Altered Stoichiometry of FKBP12.6 Versus Ryanodine Receptor as a Cause of Abnormal \(\text{Ca}^{2+}\) Leak Through Ryanodine Receptor in Heart Failure

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**Background**—In the pathogenesis of cardiac dysfunction in heart failure, a decrease in the activity of the sarcoplasmic reticulum (SR) \(\text{Ca}^{2+}\)-ATPase is believed to be a major determinant. Here, we report a novel mechanism of cardiac dysfunction revealed by assessing the functional interaction of FK506–binding protein (FKBP12.6) with the cardiac ryanodine receptor (RyR) in a canine model of pacing-induced heart failure.

**Methods and Results**—SR vesicles were isolated from left ventricular muscles (normal and heart failure). The stoichiometry of FKBP12.6 per RyR was significantly decreased in failing SR, as assessed by the ratio of the \(B_{\text{max}}\) values for \([\text{H}]\text{dihydro-FK506}\) to those for \([\text{H}]\text{ryanodine}\) binding. In normal SR, the molar ratio was 3.6 (≈1 FKBP12.6 for each RyR monomer), whereas it was 1.6 in failing SR. In normal SR, FK506 caused a dose-dependent \(\text{Ca}^{2+}\) leak that showed a close parallelism with the conformational change in RyR. In failing SR, a prominent \(\text{Ca}^{2+}\) leak was observed even in the absence of FK506, and FK506 produced little or no further increase in \(\text{Ca}^{2+}\) leak and only a slight conformational change in RyR. The level of protein expression of FKBP12.6 was indeed found to be significantly decreased in failing SR.

**Conclusions**—An abnormal \(\text{Ca}^{2+}\) leak through the RyR is present in heart failure, and this leak is presumably caused by a partial loss of RyR-bound FKBP12.6 and the resultant conformational change in RyR. This abnormal \(\text{Ca}^{2+}\) leak might possibly cause \(\text{Ca}^{2+}\) overload and consequent diastolic dysfunction, as well as systolic dysfunction. (Circulation. 2000;102:2131-2136.)

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

In cardiac muscle, a small amount of \(\text{Ca}^{2+}\) entering through the L-type \(\text{Ca}^{2+}\) channel induces the release of a large amount of \(\text{Ca}^{2+}\) from the sarcoplasmic reticulum (SR) through the \(\text{Ca}^{2+}\) release channel, also referred to as the ryanodine receptor (RyR).1–4

An associated protein, FKBP12, has been found to be copurified with RyR during sucrose density gradient centrifugation.5 The physiological function of FKBP12 is modulation of RyR-1, the skeletal muscle isoform of the \(\text{Ca}^{2+}\) release channel, possibly by enhancing cooperation among its 4 subunits.6–7 Recently, a novel FKBP with a different electrophoretic mobility (FKBP12.6) was found to be specifically associated with RyR-2, the cardiac muscle isoform of the \(\text{Ca}^{2+}\) release channel.8,9 FKBP12.6 has 85% homology with FKBP12.10 The stoichiometry of binding is ≈4 mol FKBP per RyR tetramer (or 1 FKBP to 1 RyR monomer) in both skeletal muscle and cardiac muscle. However, in contrast to the effects of FKBP12 on RyR-1, there is controversy as to the modulatory influence exerted by FKBP12.6 over RyR-2. Kaftan et al11 found that rapamycin, a drug that inhibits the prolyl isomerase activity of FKBP12.6 and dissociates FKBP12.6 from the canine RyR-2 channel, increases the open probability and reduces the current amplitude of cardiac \(\text{Ca}^{2+}\) release channels. Conversely, Timerman et al12 showed that removal of FKBP12.6 from the canine RyR-2 by FK590 (FK506 analogue) produced no appreciable effect on the sensitivity of the channel to the activating \(\text{Ca}^{2+}\).

Previously,12 we demonstrated that in a pacing-induced canine heart failure model, the rate of polylysine-induced \(\text{Ca}^{2+}\) release from the SR vesicles was significantly decreased, suggesting that the gating function of the SR \(\text{Ca}^{2+}\) release channel in response to the release trigger polylysine is
altered in heart failure. When we found that the addition of FK506 to normal SR vesicles decreased the rate of drug-induced Ca\(^{2+}\) release toward that seen in failing SR vesicles (unpublished data), we hypothesized that the mode of interaction between FKBP12.6 and the RyR-2 may be altered in heart failure and that this might be the major cause of the impaired channel gating function. The goal of this study was to investigate this hypothesis.

**Methods**

**Materials**
Fluo 3 was obtained from Molecular Probes, and sulfo succinimidyl 3-(2-(7-azido-4-methylcoumarin-3-acetamidomethyl)dithio)propionate (SAED) was from Pierce. FK506 was provided by Fujisawa Pharmaceutical Co. Ltd. [\(^{3}\)H]ryanodine and [\(^{3}\)H]dihydro-FK506 were purchased from DuPont NEN. A monoclonal antibody against the canine cardiac ryanodine receptor was from Affinity Bioreagents, Inc. Rat recombinant FKBP12.6 and rabbit anti-FKBP12.6 antiserum directed against the sequence KKFDSSRDRNKPFK were used for immunoblot analysis.

**Production of Pacing-Induced Heart Failure**
In 12 beagle dogs of either sex weighing 10 to 14 kg, heart failure was induced by 21 day of rapid ventricular pacing at a rate of 250 bpm with an externally programmable miniature pacemaker (Medtronic Inc), as described previously. Approximately 1 hour after termination of rapid ventricular pacing, left ventricular (LV) pressure was measured under anesthesia by way of a 7F micromanometer (Millar) inserted percutaneously via the carotid artery, and 2D short-axis echocardiograms were obtained at the level of the papillary muscle.

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**
SR vesicles were prepared as described previously, according to the method of Kranias et al. \(^{15}\)

**[\(^{3}\)H]Dihydro-FK506 and [\(^{3}\)H]Ryanodine Binding Assays**
[\(^{3}\)H]Dihydro-FK506 binding was performed in CHAPS-solubilized SR by the LH-20 column method, with some modifications. The SR vesicles (0.1 mg/mL) were first solubilized in FK506-binding buffer (20 mmol/L NaPO\(_4\) [pH 7.2], 0.5% CHAPS, 2 mmol/L DTT, 5 mg/mL BSA, and 0.02% NaN\(_3\)). Then, the solubilized vesicles were incubated for 30 minutes at 37°C in a binding mixture containing 1.25 to 20 mmol/L [\(^{3}\)H]dihydro-FK506 (55 000 cpm/\(\mu\)mol). After this incubation, the samples were applied to a 3-mL Sephadex LH-20 column equilibrated in LH-20 column buffer (10 mmol/L NaPO\(_4\), 1 mmol/L DTT, 0.25% CHAPS, and 0.01% NaN\(_3\)) to separate free from bound ligand. Nonspecific binding was determined by the addition of 30 \(\mu\)mol/L unlabeled FK506.

The density of high-affinity [\(^{3}\)H]ryanodine binding sites in SR vesicles was determined by Scatchard analysis of [\(^{3}\)H]ryanodine binding isotherms, as described previously.\(^{12,14}\) The stoichiometry of FKBP per RyR was calculated directly from the ratio of the maximal number of binding sites (\(B_{max}\)) values for [\(^{3}\)H]dihydro-FK506 binding and [\(^{3}\)H]ryanodine binding.\(^{8,17}\)

**Ca\(^{2+}\) Uptake and Leak Assays**
SR vesicles (0.2 mg/mL) were incubated in 0.5 mL of solution containing 0.15 mol/L potassium gluconate, 1 mmol/L MgCl\(_2\), 0.2 mmol/L EGTA-calcium buffer (free [Ca\(^{2+}\)] = 0.3 \(\mu\)mol/L), 10 mmol/L NaNO\(_3\), and 20 mmol/L MOPS, pH 6.8. Ca\(^{2+}\) uptake was initiated by the addition of 0.5 mmol/L ATP into the cuvette, 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, various concentrations of FK506 were added in the presence of 1 \(\mu\)mol/L thapsigargin to inhibit SR Ca\(^{2+}\)-ATP activity, and the resultant Ca\(^{2+}\) leak was monitored.

**Site-Directed Labeling of RyR With a Fluorescent Conformational Probe**
Specific fluorescence labeling of the RyR in SR vesicles was performed with the cleavable heterobifunctional cross-linking reagent SAED with the aid of polylysine as a site-specific carrier, as described previously.\(^{16-20}\) The specific methyl-coumarin-acetamido (MCA) labeling of the RyR in cardiac SR vesicles was confirmed by a fluorometric scan (excitation at 360 nm, emission at 440 nm) with a fluorescence spectrophotometer (F-2000; Hitachi) after photophoresis had been carried out on 4% SDS-polyacrylamide gel.

**Fluorescence Assays of the Protein Conformational Change**
The time course of the FK506-induced changes in the fluorescence intensity of the RyR-bound MCA probe (excitation at 360 nm, emission at 440 nm) was monitored under the same conditions as those used for the Ca\(^{2+}\) leak assay (except that there was no fluo 3 in the reaction solution) with a fluorescence spectrophotometer (F-2000; Hitachi).

**Immunoblot Analysis**
Immunoblot analysis for FKBP12.6 was performed as previously described.\(^{13}\) Cardiac SR vesicles were electrophoresed on 15% gel for FKBP12.6 with Laemmli’s buffer system. The amount of protein recognized by anti-FKBP12.6 antiserum was measured by quantitative densitometry of immunoblots with NIH Image (National Institutes of Health) image-analysis software. Recombinant FKBP12.6 was loaded in the range 10 to 50 ng. Within this range, a linear increase in the density of FKBP12.6 was obtained by quantitative densitometry. The relative activity associated with FKBP12.6 and [\(^{3}\)H]ryanodine were determined by dividing its activity by that of the positive control (20 ng of recombinant FKBP12.6).

**Statistics**
Differences between 2 groups were analyzed with an unpaired \(t\) test. Statistical significance was taken at a value of \(P<0.05\).

**TABLE 1. Hemodynamic Data**

<table>
<thead>
<tr>
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<th>HR, bpm</th>
<th>LVPSP, mm Hg</th>
<th>LVEDP, mm Hg</th>
<th>(+)dP/dt, mm Hg/s</th>
<th>(r), ms</th>
<th>LVDd, mm</th>
<th>LVDs, mm</th>
<th>FS, %</th>
</tr>
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<tr>
<td>Normal (n=6)</td>
<td>90 ± 7</td>
<td>121 ± 20</td>
<td>5.5 ± 3.0</td>
<td>2188 ± 650</td>
<td>29 ± 5</td>
<td>28 ± 3</td>
<td>19 ± 2</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>Heart failure (n=6)</td>
<td>97 ± 10</td>
<td>99 ± 22</td>
<td>18.0 ± 7.1*</td>
<td>1088 ± 411*</td>
<td>51 ± 4*</td>
<td>31 ± 2*</td>
<td>28 ± 2*</td>
<td>10 ± 4*</td>
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HR indicates heart rate; LVPSP, LV peak systolic pressure; LVEDP, LV end-diastolic pressure; (+)dP/dt, peak positive dP/dt of LV pressure; \(r\), time constant of LV pressure decay during isovolumic relaxation period; LVDd, LV end-diastolic diameter; LVDs, LV end-systolic diameter; and FS, fractional shortening ([LVd - LVDd]/LVDd × 100]. Data represent mean ± SD. 

\(P<0.05\) vs control.
SR vesicles have a molar ratio of FKBP per Ca$^{2+}$ of 3.6, indicating that the ratio was 1.6 in the failing SR vesicles, indicating that approximately 2 to 3 mol FKBP have been lost from each tetramer of the Ca$^{2+}$ release channel. In contrast, the molar ratio was 1.6 in the failing SR vesicles, indicating that approximately 2 to 3 mol FKBP have been lost from each tetramer of the Ca$^{2+}$ release channel.

### Results

#### Characteristics of Animal Models

There were no significant differences between normal and heart failure groups in body weight (kg; normal 11±1, heart failure 11±3), LV weight (g; normal 56±7, heart failure 65±11), or protein yield with respect to SR vesicles (mg protein/g; normal 1.4±0.4, heart failure 1.2±0.2).

Hemodynamic data are summarized in Table 1. In the heart failure group, LV end-diastolic pressure was significantly elevated, and both the peak +dP/dt of LV pressure and the fractional shortening were decreased, whereas the time constant of the LV pressure decay during the isovolumic relaxation period (τ) was increased. These data indicate that both systolic and diastolic functions were impaired in the heart failure group.


As shown in Figure 1 and summarized in Table 2, the $B_{\text{max}}$ values for both $[^3]$H]ryanodine binding and $[^3]$H]dihydro-FK506 binding in SR vesicles were significantly lower in the failing group than in the normal group. Because the RyR contains a single high-affinity ryanodine binding site per tetramer, the ratio of binding ($B_{\text{max}}$) for $[^3]$H]dihydro-FK506 to $[^3]$H]ryanodine binding to SR vesicles was measured as described under Methods. The ratio of the $B_{\text{max}}$ values for $[^3]$H]dihydro-FK506 and $[^3]$H]ryanodine binding

#### Effects of FK506 on Ca$^{2+}$ Leak and Conformational Change of RyR

Figure 2A shows the time course of the Ca$^{2+}$ leak induced by FK506 (30 μmol/L) plus 1 μmol/L thapsigargin, after the Ca$^{2+}$ uptake induced by MgATP. We confirmed that 1 μmol/L thapsigargin completely inhibited the Ca$^{2+}$ uptake when it was added together with MgATP to the priming solution containing SR vesicles. In accord with our previous finding, the Ca$^{2+}$ leak was observed even without FK506, and FK506 had no further effect on this spontaneous Ca$^{2+}$ leak. Ruthenium red 10 μmol/L plus Mg$^{2+}$ 10 mmol/L (which inhibits Ca$^{2+}$ release and drug-induced Ca$^{2+}$ release through the RyR) completely almost completely inhibited the FK506-induced Ca$^{2+}$ leak in normal SR vesicles and also the spontaneous Ca$^{2+}$ leak in failing SR vesicles. When FK506 was added before the addition of MgATP, the amount of Ca$^{2+}$ uptake was reduced in normal SR vesicles, but it was unchanged in failing SR vesicles. In both groups, the amount of Ca$^{2+}$ uptake (nmol · mg$^{-1}$ · min$^{-1}$; normal 13.6±2.9, failure 8.9±1.3) was not significantly influenced by 30 μmol/L FK506 (nmol · mg$^{-1}$ · min$^{-1}$; normal 14.2±2.0, failure 8.3±1.1) in the presence of 10 mmol/L Mg$^{2+}$ and 10 μmol/L ruthenium red. These data indicate that the Ca$^{2+}$ leak is solely ascribable to the problem with RyR and that FK506 does not have a direct effect on SR Ca$^{2+}$ uptake.

Figure 2B shows the concentration-dependence of the FK506 (0 to 100 μmol/L)–induced Ca$^{2+}$ leak in the 2 types of SR vesicles. After the addition of FK506, a Ca$^{2+}$ leak was induced in a dose-dependent manner in normal SR vesicles. In failing SR vesicles, however, a prominent Ca$^{2+}$ leak was observed even in the absence of FK506, and the addition of FK506 produced little or no further increase in Ca$^{2+}$ leak.

Figure 3 shows the specific incorporation of MCA into the RyR moiety of the SR, as determined by fluorometry of the electrotherapeutically separated proteins. Fluorometric scanning (excitation at 360 nm, emission at 440 nm) revealed that MCA


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<tr>
<td></td>
<td>$B_{\text{max}}$ pmol/mg</td>
<td>$K_D$ nmol/L</td>
<td>$B_{\text{max}}$ pmol/mg</td>
</tr>
<tr>
<td>Normal (n=6)</td>
<td>2.50±0.44</td>
<td>0.95±0.06</td>
<td>8.82±1.28</td>
</tr>
<tr>
<td>Heart failure (n=6)</td>
<td>1.25±0.23*</td>
<td>1.01±0.36</td>
<td>2.01±0.55*</td>
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*P<0.05 vs. control.
fluorescence was clearly localized in RyR in both normal and failing SR vesicles, no other bands being fluorescently labeled. Figure 4A shows typical examples of the changes in MCA fluorescence produced by the addition of FK506 under the same conditions as those used for the Ca2\textsuperscript{+} leak assay. After Ca2\textsuperscript{+} uptake, the addition of FK506 induced an increase in MCA fluorescence at a faster rate than that of the Ca2\textsuperscript{+} leak in normal SR vesicles (compare Figure 4A with Figure 2A), whereas it produced only a slight increase in MCA fluorescence in failing SR vesicles. As shown in Figure 4B, in normal SR vesicles, the amplitude of the FK506-induced change in MCA fluorescence increased as the concentration of FK506 was increased. In failing SR vesicles, however, FK506 produced only a small effect on MCA fluorescence even at its highest concentrations.

FK506 had no effect on the background fluorescence of unlabeled SR vesicles in either normal or failing SR vesicles. Changes in MCA fluorescence did not occur during an ionomycin-induced Ca2\textsuperscript{+} leak in either normal or failing SR vesicles (not shown), suggesting that the conformational change in RyR mediating the FK506-induced or spontaneous Ca2\textsuperscript{+} leak is not under the control of changes in intravesicular [Ca2\textsuperscript{+}]. Furthermore, even when the extravesicular [Ca2\textsuperscript{+}] was strictly buffered to 0.3 \textmu mol/L (with 5 mmol/L EGTA-calcium buffer) during the FK506-induced Ca2\textsuperscript{+} leak, the change in MCA fluorescence was still observed (not shown), indicating that the FK506-induced conformational change in RyR is not under the control of changes in extravesicular [Ca2\textsuperscript{+}] either.

FKBP12.6 Expression in Normal and Failing SR Vesicles

As shown in Figure 5, an immunoreactive band for FKBP12.6 was detected in both normal and failing SR vesicles. However, the expression level of FKBP12.6 was significantly lower (0.80 \pm 0.43) in failing SR vesicles than in normal SR vesicles (2.34 \pm 0.37).

**Discussion**

The major findings of this study are as follows. First, the stoichiometry of FKBP per RyR was significantly decreased in failing SR vesicles. Second, FK506, which binds to the FKBP12.6 associated with the RyR, caused a dose-dependent Ca2\textsuperscript{+} leak in normal SR vesicles that showed a close parallelism with the conformational change in RyR. Third, in failing SR vesicles, a prominent Ca2\textsuperscript{+} leak was observed even in the absence of FK506, and FK506 produced little or no further increase in Ca2\textsuperscript{+} leak and only a slight conformational change in RyR. Fourth, as we expected in view of the above results, the level of protein expression of FKBP12.6 was indeed significantly decreased in failing SR vesicles.

The most important new concept to emerge from these findings is that the abnormal Ca2\textsuperscript{+} leak observed in failing SR vesicles is due to a decreased stoichiometry of FKBP/RyR and
the resultant conformational change in RyR, which leads to RyR instability. We interpret the time course of the FK506-induced change in MCA fluorescence as indicating that a conformational change in RyR occurred as a consequence of the dissociation of FKBP12.6 from RyR. Our results indicate that $50\%$ of the FKBP12.6 molecules have already been lost from RyR in heart failure, whereas $4$ FKBP12.6 molecules to be dissociated remain to be bound in the normal heart. Hence, treatment with FK506 would be expected to produce a much more conspicuous RyR conformational change in normal SR vesicles than in failing SR vesicles (in which a considerable portion of the RyR-bound FKBP12.6 has already been dissociated). Furthermore, the fact that the time course of the change in MCA fluorescence is much faster than that of the Ca$_{\text{2+}}$ leak suggests that dissociation of the RyR-bound FKBP12.6 and the resultant RyR conformational change is a causative mechanism for the observed Ca$_{\text{2+}}$ leak.

Our view, as deduced from the above findings, is schematically illustrated in Figure 6. When sufficiently high concentrations of FK506 (or rapamycin) are applied to cardiac myocytes, cooperation among the 4 RyR subunits is disrupted, thus destabilizing the channel and in turn inducing an abnormal Ca$_{\text{2+}}$ leak. Presumably, equivalent phenomena are occurring in failing hearts even without the addition of FKBP-dissociating agents. This Ca$_{\text{2+}}$ leak will decrease SR Ca$_{\text{2+}}$ loading and elevate basal cytosolic Ca$_{\text{2+}}$ levels during diastole, leading to contractile and relaxation dysfunction.

Before we can firmly advance this as an explanation, several questions remain to be resolved. Because FK506 binds to FKBP12 as well as to FKBP12.6, we need to examine the possibility that the B$_{\text{max}}$ of [3H]dihydro-FK506 binding may indicate mixed binding of FK506, that is, to both FKBP12.6 and FKBP12. In this regard, we should note that in cardiac muscle, the type of FKBP bound to RyR is solely FKBP12.6 and that FKBP12 exists as a soluble form in the cytoplasm. Moreover, Lam et al showed that only FKBP12.6 was...
clearly detected (with no FKBP12) in a cardiac SR vesicle preparation that was similar to our preparation (see their Figure 5). In addition, in our study, the binding isotherm of [3H]dihydro-FK506 to cardiac SR vesicles is a simple hyperbola (yielding a straight line in a Scatchard analysis), a curve that is indicative of a single class of FK506 binding site. Taken together, the above evidence relating to [3H]dihydro-FK506 binding in cardiac SR vesicles indicates specific [3H]dihydro-FK506 binding to RyR-associated FKBP12.6.

The presumed stoichiometry of 4 mol FKBP12.6 per RyR tetramer relies on the assumption that the RyR is the predominant or only SR protein that binds FKBP12.6. In fact, Timerman et al have already confirmed that this is the case on the basis of probability of RyR is 3 to 20 for [3H]dihydro-FK506 binding assays.

We thank Dr Noriaki Ikemoto (Department of Muscle Research, Boston Biomedical Research Institute, Boston, Mass) for a critical reading of the manuscript and helpful suggestions.

Acknowledgments

This work was supported by a grant-in-aid for scientific research from the Ministry of Education in Japan (grant C 11670684) and by a Health Sciences Research Grant for Comprehensive Research on Aging and Health from the Ministry of Health and Welfare, Japan. We thank Dr Noriaki Ikemoto (Department of Muscle Research, Boston Biomedical Research Institute, Boston, Mass) for a critical reading of the manuscript and helpful suggestions.

References

1. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol. 1983;245:C1–C14.
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_Circulation_. 2000;102:2131-2136
doi: 10.1161/01.CIR.102.17.2131

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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