Cell, Vol. 101, 365–376, May 12, 2000, Copyright ©2000 by Cell Press

PKA Phosphorylation Dissociates FKBP12.6 from the Calcium Release Channel (Ryanodine Receptor): Defective Regulation in Failing Hearts

Steven O. Marx,*†‖ Steven Reiken,*†‖ Yuji Hisamatsu,*† Thotalla Jayaraman,*† Daniel Burkhoff,*† Nora Rosemblit,*† and Andrew R. Marks*†‡§
*Center for Molecular Cardiology
†Department of Medicine
‡Department of Pharmacology
College of Physicians and Surgeons of Columbia University
New York, New York 10032

Summary

The ryanodine receptor (RyR)/calcium release channel on the sarcoplasmic reticulum (SR) is the major source of calcium (Ca^{2+}) required for cardiac muscle excitation-contraction (EC) coupling. The channel is a tetramer comprised of four type 2 RyR polypeptides (RyR2) and four FK506 binding proteins (FKBP12.6). We show that protein kinase A (PKA) phosphorylation of RyR2 dissociates FKBP12.6 and regulates the channel open probability (P_o). Using cosedimentation and coimmunoprecipitation we have defined a macromolecular complex comprised of RyR2, FKBP12.6, PKA, the protein phosphatases PP1 and PP2A, and an anchoring protein, mAKAP. In failing human hearts, RyR2 is PKA hyperphosphorylated, resulting in defective channel function due to increased sensitivity to Ca^{2+}-induced activation.

Introduction

Calcium (Ca^{2+}) release channels (ryanodine receptor) on the sarcoplasmic reticulum (SR) of striated muscles are required for EC coupling. In cardiac muscle, the type 2 ryanodine receptor (RyR2) is a tetramer comprised of four 565,000 dalton RyR2 polypeptides and four 12,000 dalton FK-506 binding proteins (FKBP12.6). FKBP12s are regulatory subunits that stabilize RyR channel function (Brillantes et al., 1994) and facilitate coupled gating between neighboring RyR channels (Marx et al., 1998) which are packed into dense arrays in specialized regions of the SR that release intracellular stores of Ca^{2+} that trigger muscle contraction.

RyRs are ligand-activated channels and Ca^{2+} is the important physiological ligand that activates the channels in cardiac muscle during EC coupling. The Ca^{2+} dependence of RyR channel activity is biphasic such that low cytosolic [Ca^{2+}] (μM) activates the channels and high cystolic [Ca^{2+}] (mM) inactivates the channels (Bezprozvanny et al., 1991). One FKBP12 molecule is bound to each RyR subunit, and dissociation of FKBP12 significantly alters the biophysical properties of the channels, resulting in the appearance of subconductance states and increased P_o due to an increased sensitivity to Ca^{2+}-dependent activation (Brillantes et al., 1994; Kaftan et al., 1996). In addition, dissociation of FKBP12 from RyR channels inhibits coupled gating, resulting in channels that gate stochastically rather than as an ensemble (Marx et al., 1998). Coupled gating of arrays of RyR channels is thought to be important for efficient EC coupling that regulates muscle contraction (Marx et al., 1998).

FKBPs are *cis-trans* peptidyl-prolyl isomerases that are widely expressed and subserve a variety of cellular functions (Marks, 1996). FKBP12s are tightly bound to and regulate the function of the skeletal (RyR1) (Jayaraman et al., 1992; Brillantes et al., 1994) and cardiac (RyR2) (Kaftan et al., 1996) muscle Ca^{2+} release channels, as well as related intracellular Ca^{2+} release channels: the type 1 inositol 1,4,5-trisphosphate receptor (IP3R1) (Cameron et al., 1997) and the type I TGFβ receptor (TβRI) (Chen et al., 1997).

In the present study, we demonstrate that PKA phosphorylation regulates the binding of FKBP12.6 to the channel both in vitro and in vivo. PKA phosphorylation of the cardiac SR Ca^{2+} release channel/RyR2 dissociates the regulatory subunit FKBP12.6 from the channel, resulting in altered channel function manifested as an increased P_o, increased sensitivity to Ca^{2+}-induced activation, and destabilization of the channel resulting in subconductance states. RyR2 PKA phosphorylation is physiologically regulated in vivo. Channels from failing hearts were PKA hyperphosphorylated. RyR2 channels from failing hearts exhibited decreased binding of the FKBP12.6 regulatory subunit, resulting in the same severe defects in single channel properties observed in in vitro PKA hyperphosphorylated channels. We show that the RyR2 channel comprises a macromolecular complex that includes FKBP12.6, PKA, RII, the phosphatases PP1 and PP2A, and an anchoring protein, mAKAP. Taken together, these data demonstrate that local regulation of the RyR2 channel via PKA phosphorylation is a potent mechanism for modulating SR Ca^{2+} release. Dysregulation of this control mechanism occurs in failing hearts and can explain the observed defects in EC coupling that contribute to cardiac dysfunction.

Results

PKA Phosphorylates RyR2

The 565,000 dalton RyR2 polypeptide was PKA phosphorylated in in vitro kinasing reactions (Figure 1A). To confirm the identity of the PKA phosphorylated high molecular weight protein as RyR2, the phosphorylated band was immunoblotted with anti-RyR antibody. The specificity of the phosphorylation was demonstrated using a PKA inhibitor (PKI) (Figure 1A). Addition of phosphorylation buffer, including cAMP without exogenous PKA, also resulted in phosphorylation of RyR2 that was inhibited by PKI, indicating that endogenous PKA was associated with RyR2 (Figure 1A). The stoichiometry of

§To whom correspondence should be addressed (e-mail: arm42@columbia.edu).
‖These authors contributed equally to this work.

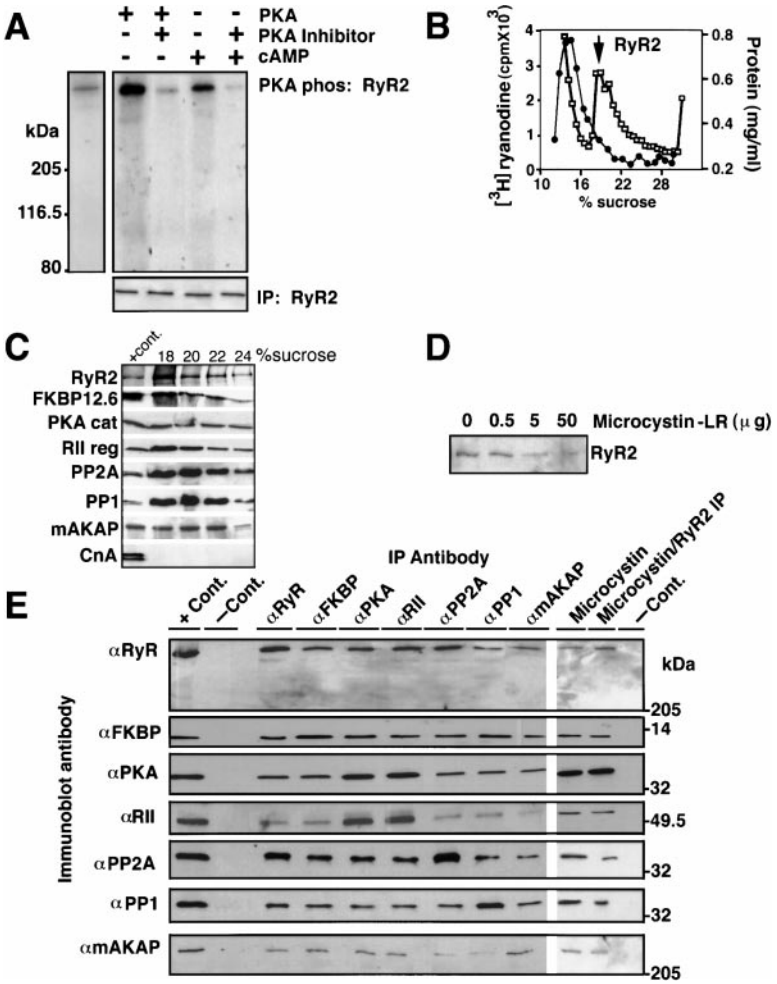

Figure 1. PKA Phosphorylation of RyR2, a Macromolecular Signaling Complex

(A) Immunoprecipitated RyR2 was phosphorylated with PKA (5 units), or cAMP (10 μM), and PKA inhibitor PKI_{5-24} (500 nM) inhibited the phosphorylation. Equivalent amounts of RyR2 protein were used in each reaction as shown by immunoblotting.

(B) RyR2 channels were isolated using [^{3}H]ryanodine by sucrose density gradient centrifugation as described (Brillantes et al., 1994). [^{3}H]ryanodine binding (open squares) and total protein (filled circles) were plotted. Individual RyR2 channels sediment at 30S (arrow), and 2 or more physically attached RyR2 channels sediment in higher sucrose fractions as previously reported for RyR1 channels (Marx et al., 1998).

(C) Immunoblotting gradient fractions with specific antibodies showed that FKBP12.6, PKA catalytic subunit, PKA regulatory subunit (RII), PP2A, PP1, and mAKAP, but not CnA were detected in all fractions containing RyR2.

(D) RyR2 binding to microcystin-Sepharose beads was competed using free microcystin-LR. Samples were pelleted and analyzed by SDS-PAGE and immunoblotting with anti-RyR antibody.

(E) Components of the RyR2 complex (FKBP12.6, PKA, RII, PP2A, PP1, and mAKAP) were coimmunoprecipitated from cardiac SR (200 μg). The RyR2 complex was sedimented using microcystin-Sepharose, the complex was competed off with free microcystin-LR followed by immunoprecipitation with anti-RyR antibody (αRyR) and immunoblotting. Positive controls (+Cont.) were recombinant or purified proteins as indicated, negative controls (−Cont.) were blocking peptides or IgG. In all cases data shown are representative of more than three similar experiments.

PKA phosphorylation was determined by immunoprecipitating RyR2 from cardiac muscle SR, fully dephosphorylating the RyR2 protein with alkaline phosphatase, and then phosphorylating it with PKA and [γ^{32}P]ATP. The stoichiometry of PKA phosphorylation was 3.8 ± 0.1 moles of phosphate per mole of channel (each channel comprises four RyR2 subunits), or one per RyR2 subunit, indicating that each RyR2 protein is PKA phosphorylated on a single amino acid residue.

RyR2 Macromolecular Complex Includes FKBP12.6, PKA, PP1, PP2A, and mAKAP

RyR2 was isolated by sucrose density gradient centrifugation using [^{3}H]ryanodine (Figure 1B) as described (Marx et al., 1998). Individual tetrameric RyR2 channels sediment as 30S complexes and multiple channels (two or more) sediment as denser complexes (Marx et al., 1998). The muscle A kinase anchoring protein (mAKAP) that binds PKA and targets it to substrates has been localized to cardiac SR (Yang et al., 1998; Kapiloff et al., 1999). The major protein phosphatases in cardiac muscle are protein phosphatase 2A (PP2A), protein phosphatase 1 (PP1) (MacDougall et al., 1991), and calcineurin (CnA). Fractions from the sucrose gradient were immunoblotted with either anti-RyR antibody or with antibodies that recognize FKBP12.6, the catalytic subunit of PKA, the PKA regulatory subunit (RII), PP2A, PP1, mAKAP, or CnA, (Figure 1C) all of which (with the exception of CnA) were detected in all fractions containing RyR2. These data are consistent with a high molecular weight complex comprised of RyR2, FKBP12.6, PKA, RII, PP1, PP2A, and mAKAP.

The phosphatase inhibitor microcystin binds to PP1 and PP2A. RyR2 was sedimented by binding to microcystin-Sepharose beads and the specificity of this interaction was demonstrated by competing off RyR2 using free microcystin-LR (Figure 1D). Coimmunoprecipitations were performed showing that FKBP12.6, PKA, RII, PP2A, PP1, and mAKAP all coimmunoprecipitated with RyR2, indicating physical association of these proteins with the SR Ca^{2+} release channel (Figure 1E). The existence of a macromolecular complex was shown independently by first sedimenting the complex with microcystin-Sepharose beads, followed by competing the complex off from the beads with free microcystin-LR, and then immunoprecipitating each of the components of the complex (Figure 1E, last three lanes). Taken together, these data show that FKBP12.6, PKA, RII, PP1,

PP2A, and mAKAP comprise a macromolecular complex with RyR2.

PKA Hyperphosphorylation of RyR2 in Failing Heart Muscle

Increased sympathetic activity is an important physiologic response to stress, resulting in activation of the adrenergic signaling pathway that generates increased cAMP levels and activates PKA. In failing hearts (regardless of the etiology of the damage to the heart), circulating catecholamine levels are markedly increased. We examined specific PKA phosphorylation of RyR2 in normal and failing hearts using both back-phosphorylation with [γ^{32}P]ATP and anti-phosphoserine immunoblots (Figures 2A and 2B).

Strikingly, PKA phosphorylation of RyR2 was significantly elevated in failing hearts from humans and from animal models (dogs with pacing-induced heart failure) compared to nonfailing hearts (Figures 2A and 2B). PKA phosphorylation of RyR2 channels from failing hearts was increased ~4-fold compared to RyR2 channels from nonfailing hearts. The stoichiometry of PKA back-phosphorylation of RyR2 channels isolated from failing hearts was 0.7 ± 0.3 moles of phosphate transferred per mole of channel ($n = 8$) compared to 3.1 ± 0.1 moles of phosphate transferred per mole of channel from normal nonfailing hearts ($n = 6$, $p < 0.0001$). These data suggest that in failing hearts, approximately three of the four PKA sites on the tetrameric RyR2 channel are phosphorylated in vivo, whereas only one site is phosphorylated in vivo on RyR2 isolated from normal nonfailing hearts.

This increase in PKA phosphorylation of RyR2 was not due to an increase in the levels of PKA protein associated with RyR2 in failing hearts as determined by coimmunoprecipitation of PKA with RyR2 (Figure 2A). PKA back-phosphorylation was performed using immunoprecipitated RyR2 to ensure that the phosphorylation signal which was measured represented specifically RyR2 PKA phosphorylation. We have previously reported that the levels of RyR2 are decreased in failing hearts (Go et al., 1995). PKA phosphorylation of RyR2 was normalized to the amount of immunoprecipitated RyR2 protein to enable valid comparisons of the amount of PKA phosphorylation per RyR2 molecule from normal and failing hearts (Figures 2A and 2B). Moreover, identical results were obtained when immunoprecipitated RyR2 was immunoblotted with an anti-phosphoserine antibody (e.g., see Figure 2B, inset) confirming that the RyR2 channels from failing hearts were PKA hyperphosphorylated compared to channels from nonfailing hearts.

Left ventricular assist devices (LVADs) are used as a bridge to transplantation when donor hearts are not available. Studies have shown that the hemodynamic unloading of the left ventricle provided by LVADs results in a significant improvement in cardiac contractile function when the device is implanted in failing hearts (Levin et al., 1995). At the time of LVAD insertion, a tissue core is removed from the patient's left ventricle and this can then be compared to tissue from the explanted heart that becomes available at the time of transplantation. Thus, the pre-LVAD sample comes from failing hearts and the post-LVAD sample comes from hearts with improved function. PKA phosphorylation of RyR2 was significantly increased in pre-LVAD heart samples compared to samples from nonfailing hearts and returned to normal levels following LVAD treatment (Figures 2A and 2B). Taken together, these data show: (1) PKA phosphorylation of RyR2 is regulated physiologically in vivo, (2) heart failure is associated with increased PKA phosphorylation of RyR2, and (3) the PKA phosphorylation of RyR2 returns to normal levels when the cardiac function is improved by an LVAD.

Many patients with end-stage heart failure are treated with β-adrenergic agonists (e.g., dobutamine) prior to cardiac transplantation, while some patients are admitted directly from home when a donor heart becomes available (and therefore are not receiving β-adrenergic agonists). PKA phosphorylation of RyR2 was significantly elevated in the hearts from patients not on β-adrenergic agonists compared to normals (Figures 2A and 2B). PKA phosphorylation of RyR2 was significantly further increased in hearts from those patients treated with β-adrenergic agonists prior to cardiac transplantation (Figures 2A and 2B). These data indicate that exogenous administration of β-adrenergic agonists to patients with heart failure can further increase the PKA phosphorylation of RyR2 in the heart.

To determine whether the increased PKA phosphorylation of RyR2 observed in failing hearts was explained by an increase in PKA activity or by a decrease in phosphatase activity, the amounts of PP1 and PP2A physically associated with RyR2 in failing hearts were compared to those in normal hearts from humans and dogs (Figures 2C and 2D). There was a significant decrease in the levels of PP1 and PP2A that coimmunoprecipitated with RyR2 from failing hearts (Figures 2C and 2D). The decrease in the amount of PP1 (but not of PP2A) associated with RyR2 was restored to normal by LVAD treatment (Figures 2C and 2D). These data suggest that, at least in part, the PKA hyperphosphorylation of RyR2 is due to a decrease in phosphatase bound to the RyR2 channel macromolecular complex.

Mapping Signaling Complex Binding Sites on RyR2

The FKBP12.6 binding site on RyR2 was identified using a yeast two-hybrid protein interaction screen in which yeast were transformed with vectors containing either fragments of RyR2 or the full-length FKBP12.6 fused to the Gal4 activation domain or DNA binding domain. One RyR2 fragment corresponding to amino acid residues 2361–2496 resulted in a positive interaction with FKBP12.6 (Figure 3A). Rapamycin inhibited the interaction between FKBP12.6 and RyR2 (Figure 3A) in a concentration-dependent manner in rapamycin-resistant yeast (Lorenz and Heitman, 1995), indicating that the interaction between FKBP12.6 and RyR2 was specific. The hydrophobic FKBP12.6 binding site comprised of isoleucine 2427 and proline 2428 is homologous to the FKBP12 binding site in RyR1, IP3R1 (Cameron et al., 1997), and TβRI (Figure 3A). Using GST-RyR2 fusion proteins in pull-down assays with cardiac SR, binding domains for PP1 (residues 513–808) and PP2A (residues 1451–1768) were mapped (Figure 3B). Interestingly, both

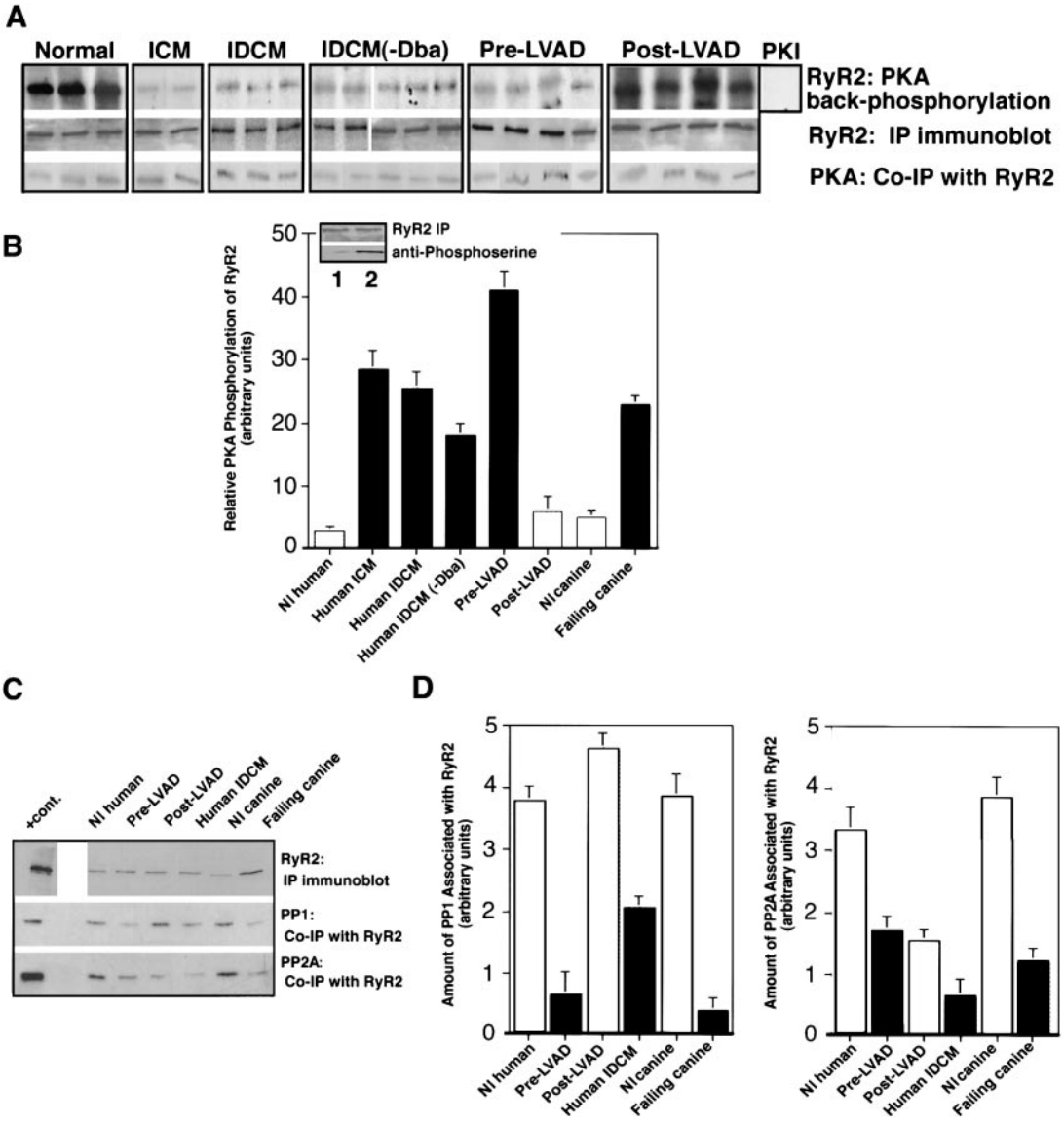

Figure 2. RyR2 PKA Phosphorylation during Heart Failure

(A) PKA back-phosphorylation of RyR2 protein immunoprecipitated from the indicated tissues is shown in the top row; the middle row shows the amount of RyR2 immunoprecipitated in each reaction; and the bottom row shows the amount of PKA coimmunoprecipitated with RyR2 from each sample. Normal, nonfailing human heart; ICM, end-stage failing human heart with ischemic cardiomyopathy; IDCM, end-stage failing human heart with idiopathic dilated cardiomyopathy; IDCM(−Dba), samples from patients not treated with a β-adrenergic agonist, Dba (dobutamine); Pre-LVAD, left ventricular sample taken from a human heart with end-stage failure during insertion of an LVAD; Post-LVAD, sample from the same human heart after LVAD treatment; PKI, representative negative control showing that PKA phosphorylation was inhibited by PKI.

(B) Quantitation of RyR2 back-phosphorylation studies shown in (A). The inset shows PKA hyperphosphorylation of RyR2 confirmed by anti-phosphoserine immunoblotting: (1) top row, RyR2 immunoblot (top lanes); (2) bottom row, anti-phosphoserine immunoblotting of the same samples. Lane 1, normal human heart; lane 2, failing human (ICM) heart. For each condition, a minimum of three experiments using tissue from three different hearts were performed; error bars represent standard deviation of the mean.

(C) PP1 and PP2A coimmunoprecipitated with RyR2 from normal and failing hearts. Following immunoprecipitation with anti-RyR antibody, immunoprecipitates were size-fractionated and immunoblotted with: anti-RyR2 (top panel), anti-PP1 (middle panel), or anti-PP2A (bottom panel) antibodies. Data shown are representative of three similar experiments.

(D) The amount of PP1 and PP2A coimmunoprecipitating with RyR2 was determined by densitometric quantitation of the immunoblots and normalized for the amount of RyR2 coimmunoprecipitated. Less PP1 and PP2A was associated with RyR2 in all of the heart failure samples. Data shown are representative of three similar experiments.

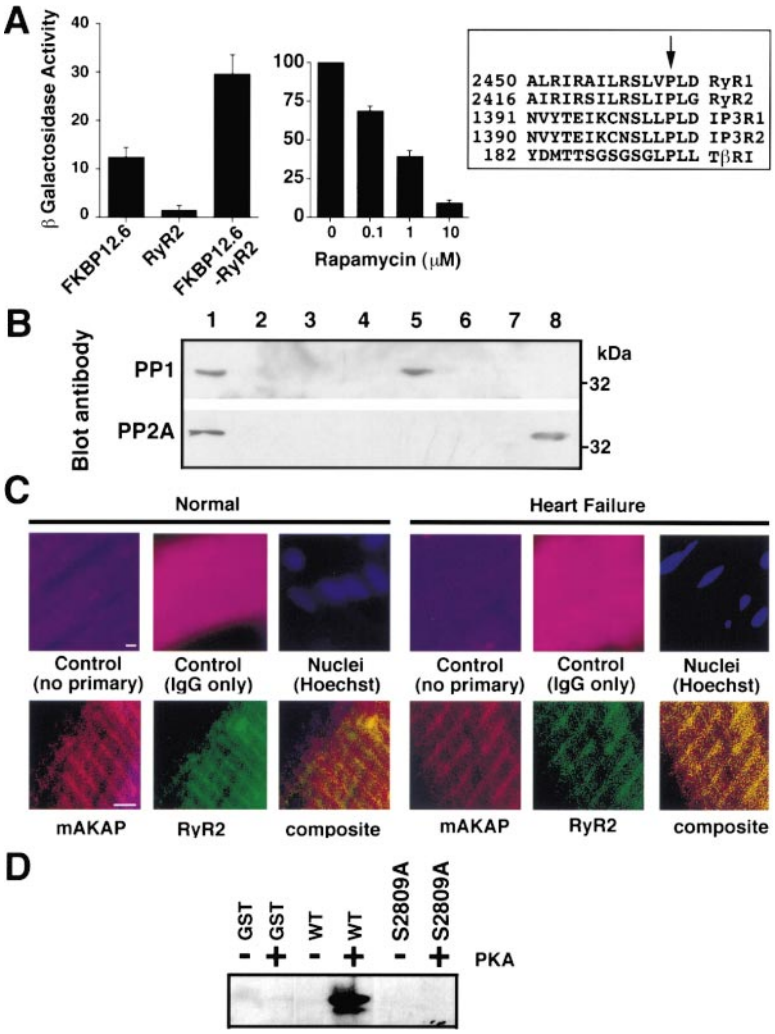

Figure 3. Mapping Signaling Complex Binding Sites on RyR2

(A) The FKBP12.6 binding site in RyR2 was identified using a yeast two-hybrid interaction screen. Left bar graph shows β-galactosidase activity for yeast transformed with: (1) FKBP12.6/activation domain fusion protein alone, (2) RyR2 (residues 2361–2496)/DNA binding domain alone, and (3) both. Interaction between FKBP12.6 and an RyR2 fragment (residues 2361–2496) activates Gal-4 transcription resulting in increased β-galactosidase activity. The right bar graph shows normalized β-galactosidase activity for rapamycin-resistant yeast transformed with FKBP12.6 and the RyR2 fragment treated with the indicated concentrations of rapamycin which competes FKBP12.6 off from RyR2. The FKBP12/12.6 binding site is defined by isoleucine 2427 and proline 2428 (arrow). Sequences of FKBP12 binding sites in RyR1, IP3R1, IP3R2, and TβRI are shown in the box.

(B) GST-RyR2 fusion proteins bound to Sepharose beads were incubated with cardiac SR (200 μg), pelleted, size fractionated by SDS-PAGE, and immunoblotted with the indicated antibodies. Lane 1, positive control (recombinant proteins); lane 2, Sepharose beads (negative control); lane 3, GST (negative control); lane 4, GST-RyR2–1–334 (amino acid residues 1–334); lane 5, GST-RyR2–513–808; lane 6, GST-RyR2–1027–1304; lane 7, GST-RyR2–1251–1500; lane 8, GST-RyR2–1451–1768.

(C) Immunohistochemistry showing colocalization of mAKAP and RyR2 to cardiac SR in normal and failing human hearts. Bars: long, 1.5 mm; short, 5 mm.

(D) In vitro kinasing reactions using GST-RyR2 fusion proteins containing the wild-type (wt) and mutant (S2809A) PKA site. PKA phosphorylation was performed with [γ^{32}P]ATP followed by size-fractionation on SDS-PAGE and autoradiography.

contain leucine/isoleucine zippers. Immunohistochemistry showed that mAKAP is present in the cardiac SR, the same cellular location as RyR2, in both normal and failing human hearts (Figure 3C). Wild-type and mutant GST-RyR2 fusion proteins were used to determine the site of PKA phosphorylation (serine 2809) (Figure 3D).

PKA Phosphorylation of RyR2 Inhibits FKBP12.6 Binding

PKA phosphorylation of immunoprecipitated RyR2 (there was no PKA phosphorylation of FKBP12.6) resulted in a decrease ($\sim$90 $\pm$ 9% reduction, $n = 8$, $p < 0.001$) in the amount of FKBP12.6 coimmunoprecipitating with RyR2 (Figure 4A). No dissociation of FKBP12.6 from RyR2 was observed in the following negative controls: (1) including PKI in the reaction, (2) boiling the PKA, or (3) omitting ATP. Neither Ca^{2+} calmodulin kinase (CaMKII) nor protein kinase C (PKC) caused the dissociation of FKBP12.6 from RyR2, indicating that the PKA phosphorylation-induced dissociation of FKBP12.6 from RyR2 is specific (Figure 4A). Furthermore, less FKBP12.6 coimmunoprecipitated with RyR2 from failing hearts compared to normal hearts in both humans (65% $\pm$ 11% reduction, $n = 4$, $p < 0.005$) and in dogs with pacing-induced heart failure (50% $\pm$ 8% reduction, $n = 3$, $p < 0.005$) (Figure 4B). Total FKBP12.6 levels in homogenates from normal and failing hearts were the same (data not shown). These data show that PKA phosphorylation of RyR2 provides a mechanism for the physiologic and pathophysiologic regulation of FKBP12.6 binding to RyR2.

RyR2 PKA Phosphorylation Increases P_o and Induces Subconductance States

We have previously shown that dissociation of FKBP12/12.6 from RyR1 or RyR2 increases the channel P_o by shifting the Ca^{2+} dependence for activation to the left (Brillantes et al., 1994; Kaftan et al., 1996). A second effect of dissociation of FKBP12/12.6 from the channels is to induce subconductance states consistent with a destabilization of the tetrameric channel structure (Brillantes et al., 1994). PKA phosphorylation of RyR2 in planar lipid bilayers resulted in a significant increase in P_o from 0.10 $\pm$ 0.03 to 0.35 $\pm$ 0.06 ($n = 4/4$, $p < 0.001$) (e.g., Figure 5A). PKA phosphorylation of RyR2 also induced subconductance states ($n = 4$) (e.g., Figure 5B)

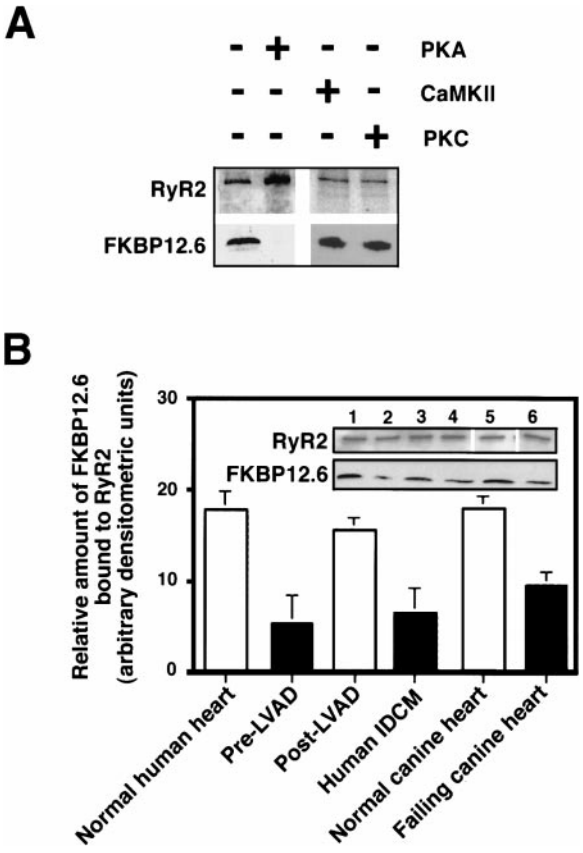

Figure 4. PKA Phosphorylation of RyR2 Inhibits FKBP12.6 Binding

(A) FKBP12.6 was coimmunoprecipitated from cardiac SR using an anti-RyR antibody followed by immunoblotting with either anti-RyR (top panel) or anti-FKBP (bottom panel) antibodies. The indicated immunoprecipitates were phosphorylated with PKA prior to size-fractionation by SDS-PAGE. Coimmunoprecipitation of FKBP12.6 with RyR2 was significantly reduced in the PKA phosphorylated samples but not in CaMKII or PKC phosphorylated RyR2 samples. (B) Quantitation of FKBP12.6 coimmunoprecipitating with RyR2 from the indicated samples. Normal human heart; pre-LVAD, left ventricular sample taken from a human heart with end-stage failure during insertion of a left ventricular assist device; Post-LVAD, sample from the same human heart after LVAD treatment; IDCM, end-stage failing human heart with idiopathic dilated cardiomyopathy; normal nonfailing canine heart; canine rapid pacing-induced heart failure model. Inset shows representative coimmunoprecipitations of RyR2 and FKBP12.6 using an anti-RyR antibody: lane 1, normal human heart; lane 2, pre-LVAD; lane 3, post-LVAD; lane 4, human IDCM; lane 5, normal canine heart; lane 6, failing canine heart. There was significantly less FKBP12.6 coimmunoprecipitated with RyR2 in each of the failing hearts compared to normals. Data shown are representative of three or more similar experiments. FKBP12.6 amounts were quantified using densitometry.

similar to those seen after dissociation of FKBP from the RyR channels (Brillantes et al., 1994; Kaftan et al., 1996; Marx et al., 1998). PKA phosphorylation of RyR2 channels did not alter the mean open time (τ_o) of the full conductance state (control channels $\tau_o = 2.1 \pm 0.8$ ms vs. 2.6 ± 0.6 ms following PKA, n = 4, p = NS). However, long-lasting subconductance states ($\tau_o = 502.1 \pm 40.8$ ms) were observed following PKA phosphorylation of RyR2 channels in the bilayer (Figure 5B). The phosphatase inhibitor okadaic acid (1 μM) increased RyR2 P_o from 0.3 ± 0.1 to 0.8 ± 0.1 (n = 5/6, $p < 0.001$). These data suggest that PKA phosphorylation activates RyR2 by dissociating FKBP12.6 from the channel resulting in increased sensitivity to Ca^{2+}-induced activation.

Heart Failure and PKA Hyperphosphorylation Produce the Same RyR2 Defects

Single channel recordings of RyR2 channels from human (n = 21, 13 channels from 3 patients with heart failure including 3 channels from pre-LVAD treatment heart samples, 4 channels from nonfailing hearts, and 4 channels from hearts post-LVAD treatment) and canine (n = 27, 14 channels from 2 dogs with pacing-induced heart failure and 13 channels from nonfailing hearts) hearts revealed that the RyR2 channels from failing hearts exhibited the same alterations in single channel properties (Figures 6A and 6B) as the PKA phosphorylated channels (Figure 5B). RyR2 channels from failing hearts exhibited an increased P_o at low *cis* (cytosolic) [Ca^{2+}] (50 nM, 0.24 ± 0.21 versus 0.002 ± 0.001, n = 27 failing hearts, n = 21 nonfailing and post-LVAD hearts, $p < 0.0001$). At 50 nM *cis* [Ca^{2+}], 70% of the RyR2 channels from failing hearts (19/27) exhibited increased P_o ($P_o > 0$) compared with 9.5% (2/21) channels from nonfailing hearts. Moreover, there were two types of behavior exhibited by RyR2 channels that were active at 50 nM *cis* [Ca^{2+}]. 56% of RyR2 channels from failing hearts exhibited low levels of activity (n = 15/27, $P_o \approx 0.03$), whereas channels from normal hearts are almost always completely inactive at 50 nM *cis* [Ca^{2+}] (Figure 6B). Strikingly, 15% of the RyR2 channels from failing hearts (n = 4/27) exhibited a second type of behavior that was never observed in channels from normal hearts: a long-lasting subconductance state at 50 nM *cis* [Ca^{2+}] with $P_o \approx 1.0$ (Figure 6B) similar to those observed following PKA phosphorylation of RyR2 channels in the bilayer (e.g., see Figure 5B). These subconductance states had markedly increased open times ($\tau_o = 802.1 \pm 66.7$ ms) compared to the RyR2 channels from nonfailing hearts ($\tau_o = 2.2 \pm 0.7$ ms) (e.g., see Figure 6B). RyR2 channels that are active at 50 nM cytosolic [Ca^{2+}] would be expected to be open throughout the cardiac cycle (in systole and diastole).

In 52% of RyR2 channels from failing hearts, subconductance states were observed (n = 14/27) that were present in less than 5% of channels from normal hearts (n = 1/21, $p < 0.001$) (e.g., see Figure 6A). The subconductance states are similar to those observed when RyR1 channels are expressed without FKBP12 (Brillantes et al., 1994) or when FKBP12.6 is dissociated from native RyR2 channels (Kaftan et al., 1996) and in PKA phosphorylated channels (Figure 5A). As noted above, channels from failing hearts also exhibited increased PKA phosphorylation (Figures 2A and 2B) and reduced FKBP12.6 binding (Figure 4B). These data suggest that increased PKA phosphorylation of RyR2 in failing hearts results in dissociation of FKBP12.6, which causes defects in the single channel properties characterized by subconductance states and increased P_o consistent with destabilized channels and altered Ca^{2+} sensitivity (Brillantes et al., 1994).

β-Adrenergic Agonist Response Restored by LVAD Treatment

Physiologic levels of PKA phosphorylation of RyR2 would increase SR Ca^{2+} release resulting in increased

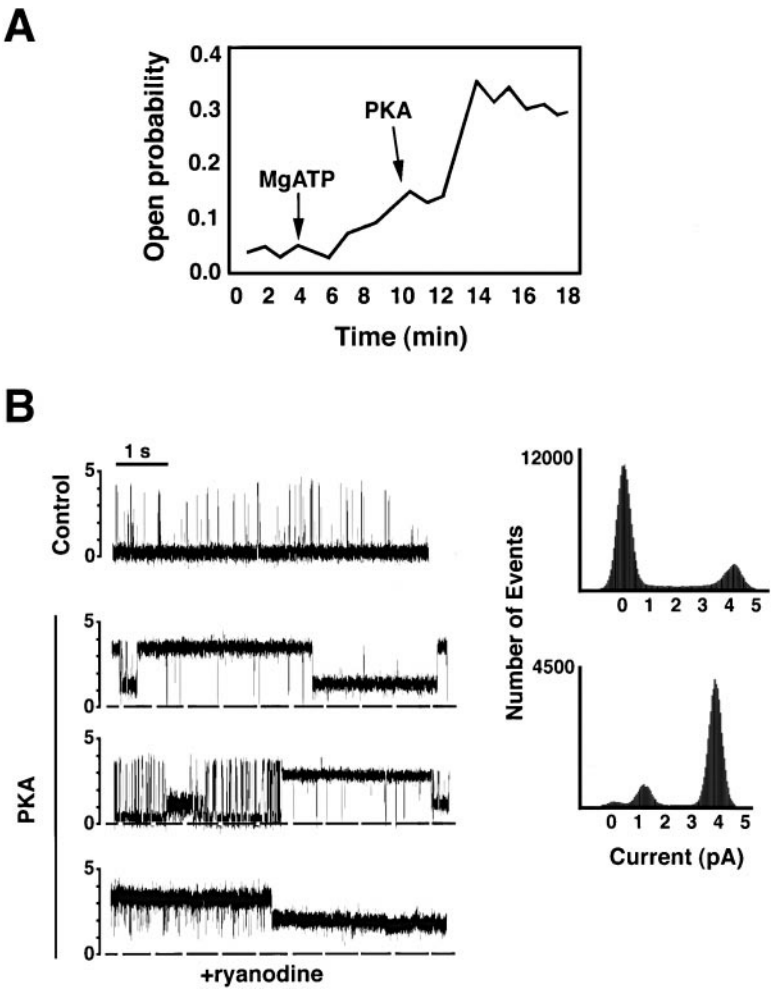

Figure 5. PKA Phosphorylation Activates RyR2 and Induces Subconductance States

(A) Open probability (P_o) of a single RyR2 channel plotted as a function of time showing the effect of MgATP (2 mM) followed by addition of PKA (2 units).

(B) Single channel tracings corresponding to the experiment shown in (A). Channel openings are in the upward direction; the current amplitude for a fully open channel under these conditions (Ba^{2+} as current carrier) was ~4 pA. Increased P_o and multiple subconductance states are seen after PKA addition. Corresponding amplitude histograms are shown at the right of the tracings for channels before and after PKA treatment. The subconductance states have current amplitudes of 1, 2, or 3 pA, corresponding to 1/4, 1/2, and 3/4 of the full conductance of the channel as previously described for channels in the absence of FKBP12 (Brillantes et al., 1994; Marx et al., 1998). The bottom tracing shows the characteristic modification of the RyR2 channels by ryanodine (1 μM). Recordings were at 0 mV potential across the lipid bilayer membrane; the dashed lines indicate the closed state of the channels. Data shown are representative of four experiments using SR microsomes containing RyR2 isolated from two different dogs (two separate isolations for each animal). Similar results were obtained using RyR2 channels isolated from normal human heart.

cardiac muscle contractility that explains, at least in part, the inotropic effects of β-adrenergic agonists. The blunting of the β-adrenergic agonist-induced increase in cardiac contractility in failing hearts has been attributed to the downregulation and desensitization of β-adrenergic receptors in failing hearts (Bristow et al., 1992).

The blunted response to β-adrenergic agonists may in part be explained by the fact that in failing heart muscle, RyR2 channels are already hyperphosphorylated (Figures 2A and 2B) and further PKA phosphorylation of RyR2 cannot occur. Interventions that decrease PKA phosphorylation of RyR2 back toward the levels observed in nonfailing hearts should restore β-adrenergic-agonist-induced increases in cardiac contractility. To test this hypothesis, we used muscle strips isolated from pre- and post-LVAD hearts placed in organ baths under conditions such that isoproterenol-induced contraction could be determined. Compared to normal hearts, the pre-LVAD (failing) muscle strips exhibited a blunted response to isoproterenol (Figure 6C) that was significantly restored following LVAD treatment. LVAD treatment restores PKA phosphorylation of RyR2 to normal levels (Figures 2A and 2B) and reverses the defect in single channel properties of RyR2/Ca^{2+} release channels (data not shown). These data suggest that restoration of sensitivity to β-adrenergic agonists observed in the post-LVAD muscle may be explained in part by the increased availability of these RyR2 channels to be physiologically PKA phosphorylated.

Discussion

This study demonstrates that PKA phosphorylation regulates FKBP12.6 binding to RyR2, providing a mechanism for modulating the SR Ca^{2+} release channel required for EC coupling. PKA hyperphosphorylation of RyR2 in failing hearts resulted in the following abnormal single channel properties: (1) increased Ca^{2+} sensitivity for activation and (2) elevated channel activity (P_o) associated with destabilization of the tetrameric channel complex (manifested as subconductance states including long-lasting partially open states never observed in channels from nonfailing hearts). Cosedimentation and coimmunoprecipitation studies were used to define an RyR2 channel macromolecular complex that includes FKBP12.6, PKA, RII, PP1 and PP2A, and mAKAP, suggesting that phosphorylation of the channel is locally controlled (Figure 7).

FKBP12.6 and FKBP12 are integral components of the cardiac muscle RyR2 and skeletal muscle RyR1 SR Ca^{2+} release channels, respectively (Jayaraman et al., 1992; Marks, 1996), required for normal channel gating (Brillantes et al., 1994; Kaftan et al., 1996; Marx et al., 1998). Dissociation of FKBP12/12.6 from RyR1 or RyR2

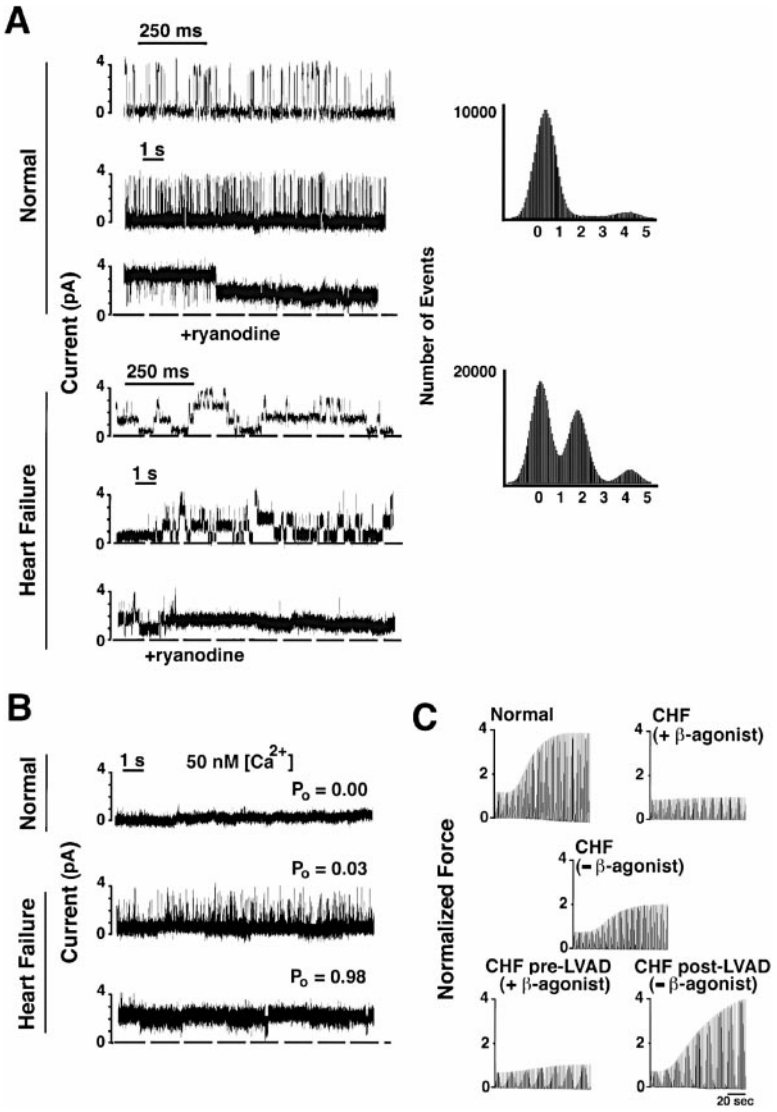

Figure 6. Defective RyR2 Channels and Contractility in Failing Heart Muscle

(A) Single channel tracings of RyR2 from: normal canine heart (top three tracings); failing canine heart (bottom three tracings). Corresponding amplitude histograms are at right. The bottom tracing in each set of three shows the characteristic modification of the RyR2 channels by ryanodine (1 μM). Recordings were at 0 mV; the dashed lines indicate the closed state of the channels. Similar results were obtained using RyR2 channels isolated from failing human hearts (see text for details).

(B) RyR2 channels from failing canine hearts exhibited increased sensitivity to Ca^{2+}-dependent activation compared to channels from normal hearts, which were generally inactive at ≤50 nM free Ca^{2+} in the *cis* (cytoplasmic) chamber (top tracing). RyR2 channels from failing hearts exhibited two types of Ca^{2+}-dependent activation at ≤50 nM free Ca^{2+}. Some channels from failing hearts ($n = 15$) were active with a low P_o (second tracing) at ≤50 nM free Ca^{2+}, while others ($n = 4$) were extremely active at ≤50 nM free Ca^{2+}, remaining stably open in a subconductance state (bottom tracing). Similar results were obtained using RyR2 channels isolated from failing human hearts.

(C) Continuous force tracings from human left ventricular trabeculae during exposure to isoproterenol (4 μM). Normal heart sample showed >3-fold increase in force following isoproterenol. Muscles from cardiomyopathic hearts showed either no response in patients receiving β-agonists prior to transplant or a blunted response (~2-fold increase) in patients not receiving β-agonists prior to transplant. Muscle obtained from the apical core tissue of an LVAD recipient receiving β-agonist prior to surgery showed almost no response to isoproterenol. However, muscle obtained from the same patient after 64 days of LVAD support shows a >5-fold increase in response to isoproterenol ($n = 3$, $p < 0.01$).

results in three defects in channel function: (1) subconductance states equal to 1/4, 1/2, and 3/4 of the fully open channel, (2) increased P_o, and (3) increased sensitivity to Ca^{2+}-dependent activation (Brillantes et al., 1994; Kaftan et al., 1996; Marx et al., 1998). The increased P_o following removal of FKBP12/12.6 is explained by the increased sensitivity to Ca^{2+}-dependent activation (Brillantes et al., 1994) due to a shift to the left of the ascending portion of the bell-shaped curve describing the Ca^{2+} dependence of the RyR channels (Bezprozvanny et al., 1991). Increased P_o at low cytosolic Ca^{2+} (e.g., 50 nM [Ca^{2+}]) (see Figure 6B) would result in inappropriately active SR Ca^{2+} release channels and depletion of SR Ca^{2+} that might impair systolic function of the heart (by diminishing the Ca^{2+} signal that activates muscle contraction). Inappropriate SR Ca^{2+} release channel activation at low cytosolic Ca^{2+} might also contribute to early and delayed afterdepolarizations that trigger fatal cardiac arrhythmias and cause sudden cardiac death (Fozzard, 1992).

Alterations in RyR2 single channel function induced by PKA phosphorylation correspond to those observed when FKBP12.6 is dissociated from the channel (Figures 5A and 5B). In agreement with our findings, it has been reported that PKA phosphorylation of RyR2 increases the activity of the channel (Hain et al., 1995; Valdivia et al., 1995). RyR2 channels isolated from failing hearts were PKA hyperphosphorylated (Figures 2A and 2B) and exhibited the same alterations in function observed in in vitro PKA phosphorylated channels (Figures 6A and 6B). These data show that PKA hyperphosphorylation of RyR2 in failing hearts causes a defect in channel function due to the dissociation of the regulatory subunit FKBP12.6. Treatment of heart failure with an LVAD that improves heart function was associated with a decrease in RyR2 PKA phosphorylation to levels observed in normal human hearts (Figures 2A and 2B). In addition, LVAD treatment resulted in normalized RyR2 single channel function (i.e., reduction in subconductance states, normalization of the Ca^{2+} sensitivity for activation, and decreased P_o).

An additional effect of dissociation of FKBP12 from RyR1 is to uncouple gating of neighboring channels (Marx et al., 1998). We have recently found that

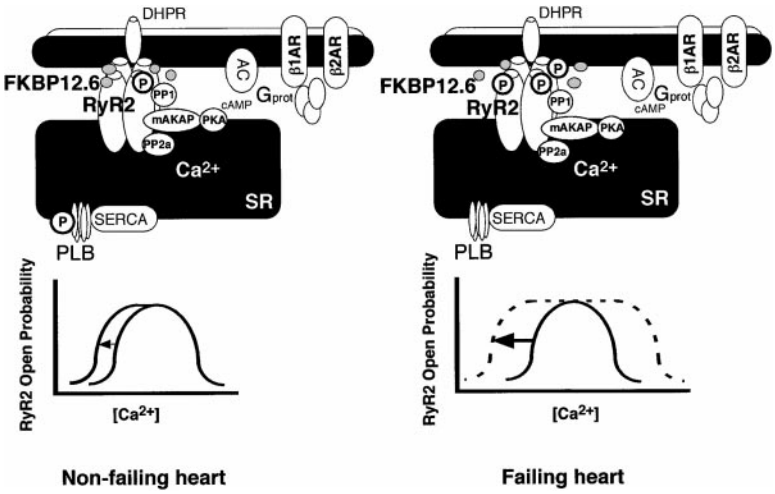

Figure 7. Model of the Effects of PKA Phosphorylation of RyR2 in the Heart

In the nonfailing heart (left panel) β-agonists bind to receptors (β1 and β2 adrenergic receptors, AR) coupled to heterotrimeric G-proteins (Gprot), which in turn activate adenylyl cyclase (AC), raising cyclic AMP (cAMP) levels and activating PKA. In this model, PKA phosphorylation of RyR2 induces dissociation of one FKBP12.6 from the channel, shifting the Ca^{2+} dependence for activation to the left, increasing the sensitivity of the RyR2 to activation by Ca^{2+} influx via the voltage-gated Ca^{2+} channel in the T tubule (dihydropyridine receptor, DHPR), and increasing RyR2 channel P_o. The result is increased SR Ca^{2+} release and cardiac contractility. The tetrameric RyR2 channel is part of a macromolecular signaling complex that includes four molecules each of RyR2, FKBP12.6, PKA, PP1 and PP2A, and mAKAP (all components of the macromolecular complex are shown for only one of the four RyR2 subunits). Ca^{2+} reuptake into the SR occurs via the SR Ca^{2+}-ATPase (SERCA) and its associated regulatory protein phospholamban (PLB).

In failing hearts (right panel), PKA hyperphosphorylation of RyR2 may contribute to the blunted response to β-agonists observed in failing heart muscle because the channels cannot be further PKA phosphorylated. RyR2 channels in failing hearts exhibit a shift in the Ca^{2+}-dependence for activation such that they are activated at resting levels of cytosolic Ca^{2+}.

FKBP12.6 is required for coupled gating between RyR2 channels (S. O. M. et al., submitted). Coupled gating provides a mechanism whereby all of the RyR2 channels in a T tubule/SR junction can be uniformly activated, resulting in an optimal Ca^{2+} signal to trigger cardiac muscle contraction. One consequence of uncoupling RyR2 channels would be a loss of EC coupling gain, which has been observed experimentally in cardiomyopathic hearts (Gomez et al., 1997).

In the present study, we show that mAKAP, which has been localized to cardiac SR as well as the perinuclear region (McCartney et al., 1995; Yang et al., 1998), cosediments and coimmunoprecipitates with RyR2. mAKAP could bind directly to RyR2, similar to yotia, which binds directly to the NMDA receptor (Westphal et al., 1999) or via an adaptor. The PKA regulatory subunit RII binds directly to AKAPs (Fraser and Scott, 1999) and anchors the PKA catalytic subunit. PP1 and PP2A may interact with RyR2 directly or via their own regulatory/targeting proteins, possibly by binding to leucine/isoleucine zippers present in RyR2.

β-adrenergic signaling cascade components (the stimulatory G protein Gs and adenylyl cyclase) have been localized to the transverse tubular network in rat ventricular myocytes (Laflamme and Becker, 1999). Thus, one important consequence of anchoring PKA, RII, PP1, and PP2A to the RyR2 complex and localizing upstream components of the β-adrenergic signaling cascade to the T tubule–SR junction, is that phosphorylation/dephosphorylation of RyR2 can be regulated locally at the site of EC coupling. The stoichiometry of PKA back-phosphorylation for the channels from failing hearts was 0.7 (compared to 3.8 for fully dephosphorylated channels and 3.1 for RyR2 from nonfailing hearts), indicating that approximately 3–4 PKA sites on RyR2 were phosphorylated in failing hearts compared to <1 on RyR2 from nonfailing hearts. RyR2 PKA hyperphosphorylation explains the ~60% decrease in the amount of FKBP12.6 bound to the RyR2 channels from failing hearts compared to channels from normal hearts (Figure 4B). This decrease in FKBP12.6 binding to RyR2 channels may account for the ~70% of RyR2 channels from failing hearts that exhibited altered single channel properties similar to those observed when FKBP12.6 is competed off from the channel using rapamycin or FK506 (Brillantes et al., 1994; Kaftan et al., 1996; Marx et al., 1998). Moreover, 15% of channels from failing hearts exhibited the most severe defect (long-lasting subconductance states with $P_o \approx 1$ at 50 nM cytosolic [Ca^{2+}]) (e.g., Figure 6B) suggesting that these channels have <1 FKBP12.6 bound.

Heart failure is the leading cause of mortality and morbidity in the United States, accounting for ~400,000 deaths annually with ~50% of these deaths caused by disturbances in the cardiac rhythm referred to as sudden cardiac death (SCD). A common feature of human heart failure and of many animal models of heart failure is that a hyperadrenergic state and elevated levels of circulating catecholamines are a marker for increased mortality in heart failure patients (Cohn et al., 1984).

Downregulation of β-adrenergic receptors in failing heart muscle and desensitization of these receptors attributable to uncoupling from their downstream signaling molecules, G proteins, have been documented (Bristow et al., 1982), yet β-adrenergic blockers have proven to be one of the most important treatments for heart failure (CIBIS-II, 1999; MERIT-HF, 1999). cAMP levels and PKA activity were unchanged in failing human hearts (Regitz-Zagrosek et al., 1994; Kirchhefer et al., 1999) or cAMP levels were reduced but PKA activity unchanged (Bohm et al., 1994). β-adrenergic blockade has been viewed as counterintuitive in heart failure therapy since the adrenergic system may be downregulated and negative inotropic drugs are considered potentially dangerous to patients. Therefore, a mechanistic understanding of the molecular basis for the therapeutic benefit afforded by β-adrenergic blockers in heart failure would be an important advance.

In the present study, we show that the SR Ca^{2+} release channel RyR2 is unexpectedly PKA hyperphosphorylated in failing hearts. These data raise the concept that local signaling may increase rather than decrease phosphorylation of PKA substrates in cardiomyocytes from failing hearts.

One explanation for the surprising finding of PKA hyperphosphorylation of RyR2 is that targeting of phosphatases to RyR2 may be downregulated in failing hearts. Indeed, we found that the levels of PP1 and PP2A associated with RyR2 were significantly decreased in failing hearts (Figures 2C and 2D). Cellular PP1 levels are increased in failing hearts (Neumann et al., 1997); thus, the decrease in RyR2-associated PP1 must be due to a specific decrease in PP1 association with RyR2 that cannot be explained by a generalized decrease in PP1 levels in the heart.

Defects in Ca^{2+} regulation that could explain the decreased contractility observed in failing hearts, including a reduced amplitude and slowed decay of the Ca^{2+} transient, have been described (Morgan et al., 1990; Beuckelmann et al., 1992). The release and reuptake of Ca^{2+} from the SR controls the force of contraction during systole in the heart. SR Ca^{2+} release occurs via activation of RyR2, and Ca^{2+} reuptake occurs via the SR Ca^{2+}-ATPase which in turn is regulated by phospholamban. PKA has multiple substrates in cardiomyocytes including phospholamban, the L-type Ca^{2+} channel on the sarcolemma and components of the contractile apparatus. It has been appreciated for some time that β-adrenergic agonists can modulate the activity of molecules involved in regulating cardiac contractility. Clearly, a disease as complex as heart failure involves an interplay between a number of molecules and signaling pathways that contribute to the regulation of Ca^{2+} homeostasis. One key point distinguishing the present study is the identification of a functional defect in a Ca^{2+} handling molecule that occurs not only in animal models (e.g., the paced dog model) but also in human failing hearts and is reversed by treatment of the heart failure (e.g., with an LVAD) in humans.

The present study provides a novel mechanism for modulating RyR2 channel function by physiologically controlling the binding of FKBP12.6 to the channel via PKA phosphorylation. Furthermore, the finding of PKA hyperphosphorylated channels with defective function in failing hearts provides a mechanism for cardiac dysfunction in heart failure.

Experimental Procedures

Immunoprecipitation and Back-Phosphorylation of RyR2

Cardiac SR (200 μg) or homogenates (500 μg), prepared from ventricular tissue as described (Kaftan et al., 1996), were suspended in 0.5 ml of buffer (50 mM Tris-HCl [pH 7.4], 0.9% NaCl, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.25% Triton X100, and protease inhibitors) and incubated with the indicated antibody O/N at 4°C (IgG alone was used as a negative control for each antibody, data not shown). Protein A Sepharose beads were added, incubated at 4°C for 1 hr, washed with 1× phosphorylation buffer (8 mM MgCl, 10 mM EGTA, 50 mM Tris/piperazine-N,N′-bis[2-ethanesulfonic acid] [pH 6.8]), and resuspended in 10 μl of 1.5× phosphorylation buffer containing either vehicle alone, PKA catalytic subunit (Sigma, St. Louis, MO), PKA plus PKA inhibitor (PKI_{5-24}, 500 nM, Calbiochem, San Diego, CA), or cAMP as indicated. Back-phosphorylation of immunoprecipitated RyR2 was initiated with PKA (5 units) and MgATP (33 μM) and terminated after incubation for 5 min at RT with 5 μl of stop solution (4% SDS and 0.25 M DTT). For RyR2 phosphorylation with PKC (0.05 units, Calbiochem), 1.5 mM $CaCl_2$ was added, and for phosporylation with CaMKII (0.2 mg, Upstate Biotech, Lake Placid, NY), 1.5 mM $CaCl_2$ and 5 μM calmodulin were added. In some experiments, the ATP contained 10% [γ^{32}P]ATP (NEN Life Sciences, Boston, MA). Samples were heated to 95°C, size-fractionated on 6% SDS-PAGE, and RyR2 radioactivity was quantified using a Molecular Dynamics Phosphorimager and ImageQuant software (Amersham Pharmacia Biotech, Piscataway, NJ). Nonspecific phosphorylation (not inhibited by PKA inhibitor) was subtracted, the resulting value was divided by the amount of RyR2 protein (determined by immunoblotting and densitometry or [^{3}H] ryanodine binding) and expressed as the inverse of the PKA-dependent [γ^{32}P]ATP signal. Microcystin-Sepharose (35 μl, UBI) was used to isolate RyR2 from 200 μg of cardiac SR by incubating at 4°C for 1 hr followed by washing. Beads were resuspended in 6× SDS loading buffer and boiled, and the supernatant was size-fractionated on SDS-PAGE. PP1 and PP2A bound to the microcystin-Sepharose beads were competed off with free microcystin-LR (Calbiochem). Maximum PKA-dependent phosphorylation was determined using RyR2 pretreated with alkaline phosphatase (AP, 1:100 enzyme:protein, New England Biolabs) for 20 min at 37°C. The stoichiometry of RyR2 PKA phosphorylation was determined using [γ^{32}P]ATP standards. The ^{32}P/RyR2 ratio was calculated by dividing ^{32}P-phosphorylation by the amount of high-affinity [^{3}H]ryanodine binding (one high-affinity ryanodine binding site per RyR2).

Immunoblots

Immunoblots were performed as described (Moschella and Marks, 1993) using: anti-FKBP12 (1:1000), anti-RyR (5029, 1:3000) (Jayaraman et al., 1992), anti-PP1 (1:1000), anti-PP2A (1:1000), anti-CnA (1:1000), anti-PKA catalytic subunit (1:1000, Transduction Labs, Lexington, KY), anti-phosphoserine (1 μg/ml, Zymed San Francisco, CA), and anti-mAKAP (VO56) (Kapiloff et al., 1999). After washing, membranes were incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antiserum (1:3000, Boehringer-Mannheim) for 60 min at RT, washed three times with TBS and 0.1% Tween 20, and developed using enhanced chemiluminescence (ECL, Amersham).

Yeast Two-Hybrid Assay to Identify the FKBP12.6 Binding Site

Human FKBP12.6 cDNA was cloned into the yeast two-hybrid vector pEG202 (OriGene Technologies, Rockville, MD) to make pEGFKBP12.6 (FKBP12.6 fused to the GAL4 DNA binding domain); human RyR2 cDNA fragments cloned into pJG4–5 (OriGene) were confirmed by sequencing. The yeast two-hybrid assay was performed using the DupLEX-A yeast system (OriGene). pEGFKBP12.6 and pAD-GAL4RyR2/2361–2496 were cotransformed into the rapamycin resistant mutant yeast strain Y663 (Lorenz and Heitman, 1995) and liquid β-galactosidase assays were performed in the absence or presence of rapamycin (0.1, 1.0, and 10 μM) which competes with RyR2 for binding to FKBP12.6.

Site-Directed Mutagenesis and In Vitro Mapping

pGST-RyR2 constructs were generated using rabbit or human RyR2 cDNA, and fusion proteins were expressed and purified with glutathione Sepharose beads. Site-directed mutagenesis was performed using the 5′-3′ site-directed mutagenesis kit (Amersham Pharmacia Biotech). pGST-RyR2 fusion proteins bound to Sepharose beads were incubated with canine cardiac SR (200 μg), pelleted, washed with modified RIPA buffer, size-fractionated on SDS-PAGE, and immunoblotted with the indicated antibodies.

Immunohistochemistry

Human cardiac tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (4 μM) were dried O/N at 37°C, dewaxed, rehydrated, incubated with PBS + 0.2% Tween-20 for 5 min, then incubated with 5% goat serum in PBS for 1 hr at RT. Sections were then incubated with preimmune rabbit serum (IgG) or primary antibody [mAKAP (VO56), RyR2 (monoclonal, Affinity Bioreagents); 1:50] in PBS + 3% BSA O/N at 4°C, washed in PBS, then

incubated with either FITC or rhodamine secondary antibody (1:100; Zymed) for 1 hr at RT, washed with PBS, stained with Hoechst dye (10 mg/ml) for 5 min, and washed with PBS. For double immunostaining, slides were sequentially stained with 2 individual 1° antibodies followed by simultaneous incubation with 2° antibodies. Slides were examined using a Nikon microscope (100× objective) and images were acquired with a SPOT RT camera (Diagnostic Instruments Inc) using Adobe Photoshop.

Single Channel Recordings

Single channel recordings of RyR2 were performed and analyzed as described (Brillantes et al., 1994). At the conclusion of each experiment, ryanodine and/or ruthenium red were applied to confirm the identity of channels as ryanodine receptors. Results are presented as mean ± standard deviation; the Student's t test was used for statistical analyses.

Human Heart Samples

The use of human tissues was approved by the Institutional Review Board of Columbia-Presbyterian Medical Center. Normal and failing human heart tissues were obtained as previously described from patients undergoing cardiac transplant (Go et al., 1995). LVADs (Thermo Cardiosystems Inc., Woburn, MA) were implanted in patients as a bridge to heart transplantation according to standard clinical practice (Frazier, 1994).

Muscle Strip Function

Trabeculae (diameters <1 mm, lengths >3 mm) from human left ventricular apical core samples obtained at the time of LVAD implantation or from hearts explanted at the time of orthotopic cardiac transplantation were placed in a standard muscle bath, attached to a force transducer, stimulated at 1 Hz, and left to equilibrate for 1 hr prior to study at slack length, then stretched progressively to the point of maximal tension development (L_{max}). β-adrenergic response was tested by superfusing the muscle with Krebs-Ringer solution containing isoproterenol (4 μM).

Canine Heart Failure Model

Canine heart failure was induced by rapid cardiac pacing as described previously (Wang et al., 1997). Severe heart failure was evidenced by an ~40% reduction in LV dP/dt_{max} (to ~1800 mm Hg/s), 20% reductions in peak LV and mean aortic pressure (to 100 and 85 mm Hg, respectively), 50% increase in resting heart rate (to 132 beats/min), and a rise in end-diastolic pressure to >20 mm Hg (Wang et al., 1997). All procedures were approved by the Institutional Animal Care Committee.

Acknowledgments

We thank R. Axel, R. Kass, and Q. Al-Awqati for helpful discussions, J. D'Armiento, K. Imai, L. Tsao, and J. Fallon for assistance with immunohistochemistry, J. Heitman for rapamycin resistant yeast, A. Barbone, J. Madigan, J. Holmes, and J. Wang for assistance with measurements of heart muscle force and obtaining human and canine heart samples, and M. Kapiloff for anti-mAKAP. This work was supported by grants to A. R. M. from the NIH (RO1 HL61503, RO1 HL56180, RO1 AI39794, RO3 TW00949), the AHA (A. R. M., S. O. M. and T. J.), the Whitaker Foundation (S. R.), and the Richard and Lynne Kaiser Family Foundation.

Received November 19, 1999; revised March 21, 2000.

References

Beuckelmann, D., Nabauer, M., and Erdmann, E. (1992). Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. Circulation *85*, 1046–1055.

Bezprozvanny, I., Watras, J., and Ehrlich, B. (1991). Bell-shaped calcium response curves of Ins(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature *351*, 751–754.

Bohm, M., Reiger, B., Schwinger, R.H., and Erdmann, E. (1994). cAMP concentrations, cAMP dependent protein kinase activity, and phospholamban in non-failing and failing myocardium. Cardiovasc. Res. *28*, 1713–1719.

Brillantes, A.B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M.C., Jayaraman, T., Landers, M., Ehrlich, B.E., and Marks, A.R. (1994). Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. Cell *77*, 513–523.

Bristow, M.R., Ginsburg, R., Minobe, W., Cubicciotti, R.S., Sageman, W.S., Lurie, K., Billingham, M.E., Harrison, D.C., and Stinson, E.B. (1982). Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. N. Engl. J. Med. *307*, 205–211.

Bristow, M.R., Minobe, W., Rasmussen, R., Larrabee, P., Skerl, L., Klein, J.W., Anderson, F.L., Murray, J., Mestroni, L., Karwande, S.V., et al. (1992). Beta-adrenergic neuroeffector abnormalities in the failing human heart are produced by local rather than systemic mechanisms. J. Clin. Invest. *89*, 803–815.

Cameron, A.M., Nucifora, F.C., Jr., Fung, E.T., Livingston, D.J., Aldape, R.A., Ross, C.A., and Snyder, S.H. (1997). FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine- proline (1400–1401) and anchors calcineurin to this FK506-like domain. J. Biol. Chem. *272*, 27582–27588.

Chen, Y.G., Liu, F., and Massague, J. (1997). Mechanism of TGFbeta receptor inhibition by FKBP12. EMBO J. *16*, 3866–3876.

CIBIS-II (1999). The cardiac insufficiency bisoprolol study II (CIBIS-II): a randomised trial. Lancet *353*, 9–13.

Cohn, J.N., Levine, T.B., Olivari, M.T., Garberg, V., Lura, D., Francis, G.S., Simon, A.B., and Rector, T. (1984). Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N. Engl. J. Med. *311*, 819–823.

Fozzard, H.A. (1992). Afterdepolarizations and triggered activity. Basic Res. Cardiol. *87*, 105–113.

Fraser, I.D., and Scott, J.D. (1999). Modulation of ion channels: a "current" view of AKAPs. Neuron *23*, 423–426.

Frazier, O.H. (1994). First use of an untethered, vented electric left ventricular assist device for long-term support. Circulation *89*, 2908–2914.

Go, L.O., Moschella, M.C., Watras, J., Handa, K.K., Fyfe, B.S., and Marks, A.R. (1995). Differential regulation of two types of intracellular calcium release channels during end-stage heart failure. J. Clin. Invest. *95*, 888–894.

Gomez, A.M., Valdivia, H.H., Cheng, H., Lederer, M.R., Santana, L. F., Cannell, M.B., McCune, S.A., Altschuld, R.A., and Lederer, W.J. (1997). Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. Science *276*, 800–806.

Hain, J., Onoue, H., Mayrleitner, M., Fleischer, S., and Schindler, H. (1995). Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from cardiac muscle. J. Biol. Chem. *270*, 2074–2081.

Jayaraman, T., Brillantes, A.-M.B., Timerman, A.P., Erdjument-Bromage, H., Fleischer, S., Tempst, P., and Marks, A.R. (1992). FK506 binding protein associated with the calcium release channel (Ryanodine Receptor). J. Biol. Chem. *267*, 9474–9477.

Kaftan, E., Marks, A.R., and Ehrlich, B.E. (1996). Effects of rapamycin on ryanodine receptor/Ca^{2+}-release channels from cardiac muscle. Circ. Res. *78*, 990–997.

Kapiloff, M.S., Schillace, R.V., Westphal, A.M., and Scott, J.D. (1999). mAKAP: an A-kinase anchoring protein targeted to the nuclear membrane of differentiated myocytes. J. Cell Sci. *112*, 2725–2736.

Kirchhefer, U., Schmitz, W., Scholz, H., and Neumann, J. (1999). Activity of cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase in failing and nonfailing human hearts. Cardiovasc. Res. *42*, 254–261.

Laflamme, M.A., and Becker, P.L. (1999). Gs and adenylyl cyclase in transverse tubules of heart: implications for cAMP-dependent signaling. Am. J. Phys. *277*, H1841–H1848.

Levin, H.R., Oz, M.C., Chen, J.M., Packer, M., Rose, E.A., and Burkhoff, D. (1995). Reversal of chronic ventricular dilation in patients with end-stage cardiomyopathy by prolonged mechanical unloading. Circulation *91*, 2717–2720.

Lorenz, M.C., and Heitman, J. (1995). TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. J. Biol. Chem. *270*, 27531–27537.

MacDougall, L.K., Jones, L.R., and Cohen, P. (1991). Identification of the major protein phosphatases in mammalian cardiac muscle which dephosphorylate phospholamban. Eur. J. of Biochem. *196*, 725–734.

Marks, A.R. (1996). Cellular functions of immunophilins. Physiol. Rev. *76*, 631–649.

Marx, S.O., Ondrias, K., and Marks, A.R. (1998). Coupled gating between individual skeletal muscle Ca^{2+} release channels (ryanodine receptors). Science *281*, 818–821.

McCartney, S., Little, B.M., Langeberg, L.K., and Scott, J.D. (1995). Cloning and characterization of A-kinase anchor protein 100 (AKAP100). A protein that targets A-kinase to the sarcoplasmic reticulum. J. Biol. Chem. *270*, 9327–9333.

MERIT-HF (1999). Effect of metoprolol CR/XL in chronic heart failure: metoprolol CR/XL randomised intervention trial in congestive heart failure (MERIT-HF). Lancet *353*, 2001–2007.

Morgan, J., Erny, R., Allen, P., Grossman, W., and Gwathmey, J. (1990). Abnormal intracellular calcium handling: a major cause of systolic and diastolic dysfunction in ventricular myocardium from patients with end-stage heart failure. Circulation *81* (suppl III), 21–32.

Moschella, M.C., and Marks, A.R. (1993). Inositol 1,4,5-trisphosphate receptor expression in cardiac myocytes. J. Cell Biol. *120*, 1137–1146.

Neumann, J., Eschenhagen, T., Jones, L.R., Linck, B., Schmitz, W., Scholz, H., and Zimmermann, N. (1997). Increased expression of cardiac phosphatases in patients with end-stage heart failure. J. Mol. Cell. Cardiol. *29*, 265–272.

Regitz-Zagrosek, V., Hertrampf, R., Steffen, C., Hildebrandt, A., and Fleck, E. (1994). Myocardial cyclic AMP and norepinephrine content in human heart failure. Eur. Heart J. *15* (Suppl D), 7–13.

Valdivia, H.H., Kaplan, J.H., Ellis-Davies, G.C., and Lederer, W.J. (1995). Rapid adaptation of cardiac ryanodine receptors: modulation by Mg^{2+} and phosphorylation. Science *267*, 1997–2000.

Wang, J., Yi, G.H., Knecht, M., Cai, B.L., Poposkis, S., Packer, M., and Burkhoff, D. (1997). Physical training alters the pathogenesis of pacing-induced heart failure through endothelium-mediated mechanisms in awake dogs. Circulation *96*, 2683–2692.

Westphal, R.S., Tavalin, S.J., Lin, J.W., Alto, N.M., Fraser, I.D., Langeberg, L.K., Sheng, M., and Scott, J.D. (1999). Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. Science *285*, 93–96.

Yang, J., Drazba, J.A., Ferguson, D.G., and Bond, M. (1998). A-kinase anchoring protein 100 (AKAP100) is localized in multiple subcellular compartments in the adult rat heart. J. Cell Biol. *142*, 511–522.