, in the SR vesicles from 4W-paced/CV-treated hearts, K_Q values were comparable to the values of the normal SR (Fig. 4B, bottom panel). These results suggest that domain unzipping has already taken place in failing SR vesicles (caused partly by PKA-phosphorylation mediated FKBP12.6 dissociation and oxidative stress) and that CV treatment restores the normal zipped state, thereby preventing Ca^{2+} leak. As shown in Figure 4C, both RyR2-pSer^{2808} and RyR2-bound FKBP12.6 were not altered by SIN-1, regardless of the presence of CV or DPc10.

Effects of CV on the amount of SR Ca^{2+}-ATPase, the rate of Ca^{2+} uptake, and the ratio of Ser16-phosphorylated PLB and total PLB in failing hearts. After RV pacing for 4 weeks, both the SR Ca^{2+} uptake activity and the amount of SR Ca^{2+}-ATPase were reduced, and these changes were not observed in the CV-treated group (Figs. 5A and 5B). The levels of Ser16-phosphorylated PLB (p-PLB) and total PLB (t-PLB) among these groups of SR vesicles are compared in Figure 5C (top figure: gel picture; bottom figure: the calculated values of relative phosphorylation). There was no difference in the level of total PLB among the 3 groups, but there was a significant decrease in the basal level of phosphorylated PLB in the failing SR vesicles. In SR vesicles from 4W-paced/ CV-treated hearts, the level of phosphorylated PLB was restored back toward a normal level.

Effects of CV on Ca^{2+} transient and cell shortening in normal and failing cardiomyocytes. We further assessed whether the adverse effects of oxidative stress on SR Ca^{2+} release function and its reversal by CV seen in SR vesicles can be also seen in canine cardiomyocytes. First, we deter-

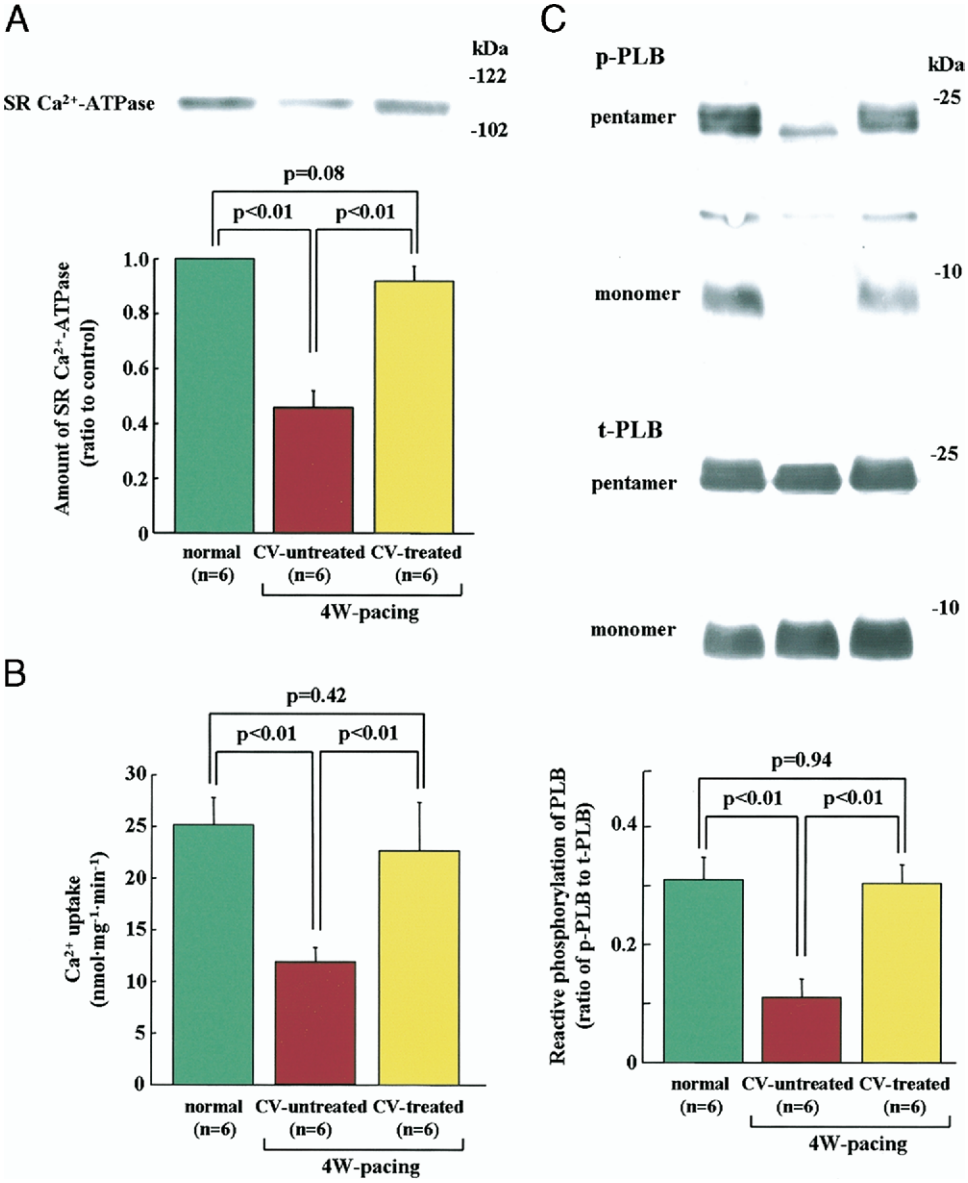

Figure 5 **SR Ca^{2+}-ATPase, Ca^{2+} Uptake, and Phospholamban**

(A) Representative Western blot of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase **(top panel)** and the densitometric analysis of the Western blot **(bottom panel)**. **(B)** The rate of Ca^{2+} uptake. **(C)** Representative Western blot of Ser^{16}-phosphorylated phospholamban (p-PLB) and total PLB (t-PLB) **(top panel)** and the densitometric analysis of the Western blot **(bottom panel)**. The p-PLB values (sum of pentamer and monomer, in arbitrary units) were normalized with respect to the t-PLB values (sum of pentamer and monomer, in arbitrary units). Data are presented as means ± SD. Abbreviations as in Figure 3.

mined the dose of CV that is sufficient for the antioxidant effect but not for the beta-blocking (inverse agonism) effect. Figure 6A shows the concentration-dependent effect of CV on cell shortening of normal and failing cardiomyocytes. Acute addition of CV (<30 nmol/l) had no appreciable effect on the cell shortening in both cardiomyocytes. Likewise, CV (30 nmol/l) had no appreciable effect on Ca^{2+} transients even after stimulation by forskolin (Fig. 6B). In contrast, in failing cardiomyocytes, incubation with CV (30 nmol/l) for 12 h resulted in a significant if not complete improvement of both the Ca^{2+} transient and cell shortening at baseline and in the presence of forskolin (Fig. 6C). However, when DPc10 was incorporated into failing cardiomyocytes by protein delivery kit (Bioporter, Gene Therapy Systems, Inc., San Diego, California), the beneficial effects of CV (30 nmol/l) were completely abolished (Fig. 6C). This finding suggests that the beneficial effect of CV on failing cardiomyocyte function is mediated through amelioration of defective interdomain interaction in the RyR2, which is induced by an inhibition of oxidative stress within

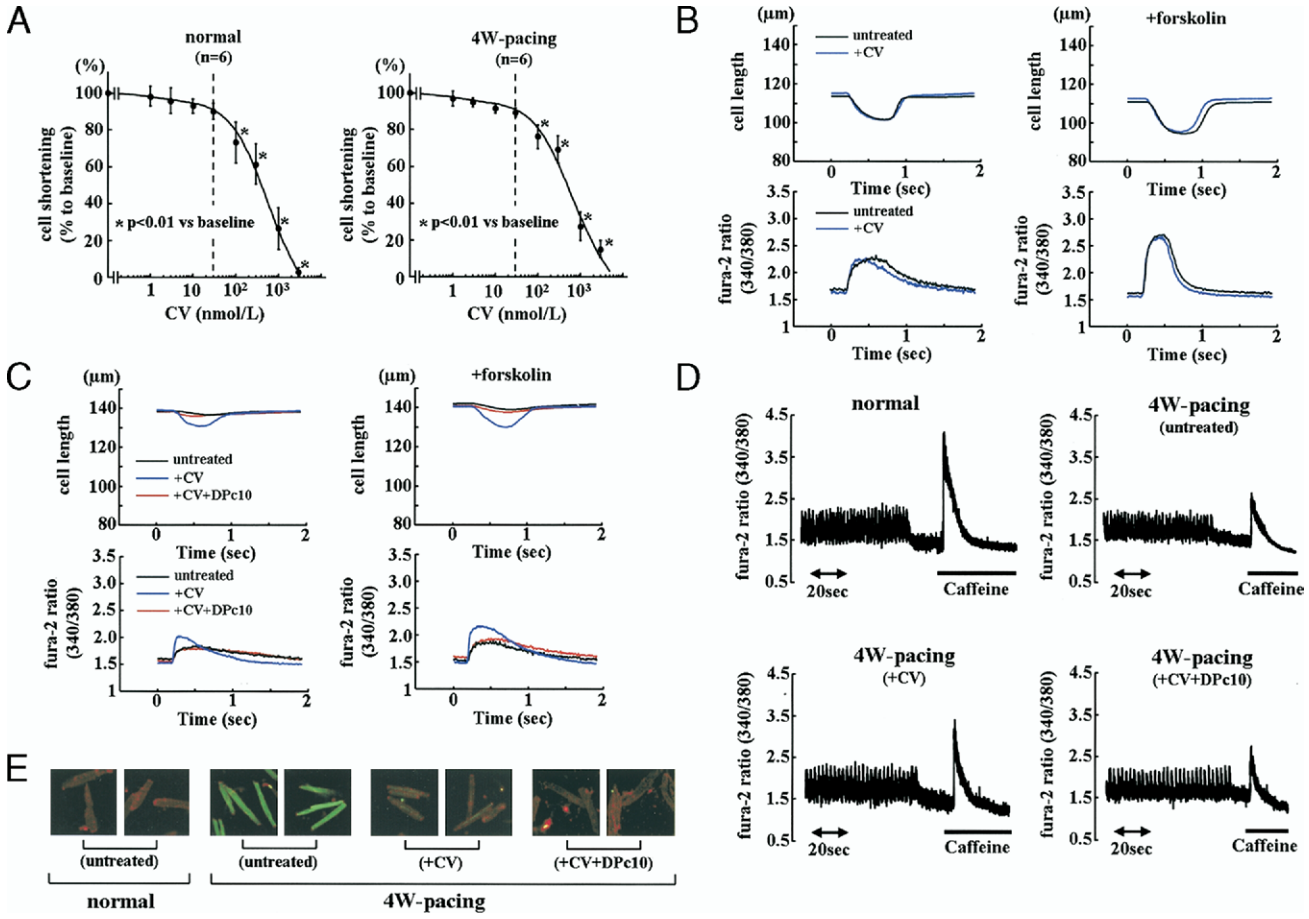

Figure 6 Cell Shortening and the Corresponding Ca^{2+} Transient in Cardiomyocytes

(A) Dose-dependent effect of CV on cell shortening in normal and failing (4W-paced) cardiomyocytes. **(B and C)** Effect of forskolin (500 nmol/l) on cell shortening and Ca^{2+} transient in normal **(B)** and failing cardiomyocytes **(C)**, in the presence of CV (30 nmol/l, incubated for 12 h) or DPc10-incorporation. **(D)** Estimated SR Ca^{2+} content by caffeine (20 mmol/l) application in the presence of CV (30 nmol/l, incubated for 12 h) or DPc10-incorporation. **(E)** 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorescence images in normal and failing cardiomyocytes in presence of CV (30 nmol/l, incubated for 12 h) or DPc10-incorporation. Confocal microscopy clearly detects the DCFH-DA fluorescence signal indicated as **green** in failing cardiomyocytes. Cell surface membrane was fluorescently labeled as **red** by wheat germ agglutinin (Alexa Fluor 633 conjugate; Molecular Probes). Abbreviations as in Figure 3.

RyR2. In contrast to the aforementioned effect of CV (30 nmol/l), metoprolol (100 nmol/l), at a dose that did not acutely change baseline cell shortening in normal cardiomyocytes but decreased cell shortening after addition of isoproterenol (50 nmol/l) ($-28 \pm 5\%$; $n = 6$, $p < 0.01$ vs. baseline, similar to the decrease by CV [30 nmol/l]: $-29 \pm 4\%$; $n = 6$, $p < 0.01$ vs. baseline), had no effect on both cell shortening and Ca^{2+} transient in failing cardiomyocytes. Table 2 summarizes the cell shortening and Ca^{2+} transient data in normal and failing cardiomyocytes. Figure 6D shows the estimated SR Ca^{2+} content by caffeine application. In failing cardiomyocytes, SR Ca^{2+} content was reduced, whereas incubation with CV for 12 h increased the SR Ca^{2+} content. However, incorporation of DPc10 into the failing cardiomyocyte eliminated the increase in SR Ca^{2+} content by CV.

Figure 6E shows the fluorescence images after application of the fluorescent probe of intracellular ROS, DCFH-DA (1 μmol/l), into the normal and failing cardiomyocytes. In failing cardiomyocytes, the fluorescence intensity was increased to a high level, but a normal level of fluorescence intensity was restored by application of 30 nmol/l CV (Fig. 6C). Introduction of DPc10 into the failing cardiomyocyte had no appreciable effect on intracellular ROS. These findings indicate that the ROS level is increased in failing cardiomyocytes, which can indeed be reversed by incubation of CV, and that domain unzipping of the regulatory domain in RyR2 does not increase ROS per se.

Discussion

The most important new aspect of this study is that the beneficial effects of CV on the in vivo cardiac function seem

Table 2 In Vivo Cell Shortening and Ca^{2+} Transient in Normal and Failing Cardiomyocytes

	Cell Shortening (% Decrease From Baseline)		Peak of Ca^{2+} Transient (% Increase From Baseline)		Time From Peak to 80% Decline ($\times 10^{-2}$ s)	
	(−)	Forskolin (500 nmol/l)	(−)	Forskolin (500 nmol/l)	(−)	Forskolin (500 nmol/l)
Normal (n = 30)						
Untreated	8.7 ± 1.2	13.2 ± 1.9	30.5 ± 4.6	54.7 ± 4.7	83.3 ± 23.5	61.4 ± 16.6
Carvedilol (30 nmol/l)	8.4 ± 1.3	13.5 ± 1.7	29.9 ± 5.1	53.5 ± 11.1	85.3 ± 18.2	64.6 ± 14.6
Heart failure (n = 30)						
Untreated	1.7 ± 0.5	2.1 ± 0.5	14.7 ± 3.7	16.3 ± 2.8	129.1 ± 17.5	115.3 ± 29.8
Carvedilol (30 nmol/l)	4.9 ± 0.9*	8.5 ± 1.7*†	25.6 ± 3.2*	40.0 ± 8.7*†	104.8 ± 18.8‡	74.5 ± 15.4*†
Carvedilol (30 nmol/l) + DPc10	1.6 ± 0.7§	1.9 ± 0.8§	15.1 ± 2.7§	16.4 ± 2.7§	123.9 ± 25.8	114.0 ± 19.4§
Metoprolol (100 nmol/l) (n = 20)	1.9 ± 0.3§	2.4 ± 0.8§	15.4 ± 2.9§	16.9 ± 3.1§	123.5 ± 10.9	113.4 ± 13.2§

*$p < 0.01$ versus untreated, †$p < 0.01$ versus forskolin (−), ‡$p < 0.05$, §$p < 0.01$ versus carvedilol 30 nmol/l.

to be attributable to the restoration of defective interdomain interaction within RyR2, either by preventing PKA hyperphosphorylation-induced dissociation of FKBP12.6 from RyR2 or by reducing the oxidative stress level within RyR2 or by both. In this study, we demonstrated that incubation of failing cardiomyocytes with low-dose CV (30 nmol/l; for 12 h), which is not sufficiently high to inhibit cell shortening acutely, significantly improved cardiomyocyte function (i.e., increase in cell shortening and peak of Ca^{2+} transient), concurrent with a reduction of ROS level in failing cardiomyocytes. This finding strongly suggests that the antioxidant effect of CV is enough to improve cardiomyocyte function as a chronic effect, even without exerting a beta-blocking effect.

Importantly, this beneficial effect of low-dose CV on cardiomyocyte function is completely abolished when domain unzipping is introduced by incorporating DPc10, a synthetic peptide corresponding to Gly^{2460}-Pro^{2495} of RyR (one of the mutable domains in CPVT), into failing cardiomyocytes. As shown in our previous study (3), DPc10 was found to induce domain unzipping and Ca^{2+} leak without causing any changes in the level of PKA phosphorylation within RyR2 and in the amount of the RyR2-bound FKBP12.6. These results suggest that the beneficial effects of low-dose of CV (30 nmol/l) on failing cardiomyocyte function is solely attributable to the restoration of defective interdomain interaction (i.e., from unzipped state to a zipped state) by reducing the oxidative stress level within the RyR2. This notion is supported by the following findings. The treatment of the SR isolated from normal hearts with $OONO^-$-donor SIN-1 resulted in a considerable reduction in the content of reactive thiols in the RyR2 moiety. In failing SR vesicles, the content of reactive thiols was considerably reduced; however, the thiol content in the SR from the CV-administered hearts was essentially identical to that of the normal control even after RV pacing for 4 weeks. Moreover, the SIN-1–induced oxidative stress within the RyR2 of normal SR resulted in an increased extent of domain unzipping (defective interdomain interaction), but CV prevented the defective interdomain interaction from occurring. In addition, the SR Ca^{2+} leak was increased by SIN-1–mediated oxidation of normal SR, but again it was reversed by CV. However, if DPc10 was used to force the domains to unzip, CV was without effect (3).

The improvement of failing cardiomyocyte function by CV might partly be attributable to the improvement of beta-adrenergic signaling by its beta-blocking effect (e.g., increases in SR Ca^{2+}-ATP activity, myofibrillar ATPase activity, creatine kinase activity) (14). Also, the fact that chronic administration of CV to failing hearts suppressed Ser 2808 RyR phosphorylation suggests that the reduced oxidation of RyR might partly be attributable to the indirect beta-blocking effect of CV rather than antioxidant effect. However, the fact that an equivalent low-dose of metoprolol, exerting a similar degree of beta-blocking action as CV, had no effect on improvement of failing cardiomyocyte function suggests that the antioxidant effect of CV plays a predominant role in improving failing cardiomyocyte function.

In this study, we used the dose of CV at which heart rate was decreased by 10% to 15%, but LV contractility was unchanged. This dose might correspond with the initial trial dose of CV used for the treatment of human heart failure. Some patients do not tolerate the incremental dosage of beta-blocker. On the basis of the present findings, a low dose of CV, which has antioxidant effects but not appreciable beta-blocking effects, might be enough to improve outcome of patients with heart failure. Indeed, in the Mucha trial (15), administration of very low doses of CV (5 mg/day) to Japanese patients with chronic heart failure decreased the risk of cardiovascular events by 71%.

Chronic administration of CV improved the Ca^{2+} uptake function, SERCA2a expression, and PLB phosphorylation. Obviously, these effects lead to increased SR Ca^{2+} load and, in turn, increased Ca^{2+} release from RyR2, leading to enhanced LV contractility. Phosphorylation of PLB at Ser16 by PKA and at Thr17 by CAMKII might also contribute to improvement of LV function in failing hearts.

In conclusion, administration of CV during RV pacing of the canine heart prevented the development of heart failure, by correcting several problems occurring in the RyR2 moiety of the SR of failing hearts, such as defective

interaction of the regulatory domains in the RyR2, partial dissociation of FKBP12.6, PKA hyperphosphorylation, and Ca^{2+} leak. Oxidation of the cysteine residues of the RyR2 with SIN-1 destabilized the interdomain interaction mimicking the situation in the failing SR, but upon treatment of the SR with CV the normal mode of interdomain interaction was restored, with concurrent inhibition of Ca^{2+} leak. The present study provides the molecular basis as to why CV is effective for improving the prognosis of patients with chronic heart failure.

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