

β -Adrenergic Receptor Blockers Restore Cardiac Calcium Release Channel (Ryanodine Receptor) Structure and Function in Heart Failure

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Background— β -Adrenergic receptor blockade is one of the most effective treatments for heart failure, a leading cause of mortality worldwide. The use of β -adrenergic receptor blockers in patients with heart failure is counterintuitive, however, because they are known to decrease contractility in normal hearts. The ryanodine receptor (RyR2) on cardiac sarcoplasmic reticulum is the key calcium release channel required for excitation-contraction coupling. In failing hearts, the stoichiometry and function of the RyR2 macromolecular complex is altered. Decreased levels of phosphatases (PP1 and PP2A) and hyperphosphorylation by protein kinase A result in dissociation of the regulatory protein FKBP12.6 and channels with increased open probability.

Methods and Results—Here, we show that systemic oral administration of a β -adrenergic receptor blocker reverses protein kinase A hyperphosphorylation of RyR2, restores the stoichiometry of the RyR2 macromolecular complex, and normalizes single-channel function in a canine model of heart failure.

Conclusions—These results may, in part, explain the improved cardiac function observed in heart failure patients treated with β -adrenergic receptor blockers. (*Circulation*. 2001;104:2843-2848.)

Key Words: heart failure ■ calcium ■ sarcoplasmic reticulum ■ ion channels ■ receptors, adrenergic, beta

Clinical trials have shown that β -adrenergic receptor blocker therapy results in a 20% to 66% reduction in mortality at 12 months in patients with heart failure (HF).¹⁻³ It is not intuitively obvious, however, how β -adrenergic receptor blockers can improve cardiac function in failing hearts, because they are known to decrease contractility in normal hearts.⁴⁻⁶

HF is a complex disease⁷ that is characterized by a hyperadrenergic state.⁸ However, β_1 -adrenergic receptors are downregulated and uncoupled from G proteins in failing hearts.^{9,10} Nevertheless, we recently showed that ryanodine receptor (RyR2) is protein kinase A (PKA)-hyperphosphorylated in HF,¹¹ indicating that the net effect of β -adrenergic signaling is upregulated in HF with respect to RyR2 as a substrate for PKA phosphorylation. It is likely that the PKA hyperphosphorylation of RyR2 is a maladaptive response,¹² because it results in the depletion of the regulatory subunit FKBP12.6,^{11,13} yielding channels that are pathologically sensitive to Ca^{2+} -induced Ca^{2+} release from the sarcoplasmic reticulum (SR).¹¹ RyR2 is a macromolecular complex that includes FKBP12.6 as well as PKA and 2 phosphatases (PP1

and PP2A) that are bound to the cytoplasmic domain of the channel via targeting proteins.^{11,14} In failing hearts, the RyR2 macromolecular complex undergoes remodeling characterized by a reduction in the amounts of PP1, PP2A, and FKBP12.6 that are bound to the cytoplasmic domain of the channel.¹¹

In the present study, we used a well-characterized canine model of pacing-induced HF to show that β -adrenergic receptor blockade both restores the normal stoichiometry of the RyR2 macromolecular complex and normalizes the function of the channel.

Methods

HF Model

Seventeen dogs weighing 28 to 30 kg were used for the study. A rapid cardiac pacing regimen that results in severe HF was used as described previously.¹⁵ Animals were assigned to 1 of 4 groups: (1) normal without heart instrumentation (n=6), (2) HF (n=10), (3) HF treated with metoprolol (n=6), and (4) normal without heart instrumentation plus metoprolol (n=2). In groups 2 and 3, after baseline measurements had been obtained, rapid left ventricular (LV) pacing was initiated at 210 bpm for 3 weeks, followed by an additional week of pacing at 240 bpm with an external pacemaker (EV4543, Pace

Received July 24, 2001; revision received September 13, 2001; accepted September 14, 2001.

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Medical, Inc). Metoprolol (25 mg PO BID, Mylan Pharmaceuticals Inc) was begun 2 weeks after initiation of pacing and continued for 2 weeks. Metoprolol was begun 2 weeks after baseline measurement for the control dogs with heart instrumentation. Hemodynamic assessment was performed at baseline and at 2, 3, and 4 weeks. Hemodynamic measurements were performed ≥ 40 minutes after the pacemaker was turned off. Baseline LV change in pressure over time (dp/dt_{max} , mm Hg/s) was 3441 ± 117 ($n=24$) and fell to 1730 ± 304 ($n=10$, $P<0.01$) in animals subjected to rapid LV pacing, consistent with the development of HF. Because rapid LV pacing was continued throughout the metoprolol treatment period, this model was not designed to assess whether β_1 -adrenergic receptor blockade restores normal hemodynamic function in failing hearts. It has previously been demonstrated, however, that β_1 -adrenergic receptor blockade, including therapy with metoprolol, improves LV function in HF in both humans and dogs^{16,17} and improves survival.²

Ryanodine Receptor Macromolecular Complex

SR membranes were prepared from canine ventricular tissue as described previously.^{11,18} Protein concentration was measured by Bradford assay. SR samples were stored at -80°C until use. Cardiac homogenates were prepared with 1.0 g of cardiac tissue homogenized in 1.0 mL of a buffer [mmol/L: Tris-HCl 50 (pH 7.4), NaCl 200, NaF 20, Na_3VO_4 1.0, and DTT 1.0, and protease inhibitors]. Samples were centrifuged at 3000g for 10 minutes and stored at -80°C until use.

Cardiac homogenates (500 μg) were suspended in 0.5 mL of modified RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 0.9% NaCl, 1.0 mmol/L NaF, 1.0 mmol/L Na_3VO_4 , 0.25% Triton X100, and protease inhibitors]. Samples were incubated with anti-RyR (5029) antibody¹¹ overnight at 4°C . Protein A sepharose beads were added, incubated at 4°C for 1 hour, washed with $1\times$ phosphorylation buffer [8 mmol/L MgCl_2 , 10 mmol/L EGTA, and 50 mmol/L Tris/piperazine- N,N' -bis(2-ethanesulfonic acid), pH 6.8], and resuspended in 10 μL of a $1.5\times$ phosphorylation buffer containing either vehicle alone, PKA catalytic subunit (Sigma), or PKA plus a PKA inhibitor (PKI₅₋₂₄, 500 nmol/L, Calbiochem). Backphosphorylation of immunoprecipitated RyR2 was initiated with 33 $\mu\text{mol/L}$ Mg-ATP containing 10% [γ -³²P]ATP (NEN Life Sciences) and terminated after 5 minutes at room temperature with 5 μL of stop solution (4% SDS and 0.25 mol/L DTT). Samples were size-fractionated on 6% SDS-PAGE, and RyR2 radioactivity was quantified with a Molecular Dynamics PhosphorImager and ImageQuant software (Amersham Pharmacia Biotech). Nonspecific phosphorylation (not inhibited by PKA inhibitor) was subtracted, and the resulting value was divided by the amount of RyR2 protein (determined by immunoblotting and densitometry) and expressed as the inverse of the specific PKA-dependent [γ -³²P]ATP signal.

Cardiac homogenates were immunoprecipitated with anti-RyR antibody, and samples were size-fractionated with SDS-PAGE and immunoblotted as previously described.¹¹ Primary antibodies used were anti-PKA catalytic subunit 1:1000, anti-PP1 1:1000, anti-RII 1:1000, and anti-PP2A 1:1000 from Transduction Laboratories; anti-FKBP 1:1000¹⁹; and anti-RyR (5029, 1:3000).¹⁹ Data shown represent results from 3 or more separate experiments.

β -Adrenergic receptor binding studies were performed as previously described²⁰: adrenergic receptor density and affinity were reduced in the failing hearts (normal hearts: $B_{max} = 36.5 \pm 0.9$ fmol/mg, $K_d = 4.3 \pm 0.3$ nmol/L versus failing hearts: $B_{max} = 29.7 \pm 0.3$ fmol/mg, $K_d = 10.0 \pm 0.8$ nmol/L, $n=6$, $P<0.01$), and both were restored to normal with metoprolol ($B_{max} = 34.9 \pm 2.1$ fmol/mg, $K_d = 6.2 \pm 1.7$ nmol/L, $n=6$, $P<0.01$ compared with failing hearts, $P=NS$ compared with normal hearts).

Single-Channel Recordings

Single-channel recordings of RyR2 were performed and analyzed under voltage-clamp conditions as described previously.¹¹ Solutions used for channel analysis were (mmol/L): *trans* solution, HEPES 250 and $\text{Ca}(\text{OH})_2$ 53, pH 7.35, and *cis* solution, HEPES 250, Tris 125, EGTA 1, and CaCl_2 0.5, pH 7.35. Free [Ca^{2+}] (*cis*) 150 nmol/L was calculated by use of Chelator software.²¹ At the conclusion of each

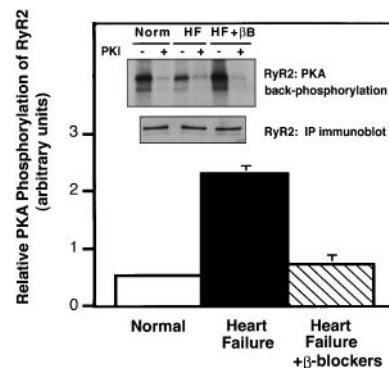


Figure 1. β -Blockers reduce PKA-mediated phosphorylation of RyR2. PKA phosphorylation of RyR2 was measured in normal canine hearts (Norm), pacing-induced HF, and HF animals treated with β -adrenergic receptor blockers (HF + β B, metoprolol 25 mg PO BID). Immunoprecipitated RyR2 was phosphorylated with PKA (5 U), and PKI₅₋₂₄ was used to demonstrate specificity of phosphorylation. Equivalent amounts of RyR2 protein were used in each kinasing reaction, as shown by immunoblotting. Relative PKA phosphorylation of RyR2 from heart homogenates from normals ($n=6$), HF animals ($n=10$), and HF + β B animals ($n=6$) was determined by dividing specific phosphorylation signal by amount of RyR2 protein (determined by immunoblotting and densitometry). Results are inverse of PKA-dependent [γ -³²P]ATP signal \pm SD.

experiment, ryanodine or ruthenium red was applied to confirm the identity of channels as RyR2. Results are presented as mean \pm SD; Student's *t* test or ANOVA (where indicated) was used for statistical analyses.

Results

HF was induced by rapid LV pacing and verified by hemodynamic measurements showing a significant increase in LV end-diastolic pressure from 5.4 ± 2.5 to 23.1 ± 4.8 mm Hg ($n=10$, $P<0.01$). After the establishment of HF, animals were treated with the β_1 -adrenergic receptor blocker metoprolol (25 mg PO BID), a dose that is comparable to or less than that used in patients¹⁶ with HF.

As we previously reported,¹¹ RyR2 was PKA-hyperphosphorylated in failing hearts (Figure 1). PKA phosphorylation of immunoprecipitated RyR2 was assessed with backphosphorylation as previously described.¹¹ Metoprolol treatment reversed the PKA hyperphosphorylation of RyR2 in failing hearts, returning the channel phosphorylation to the levels seen in normal nonfailing hearts (Figure 1). Because there is a single site on each RyR2 molecule (serine 2809) that is PKA-phosphorylated *in vivo*, there are 4 per tetrameric channel.¹¹ The stoichiometry of PKA backphosphorylation of RyR2, determined as previously described,¹¹ was 3.18 ± 0.02 moles of phosphate transferred per mole of channel from normal hearts ($n=6$), compared with 0.72 ± 0.13 moles of phosphate transferred per mole of channel from failing hearts ($n=10$, $P<0.01$ compared with normal hearts by ANOVA) and 2.94 ± 0.15 moles of phosphate transferred per mole of channel ($n=6$, $P=NS$ compared with normal hearts by ANOVA) from failing hearts in animals treated with metoprolol. These data indicate that in failing hearts, ≈ 3 of 4 PKA sites on the tetrameric RyR2 were PKA-phosphorylated *in vivo*, whereas only 1 of these sites was PKA-phosphorylated in RyR2 from normal hearts and in failing hearts in animals

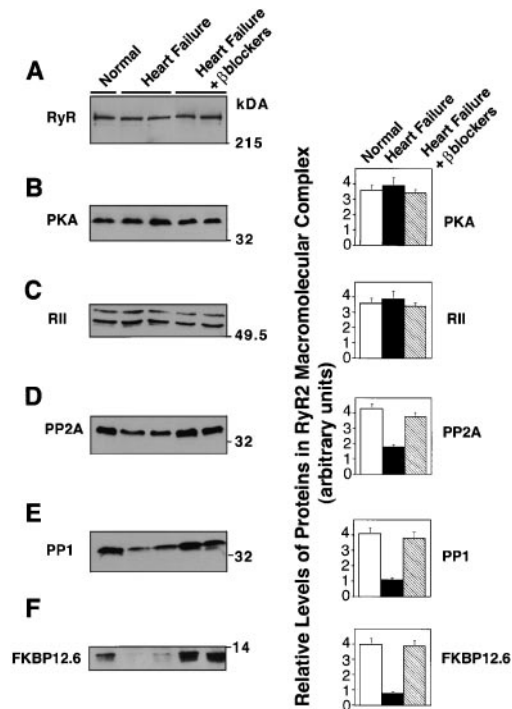


Figure 2. β -Adrenergic receptor blockade normalizes levels of proteins in RyR2 macromolecular complex. Heart homogenates were immunoprecipitated with anti-RyR antibody as described.¹¹ Representative immunoblots are shown for each component of RyR2 macromolecular complex: (A) RyR2, (B) PKA, (C) RII, (D) PP2A, (E) PP1, and (F) FKBP12.6 in hearts from normals ($n=6$), HF animals ($n=10$), and HF treated with β -adrenergic receptor blockers ($n=6$). Protein levels were quantified by densitometry of immunoblots. Results are relative amount of each component of RyR2 macromolecular complex corrected for amount of RyR2 in each immunoprecipitation.¹¹ Error bars are SD.

treated with metoprolol. β_1 -Adrenergic receptor blockade of normal animals had no effect on RyR2 PKA phosphorylation.

To determine the effects of pacing-induced HF and β -adrenergic receptor blocker treatment on the RyR2 macromolecular complex, RyR2 was immunoprecipitated from cardiac homogenates, and the levels of components of the macromolecular complex were determined by immunoblotting as previously described¹¹ (Figure 2). As previously reported,¹¹ no significant differences in the levels of PKA (Figure 2B) and RII (PKA regulatory subunit) (Figure 2C) were associated with RyR2 in the failing versus normal hearts, whereas the amount of the phosphatases PP2A (Figure 2D) and PP1 (Figure 2E) in the RyR2 macromolecular complex were significantly reduced ($P<0.01$ by ANOVA) in the failing hearts compared with normal controls (Figure 2). Treatment with metoprolol restored PP1 and PP2A levels in the RyR2 macromolecular complex in failing hearts back to those seen in normal hearts (Figure 2). Cellular PP1 levels are increased in the failing heart²²; thus, the observed decreased association of PP1 with RyR2 is probably not explained by a generalized decrease in phosphatase levels and is due to a specific reduction in the targeting of PP1 to RyR2. Targeting of PP1 and PP2A to the RyR2 macromolecular complex was restored to normal levels by β -adrenergic receptor blockade (Figure 2D and 2E).

We have previously shown that PKA hyperphosphorylation of RyR2 in failing hearts dissociates the channel regulatory protein FKBP12.6 from RyR2, resulting in defective channel function characterized by increased open probability (P_o) and increased gating frequency (f_o).¹¹ A significant ($P<0.01$ by ANOVA) decrease in the amount of PP1, PP2A, and FKBP12.6 was associated with RyR2 in failing hearts compared with normal hearts (Figure 2A through 2F). Metoprolol treatment of HF animals restored the level of FKBP12.6 bound to RyR2 to normal (Figure 2F); there is no change in the total amount of FKBP12.6 in failing versus normal hearts.¹¹ Like FKBP12, which stabilizes skeletal muscle RyR1 function^{19,23,24} and is required for coupled gating between individual channels,²⁵ FKBP12.6 plays a similar role in the RyR2 macromolecular complex.^{11,18,26}

To determine whether the β -adrenergic blockade-induced normalization of PKA phosphorylation of RyR2 and restoration of the stoichiometry of the macromolecular complex was associated with normalization of channel function, we examined the single-channel properties of RyR2 in planar lipid bilayers as previously described.¹¹ One hundred fifty-three RyR2 channels were studied, including 55 channels from 4 nonfailing canine hearts (Figure 3A), 55 channels from 5 failing canine hearts (Figure 3B), and 43 channels from 4 failing hearts from animals treated with metoprolol (Figure 3C). Compared with RyR2 from normal hearts, RyR2 channels from failing hearts exhibited significantly increased P_o and/or f_o (Table and Figure 3A, compared with Figure 3B). Both increased P_o and increased f_o are seen when FKBP12.6 is removed from RyR2.^{11,18} Compared with channels from normal hearts, in which 0 of 55 channels exhibited increased P_o or f_o , 93% (51/55) of the RyR2 channels from failing hearts were abnormal, exhibiting increased P_o and/or f_o (Table). In contrast, only 12% of the RyR2 channels (5/43, $P<0.01$) from failing hearts from animals treated with metoprolol were abnormal (Table). Although 93% of the RyR2 channels from failing hearts exhibited abnormal function in planar lipid bilayers, as previously reported,¹¹ the single-channel properties were heterogeneous (Table and Figure 3B). This heterogeneity may reflect the fact that each individual channel may have anywhere from 0 to 4 of the 4 possible serines phosphorylated and may have anywhere from 0 to 4 molecules of FKBP12.6 bound to the channel. The single-channel properties of RyR2 from failing hearts would be expected to be heterogeneous, with only a subset of channels exhibiting the most severe defects. If all of the RyR2 channels from failing hearts had increased P_o at low *cis* (cytosolic Ca^{2+}), this would be incompatible with life. To assess the heterogeneous function of RyR2 from failing hearts, the channels were segregated into 3 clearly distinguishable functional groups (Table). Group A includes channels with normal P_o and f_o , Group B includes channels with normal P_o and increased f_o , and Group C includes channels with increased P_o and f_o . Thus, group C includes channels with the most severe functional defects. There were no channels from normal hearts in groups B or C, whereas 93% of the channels from failing hearts were in groups B or C, with 45% in group C. Of the channels from HF treated with β -blockers, 12% were in groups B or C but only 6% were in group C. This heteroge-

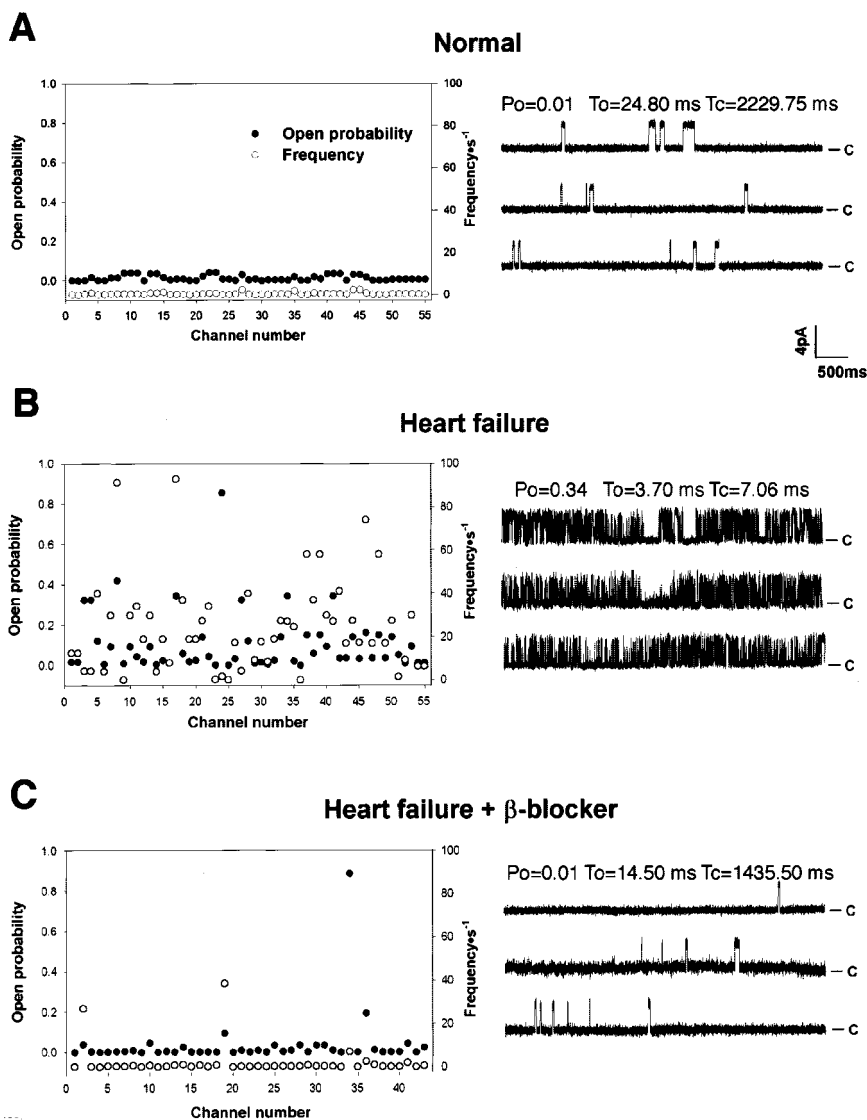


Figure 3. β -Adrenergic receptor blockade normalizes RyR2 function in failing hearts. RyR2 channels from (A) normal canine heart, (B) pacing-induced failing canine heart, and (C) failing hearts treated with metoprolol were studied in planar lipid bilayers. Recordings were at 0 mV, and closed state of channels is indicated (C). P_o (solid circles) and f_o (open circles) are shown for RyR2 channels. $[Ca^{2+}]$ in *cis* chamber was 150 nmol/L. P_o indicates open probability; T_o , open time; and T_c , closed time. Channel openings are upward.

neity in the function of RyR2 channels from failing hearts is consistent with our previous observation that $\approx 70\%$ of the channels exhibited abnormal single-channel properties, including 56% that exhibited some increase in P_o at low *cis* (cytosolic, 150 nmol/L) Ca^{2+} and 15% that exhibited a large increase in P_o at 50 nmol/L *cis* (cytosolic) Ca^{2+} .¹¹

Discussion

Taken together, these data indicate that systemic β -adrenergic blockade can reverse the defects in the structure and function of RyR2 observed in failing hearts.¹¹ Regulation of Ca^{2+} homeostasis in cardiomyocytes is a complex process that requires the integrated function of multiple Ca^{2+} -handling

Single-Channel Properties of RyR2 From Normal Hearts, Failing Hearts, and Failing Hearts From Animals Treated With β -Adrenergic Receptor Blockers (Metoprolol)

	Normal	Heart Failure			Heart Failure + β -Blockers		
		A	B	C	A	B	C
No. of channels	55	4	26	25	38	2	3
P_o	0.01 ± 0.01	0.02 ± 0.02	0.06 ± 0.16	$0.21 \pm 0.17^*$	0.01 ± 0.01	0.03 ± 0.01	$0.39 \pm 0.43^*$
f_o, s^{-1}	0.61 ± 0.59	0.58 ± 0.68	$15.98 \pm 9.63^*$	$70.1 \pm 180.2^*$	0.41 ± 0.38	$13.8 \pm 18.71^*$	$16.1 \pm 19.62^*$

RyR2 channels from failing hearts and failing hearts from animals treated with metoprolol were segregated into 3 distinct groups: group A includes channels with normal P_o and f_o , group B includes channels with normal P_o and increased f_o , and group C includes channels with increased P_o and f_o . See text for explanation of groups A, B, and C.

* $P < 0.01$ vs channels from normal hearts.

molecules.²⁷ RyR2 plays a key role in cardiac excitation-contraction coupling, and data from our laboratory and others suggest that dysregulation of SR Ca²⁺ release via RyR2 could contribute to defects in Ca²⁺ signaling in failing hearts.^{11,28–35} Many studies have shown that β -adrenergic blockade can restore function in failing hearts.^{17,30,36–39} We have proposed that hyperphosphorylated RyR2 depleted of FKBP12.6 could contribute to a reduction in SR Ca²⁺ content and uncoupling of RyR2.²⁶ The chronic alterations in RyR2 function could contribute to a reduction in excitation-contraction coupling gain and decreased Ca²⁺ transients in failing hearts and possibly produce diastolic Ca²⁺ release that can trigger delayed afterdepolarizations⁴⁰ and initiate fatal ventricular cardiac arrhythmias (sudden cardiac death).^{11,12} In agreement with these possibilities, recent studies have shown either a reduction in SR Ca²⁺ content,⁴¹ decreased Ca²⁺ transients,⁴² or decreased excitation-contraction coupling gain⁴³ in cardiomyocytes from failing hearts as well as reduced levels of FKBP12.6 in the RyR2 macromolecular complex.⁴⁴

Systemic β -adrenergic receptor blockade therapy has multiple effects on a wide range of signaling molecules both in the heart and in other tissues. The present study focuses on one of these pathways and demonstrates normalization of RyR2 structure and function in failing hearts treated with β -adrenergic receptor blockade. RyR2 is one of the first molecular targets identified for β -adrenergic receptor blockade therapy outside of the β -adrenergic receptor signaling pathway. The restoration of normal RyR2 structure and function contributes to a mechanistic understanding that may in part explain some of the beneficial effects of β -adrenergic receptor blockade observed in HF.

Acknowledgments

This study was supported by grants to Dr Marks from the National Institutes of Health, the American Heart Association, and the Richard and Lynne Kaiser Family Foundation and to Dr Reiken from the Whitaker Foundation. Dr Marks is a Doris Duke Foundation Distinguished Clinical Scientist. We thank members of the Marks laboratory for helpful discussions and comments on the manuscript.

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Circulation. 2001;104:2843-2848

doi: 10.1161/hc4701.099578

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

The online version of this article, along with updated information and services, is located on the World Wide Web at:

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