Ca\(^{2+}\)/Calmodulin–Dependent Protein Kinase Modulates Cardiac Ryanodine Receptor Phosphorylation and Sarcoplasmic Reticulum Ca\(^{2+}\) Leak in Heart Failure

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Abstract—Abnormal release of Ca from sarcoplasmic reticulum (SR) via the cardiac ryanodine receptor (RyR2) may contribute to contractile dysfunction and arrhythmogenesis in heart failure (HF). We previously demonstrated decreased Ca transient amplitude and SR Ca load associated with increased Na/Ca exchanger expression and enhanced diastolic SR Ca leak in an arrhythmogenic rabbit model of nonischemic HF. Here we assessed expression and phosphorylation status of key Ca handling proteins and measured SR Ca leak in control and HF rabbit myocytes. With HF, expression of RyR2 and FK-506 binding protein 12.6 (FKBP12.6) were reduced, whereas inositol trisphosphate receptor (type 2) and Ca/calmodulin–dependent protein kinase II (CaMKII) expression were increased 50% to 100%. The RyR2 complex included more CaMKII (which was more activated) but less calmodulin, FKBP12.6, and phosphatases 1 and 2A. The RyR2 was more highly phosphorylated by both protein kinase A (PKA) and CaMKII. Total phospholamban phosphorylation was unaltered, although it was reduced at the PKA site and increased at the CaMKII site. SR Ca leak in intact HF myocytes (which is higher than in control) was reduced by inhibition of CaMKII but was unaltered by PKA inhibition. CaMKII inhibition also increased SR Ca content in HF myocytes. Our results suggest that CaMKII-dependent phosphorylation of RyR2 is involved in enhanced SR diastolic Ca leak and reduced SR Ca load in HF, and may thus contribute to arrhythmias and contractile dysfunction in HF. (Circ Res. 2005;97:1314-1322.)

Key Words: ryanodine receptor  ■  CaMKII  ■  phosphorylation  ■  heart failure  ■  arrhythmia

Contractile dysfunction in HF is caused by diminished sarcoplasmic reticulum (SR) Ca load that could arise from enhanced activity of Na/Ca exchange (NCX), reduced SR Ca ATPase (SERCA) function, and increased diastolic SR Ca leak via ryanodine receptors (RyR), all of which have been demonstrated to occur in our arrhythmogenic rabbit model of nonischemic HF. HF is also associated with a nearly 50% incidence of sudden cardiac death from ventricular tachycardia (VT) that degenerates to ventricular fibrillation (VF). In 3D cardiac mapping studies in our HF rabbit model, we showed that spontaneously occurring VT initiates by nonreentrant mechanisms associated with delayed afterdepolarizations. These arise from spontaneous SR Ca release that activates a transient inward current (i\(_{\text{f}}\)) carried primarily by NCX. Thus abnormal SR Ca release via RyR may contribute to both contractile dysfunction and arrhythmogenesis.

The cardiac RyR (RyR2) is the center of a large macromolecular protein complex that directly or indirectly interacts with RyR2 and modulates its function. The complex includes FK506 binding protein 12.6 (FKBP12.6), calmodulin (CaM), protein kinase A (PKA), Ca/CaM–dependent protein kinase (CaMKII), protein phosphatases PP1 and PP2A, mAKAP, and other associated proteins such as spinophilin, calcseques-trin, and sorcin. It was shown in HF that PKA mediates RyR2 hyperphosphorylation at the RyR2-Ser2809 site (that is maintained by decreased amount of associated phosphatases). This could cause dissociation of FKBP12.6, increased RyR Ca sensitivity, and higher RyR channel Ca flux that could increase SR Ca leak. However, not all studies agree that RyR2 is hyperphosphorylated in HF, that FKBP12.6 dissociates from RyR2 after PKA-dependent phosphorylation, or that phosphorylation of RyR by PKA alone enhances SR Ca leak. Thus, PKA-dependent RyR phosphorylation may not be the sole modulator of SR Ca handling in HF.

CaMKII is an important regulator of cardiac myocyte Ca homeostasis and shares common functional targets with PKA with respect to E–C coupling (eg, phospholamban [PLB], Ca current, RyR2), the predominant cardiac isoform, has a catalytic domain, a central regulatory domain (including autoinhibitory and CaM-binding regions), and an association domain. Ca–CaM binding activates CaMKII and subsequent autophosphorylation causes sustained Ca-independent kinase activity. CaMKII phosphorylates RyR2...
CaMKII expression is increased in human arrhythmias. CaMKII expression is increased in human arrhythmias.22–24 CaMKII expression is increased in human arrhythmias.22–24 CaMKII overexpression, it is unknown how CaMKII modulates SR Ca
release.15,23 However, in HF that is not induced by CaMKII overexpression, it is unknown how CaMKII modulates SR Ca handling (eg, activation state of CaMKII and CaMKII-dependent modulation of RyR2 and PLB function).

Here we investigated alterations in RyR expression and diastolic function and how RyR2 modulation by PKA and CaMKII may contribute to increased SR Ca leak. Our aims were to assess: (1) expression of RyR2 as well as the cardiac type 2 inositol 1,4,5 trisphosphate receptor (IP3R2); (2) expression of RyR2 interacting proteins including FKBP12.6, PP1, PP2A, CaM, and CaMKII; (3) RyR2 and PLB phosphorylation by PKA and CaMKII; (4) expression and phosphorylation state of CaMKII; and (5) the role of CaMKII and PKA on increased SR Ca leak in HF.

Materials and Methods
Arrhythmogenic Rabbit Nonischemic HF Model
New Zealand White rabbits underwent HF induction by aortic insufficiency followed by aortic constriction.1,2,7 Rabbit hearts were rapidly excised and left ventricular (LV) free wall was flash-frozen in liquid nitrogen. LV myocytes were isolated.2 The protocol was approved by the University of Illinois at Chicago Animal Studies Committee.

Northern Blot and Real-Time PCR Analyses
Total RNA was isolated using TRizol reagent (Gibco). Synthesis of RyR2 cRNA probe and Northern hybridization were performed.26 One-step hot-start real-time RT-PCR (Qiagen, Cepheid) was performed using a LightCycler RNA Master SYBR Green I kit (Roche).

Western Blot Analysis
LV tissue and myocytes were subjected to Western blotting using primary antibodies to RyR2, phospho-CaMKII-Ser286 (CaMKII-P), and SERCA (Affinity Bioreagents); phosphorylated RyR2-Ser2815 and RyR2-Ser2809 (RyR2-P2815, RyR2-P2809; gift from Dr Andrew R. Marks, Columbia University); IP3R (gift from Dr Gregory A. Mignery, Loyola University); CaMKII-δ and FKBP12–12.6 (Santa Cruz Biotechnology); calmodulin (RDI); total PLB, PLB-Thr17, and PLB-Thr17 (PLB-Thr-17; Baddrilla); and PP1 and PP2A (BD Biosciences).

Com Immunoprecipitation
RyR2 immunoprecipitation using a specific monoclonal RyR2 antibody (Affinity Bioreagents; absence of RyR2 antibody and use of nonrelevant antibody were negative controls) was performed.26 Immunoblotting was then performed with antibodies to RyR2, RyR2-P2815, RyR2-P2809, FKBP12–12.6, CaMKII-P, CaMKII-δ, calmodulin, PP1, PP2Ac, and GAPDH as above.

PKA and CaMKII Back-Phosphorylation
LV tissue was homogenized in a phosphorylation buffer with protease inhibitors. Immunoprecipitation of RyR2 and back-phosphorylation experiments were performed as previously described.8

SR Ca Leak Assay
In isolated myocytes from HF rabbit LV, diastolic SR Ca leak was determined from reduction in [Ca2+], and increase in SR Ca content induced by abrupt block of RyR2 with 1 mmol/L tetracaine (23°C)3 (with or without CaMKII inhibitor KN-93 or PKA inhibitor H-89).

Statistical Analysis
Data are means±SEM, with significant differences assessed using Student t test at P<0.05.

Results
Echocardiographic Data
HF rabbit hearts (n=22) exhibited marked LV dilatation and systolic dysfunction compared with their baseline condition (as well as to age-matched controls [n=23]). With HF, LV end-diastolic and end-systolic dimensions increased by 57% and 79%, respectively (both P<0.001), and mean fractional shortening decreased by 41% (P<0.001).

RyR2 and IP3R2 Expression
RyR2 mRNA was detected by Northern blots as a single 1.6-kb band and its abundance was normalized to 18S ribosomal RNA (Figure 1A). There was a 50% decrease in RyR2 mRNA in HF versus controls (n=5, 5, P<0.05, Figure 1B). Similar results were found by using real-time RT-PCR (data not shown).

RyR2 and IP3R2 protein expression was assessed by Western blot. In LV homogenates from HF hearts, we found a 30% decrease in RyR2 protein expression versus controls (n=12, 12, P<0.05; Figure 1C and 1D). Nonmyocytes in LV tissue (eg, smooth muscle or endothelial cells), which constitute ~40% of ventricular protein,27 also contain significant amounts of IP3R.28 Therefore, we measured InsP3R expression in isolated LV myocytes (where settling steps remove other, smaller cell types) and found a 93% increase in IP3R2 protein expression in HF versus controls (n=6, 6, P<0.05; Figure 1E and 1F). The increase in InsP3R and decrease in RyR2 results in a 3-fold higher IP3R2:RyR2 ratio in HF versus controls (P<0.05; Figure 1F).

FKBP12/12.6 Expression and Association With RyR2
FKBP12.6 associates with the cardiac RyR2, and FKBP12 associates with the skeletal muscle RyR1 (although total FKBP12 expression exceeds FKBP12.6 in heart).29,30 Using an antibody that recognizes both FKBP12 and 12.6, Western blots show a 25% decrease in total FKBP12/12.6 in HF versus control LV tissue and isolated myocyte (n=6, 6, P<0.05; Figure 2A through 2C). To distinguish FKBP12 versus FKBP12.6 expression, real-time PCR was used to assess mRNA levels with specific primers. At the mRNA level, FKBP12.6 expression decreased by 49%, whereas FKBP12 decreased by 20% in HF LV tissue versus control (n=8, 8, P<0.05; Figure 2D and 2E). It seems likely that the modest reduction in FKBP protein measured in Figure 2A and 2B is dominated by the FKBP12 reduction, whereas FKBP12.6 protein may be reduced by a greater degree than either FKBP12 or RyR2. This would be consistent with the apparent reduction of the upper band in doublets for FKBP12/12.6 in Western blots (Figure 2A and 2B), which may reflect mainly FKBP12.6 (not quantified).
Altered RyR2 phosphorylation could influence FKBP-RyR association; therefore, we assessed how much FKBP associates with immunoprecipitated RyR2. RyR2 has much higher affinity for FKBP12.6 than for FKBP12;30 therefore, the RyR2 immunoprecipitate likely contains primarily FKBP12.6 (versus FKBP12). In HF versus control, the level of coimmunoprecipitated FKBP12.6 normalized to RyR2 decreased by 38% (n=6, P<0.05; Figure 3A through 3C).

Although this lower FKBP12.6:RyR2 ratio could be attributable to less overall FKBP12.6 expression versus RyR2 (as implied by Figure 2D and 2E; see above), we cannot exclude the possibility that there is reduced partitioning of FKBP12.6 on the RyR2 in HF.

Figure 1. RyR2 and IP3R expression in control (Ctl) and HF rabbit. A, Northern blots of RyR2 mRNA and 18S ribosomal RNA from LV tissue with summarized data (B). C, Western blots of RyR2, RyR2-P2815, RyR2-P2809, and GAPDH from LV tissue and pooled data (D) including ratios of RyR2-P2815 and RyR2-P2809 to RyR2. E, Immunoblots of IP3R2 and GAPDH in myocytes and quantitative results (F) for IP3R2 protein expression and ratio of IP3R2 to RyR2 in LV myocytes. Western blots normalized to GAPDH. *P<0.05, ***P<0.001.

Figure 2. FKBP expression. A and B, Immunoblot images of FKBP12/12.6 and GAPDH bands from control (Ctl) and HF LV tissue and myocytes with summarized data (C). D, RT-PCR of FKBP12 and FKBP12.6 and GAPDH mRNA from control and HF LV, with pooled results (E). *P<0.05.
RyR2 Phosphorylation Status

RyR2 contains multiple phosphorylation sites including RyR2-Ser2815 (phosphorylated by CaMKII) and RyR2-Ser2809 (phosphorylated by both CaMKII and PKA), and we used 2 phospho-specific antibodies (for RyR2-P2815 and RyR2-P2809) in Western blots. In HF versus control LV homogenates, RyR2 phosphorylation was increased at both Ser2815 and Ser2809 sites (by 105% and 30% respectively; \[P < 0.001\] and \[P < 0.05\]; Figure 1C and 1D). We also assessed RyR2 phosphorylation in RyR2 immunoprecipitates and found that RyR2 phosphorylation at both Ser2815 and Ser2809 were also increased by 68% and 62%, respectively (\[n = 8, P < 0.05\]; Figure 4A and 4B).

To complement these phospho-antibody studies, RyR2 back-phosphorylation assays were performed using purified CaMKII or PKA enzymes. The ability of exogenous CaMKII and PKA to phosphorylate RyR2 was lower in HF than control (Figure 4C and 4D), implying that the CaMKII and PKA sites were already more phosphorylated in HF versus controls (by 55% for CaMKII and 66% for PKA) in immunoprecipitated RyR2 (\[n = 8, P < 0.01\]; Figure 4C through 4E). Thus our findings suggest enhanced phosphorylation of RyR2 in HF that may be attributable to CaMKII as well as PKA.

Protein phosphatases PP1 and PP2A are also part of the RyR2 macromolecular complex, and higher RyR2 phosphorylation levels in HF could be partly attributable to less associated phosphatase. In our HF rabbit model, we found a 37% and 45% decrease in the amount of PP1 and PP2A that coimmunoprecipitates with RyR2 (\[n = 10, P < 0.05\]; Figure 3A and 3B), although the global expression level was increased by 250% for PP1 and decreased by 35% for PP2A (\[P < 0.01\] and \[P < 0.05\]; respectively; Figure 3D and 3E). Thus, whereas kinases (eg, CaMKII) must initiate RyR2 phosphorylation, less-colocalized phosphatase must also contribute to the enhanced RyR2 phosphorylation in HF.

CaM and CaMKII Expression and Activation

Alterations of CaMKII expression and activation state could mediate CaMKII-dependent phosphorylation of RyR2 in HF. We measured CaMKII expression and activity on both global (total tissue) and local (associated with RyR2) levels using Western blots. Total CaMKII protein expression and auto-phosphorylated CaMKII from rabbit LV tissue homogenates were 60% and 43% higher in HF versus control (\[n = 12, P < 0.05\]; Figure 5A and 5B). With HF, the ratio of CaMKII cytoplasmic form (CaMKIIc) to nuclear form (CaMKII\(\delta\)) was increased 120% (\[P < 0.05\]). In RyR2 immunoprecipitates, there was also more total CaMKII\(\delta\) and more autophosphorylated CaMKII (by 96% and 105% versus controls; \[n = 8, P < 0.05\] and \[P < 0.01\]; Figure 5C and 5D).

Although both CaMKII\(\delta\) expression and autophosphorylation are increased, the fraction autophosphorylated does not change appreciably. This is true for both CaMKII\(\delta\) in LV homogenates (Figure 5A and 5B) and that which immunoprecipitates with RyR2 (Figure 5C and 5D). Thus, there is more CaMKII and autoactivated CaMKII in HF (both globally and at the RyR2), but fractional activation may not differ from control. Indeed, all
approaches lead us to conclude that there is higher RyR2 phosphorylation by CaMKII in our HF rabbits.

CaM itself also modulates RyR gating; therefore, we measured total and RyR2-associated CaM. Whereas total CaM levels were unchanged in HF \((n=12, 12, P=NS; \text{Figure 5B})\), the level of CaM associated with RyR2 was decreased by 30% in HF versus controls \((n=8, 8, P<0.05; \text{Figure 5D})\). This reduced CaM associated with RyR2 might independently increase RyR2 open probability and SR Ca leak but does not explain more active CaMKII associated with RyR2 in HF.

Figure 4. Phosphorylation of immunoprecipitated (IP) RyR2. A, Immunoblots of phospho-RyR2 (P-RyR2) (RyR2-P2815 and RyR2-P2609) in immunoprecipitated RyR2 with pooled data (B). C and D, Back-phosphorylation of immunoprecipitated RyR2. Autoradiograms of CaMKII (with or without KN93 or CaMKII) and PKA (with or without PKI or PKA). Lower panels show equivalent amounts of RyR2 protein were used from LV homogenates. E, Summarized back-phosphorylation data (reciprocal of CaMKII- or PKA-dependent \(^{32}P\) incorporated (normalized to RyR2). \(*P<0.05\) or \(**P<0.01\).

Figure 5. CaMKII expression and phosphorylation. A, Western blot of CaMKII-\(\delta\), CaMKII-P, CaM, and GAPDH from LV tissue (normalized to GAPDH) including CaMKII-\(\delta\)/CaMKII-P and CaMKII-P/CaMKII-\(\delta\) (B). C, Immunoblots of immunoprecipitated (IP) RyR2 and coimmunoprecipitated CaMKII-\(\delta\), CaMKII-P, and CaM from LV homogenates and pooled data (D). \(*P<0.05, \ **P<0.01\).

SERCA and PLB Expression and Phosphorylation

The greater amount and more active CaMKII overall in HF myocytes raises the question as to whether other non-RyR targets of CaMKII (eg, PLB) also have altered phosphoryla-
tion levels. We found that PLB expression was not significantly reduced, in HF, but using phospho-specific antibodies, we found a 33% increase in Thr17 phosphorylation (CaMKII site) but a 30% decrease in Ser16 phosphorylation (PKA site; n=12, 12; Figure 6A and 6B). Indeed, the mean values for both SERCA and PLB in HF were 82% of that in control. Although these were not significant in Western blot analysis, it could explain our prior finding of a 24% reduction in SR Ca-ATPase function in ventricular myocytes in this HF model.1,2

Functional Roles of PKA and CaMKII to the Increased SR Diastolic Ca Leak in HF

We showed that diastolic SR Ca leak is increased in our HF rabbit model.3 Here we use that approach to assess PKA and CaMKII contributions to this enhanced SR Ca leak. Abrupt blockade of RyR leak by tetracaine causes a shift of Ca from cytosol to SR depending on how much leak there was before RyR2 block (ie, [Ca], drops and SR Ca content rises and both are measured).31 In HF, SR Ca leak was increased for any SR Ca content ([Ca]SR) and for any level of SR Ca leak [Ca]SR was reduced.31 We used these protocols in HF myocytes, which had been pretreated for >30 minutes (or not) with 1 μmol/L KN-93 to inhibit CaMKII or 1 μmol/L H-89 to inhibit PKA. Different conditioning trains were given to vary [Ca]SR under all 3 conditions before abrupt tetracaine exposure. Thus, for each train, we measure [Ca]SR and increase in [Ca]SR with tetracaine (indicative of leak).

When we group cellular data so that the SR Ca loads are the same for all 3 cases (Figure 7A, left), the SR Ca leak (at that load) is significantly reduced by CaMKII inhibition (KN-93) but not by PKA inhibition by H-89. This is true whether we measure the tetracaine-induced increase in [Ca]SR (Figure 7A, right; n=5 to 9) or the inferred rate of SR Ca leak (Figure 7B).31 Further quantitative analysis of this data31 shows that the SR Ca leak rate constant (k leak) is also reduced by CaMKII inhibition but not by H-89 (Figure 7D, n=14 to 23). Viewed another way, if we group data for comparable leak (tetracaine-induced [Ca]SR; Figure 7C, left, n=4 to 7), then CaMKII inhibition increases the SR Ca load at which that leak is achieved, whereas PKA inhibition does not (Figure 7C, right). This means that in HF, the SR Ca load can increase to a higher level when CaMKII is blocked, whereas PKA inhibition does not alter SR Ca load. These data suggest that, although both PKA and CaMKII enhance RyR2 phosphorylation in HF, only CaMKII enhances SR Ca leak via RyR2 in HF.

As a control for KN-93 experiments, we performed a series of experiments in control rabbit myocytes at moderate SR Ca content (slightly less than in HF) with or without KN-93 (see the online data supplement). KN-93 (1 μmol/L) had no significant effect on SR Ca leak (for a given load) and no effect on SR Ca content (for a given leak), suggesting that actions of KN-93 observed in HF cells are caused by CaMKII inhibition. Of note, any side effects of KN-93 on sarcolemmal ion channels are minimized in our protocols because the cells are quiescent when the SR Ca content and leak are measured.
Ca transient amplitude and [Ca]SRT are reduced in HF versus control myocytes. Blocking SR Ca leak with KN-93 substantially increased steady-state [Ca]SRT at 0.5 Hz, but steady-state twitch Ca transients were only slight increased (Figure 7E and 7F). This can be understood if one considers that CaMKII may increase SR Ca leak, but also enhance SR fractional Ca release during E–C coupling, much like low caffeine concentrations. Then there may be higher [Ca]SRT in HF after CaMKII blockade, but a smaller fraction of that Ca is released (see Trafford et al and Discussion). These results also imply that inhibiting SR Ca leak in HF (mediated by CaMKII) may enhance SR Ca content but not twitch Ca transients.

Discussion

Contractile dysfunction in rabbit, canine, and human HF is attributable largely to reduced myocyte Ca transients that ultimately arise from decreased SR Ca load (caused by enhanced Ca extrusion via NCX, reduced SR Ca uptake via SERCA2, and increased SR Ca leak via the RyR). Moreover, we have shown in our HF rabbit model that spontaneous ventricular tachycardia arises by a nonreentrant mechanism involving spontaneous SR Ca release, activation of J_Na (mediated by NCX), and delayed afterdepolarizations (cellular mediators of nonreentrant VT). Thus, SR Ca release via RyRs may be critical in both contractile dysfunction and arrhythmogenesis in HF.

Marks and colleagues showed that RyR2 can be phosphorylated by PKA at Ser2809 and that this dissociates FKBP12.6 from the RyR2 and increases single-channel open probability. They hypothesized that RyR2 hyperphosphorylation in HF was mediated by chronic PKA activation and that a resultant enhanced SR Ca leak is the main mediator of systolic dysfunction and arrhythmias in HF. This paradigm is appealing, but several results have challenged key aspects. CaMKII also phosphorylates RyR2, enhances Ca spark frequency, sensitizes RyR2 to Ca, is elevated in human HF, and can induce HF in animals. Thus, we focused here on expression and function of SR Ca release channels and CaMKII- and PKA-dependent regulation in our well-characterized model of HF in rabbits.

InsP₃R and RyR Expression in HF

RyR2 expression was modestly reduced in HF, but InsP,R2 expression was enhanced ~2-fold. This reciprocal pattern was reported in human HF, but type 2 InsP,R (the main isoform in ventricular myocytes) was not assessed there. The functional significance is unknown, but InsP,R can mediate neurohumoral-induced arrhythmias in atrial (but not control ventricular) myocytes. InsP,R2 in normal ventricular myocytes is mainly on the nuclear envelope, associated with CaMKII, and may be involved in neurohumoral and Ca-dependent nuclear transcriptional signaling. Whether upregulated ventricular InsP,R in HF contributes to arrhythm-
mogeneration or transcriptional control in HF is not yet known but merits further study. The 25% decrease in RyR2 expression in HF could reduce SR Ca release flux but may be largely offset by functional alterations in regulation of RyR2 gating (see below).

**RyR2 Complex in HF**

In the immunoprecipitated RyR2 complex in HF, we find increased CaMKII and reduced CaM, FKBP12.6, PP1, and PP2A. These changes in FKBP and phosphatases, coupled with the expected hyper-adrenergic state in HF, would explain the enhanced RyR2 phosphorylation at sites of PKA action. Although smaller in extent, these data are consistent in direction with results of Marx et al.\(^9\) in HF. Greater PKA-dependent RyR2 phosphorylation could contribute to enhanced SR Ca leak in HF based on single RyR2 channel gating results of Marx et al.,\(^9\) although other results suggest that PKA does not activate diastolic RyR2 activity in bilayers\(^9\) or in myocytes.\(^14\)

We also found more RyR2-associated CaMKII in HF, which was also more highly autophosphorylated. Increased global CaMKII expression has been previously reported in hypertrophy and HF,\(^5,25\) and can induce HF,\(^15\) but this is the first report to show both enhanced CaMKII activation and association with the RyR2 in HF. This explains our observed higher levels of RyR2 phosphorylation at CaMKII target sites. The lower phosphatase levels at the RyR2 might contribute to the enhanced phosphorylation of both RyR2-associated CaMKII and RyR2 at CaMKII sites. CaMKII-dependent phosphorylation can also enhance RyR2 open probability,\(^21\) diastolic SR Ca leak via Ca sparks,\(^40\) and fractional SR Ca release during E–C coupling (for a given \([\text{Ca}^{2+}]_{\text{SRT}}\) and Ca current trigger).\(^22\) Thus CaMKII-dependent RyR2 phosphorylation could contribute to the enhanced diastolic SR Ca leak in HF.

CaM inhibits RyR2 open probability at physiological \([\text{Ca}^{2+}]_{\text{SRT}}\),\(^9\) and the lower CaM associated with the RyR2 in HF here may thus also contribute to increased SR Ca leak in HF. It is unclear why less CaM is bound to the RyR2 in HF, because global CaM expression was unaltered. However, this is analogous to PP1, where global expression is increased in HF but less is RyR2 associated. These differences in local expression in the cell may be important in differential cellular regulation.

**PLB and Global Versus Local Phosphorylation**

PLB phosphorylation at Ser16 was reduced in HF, consistent with elevated global levels of phosphatases in HF\(^41\) (despite less phosphatase associated with the RyR2\(^9\)). Both global and RyR2-localized CaMKII expression and activation state were higher in HF, and this may explain higher PLB Thr17 phosphorylation in HF here (despite overall increased phosphatase expression). The net effect of higher phospho-Thr17 and lower phospho-Ser16 on PLB in HF may by itself have little net functional effect, although it may mean there is less reserve of Thr17 phosphorylation in HF. Overall, mean levels of SERCA and PLB expression were both decreased by 18% (not significant), but this could suffice to explain the 24% decrease in SERCA function previously shown in this HF model.\(^1\)

**Enhanced SR Ca Leak in HF Myocytes**

There is considerable controversy about which RyR2 sites are phosphorylated by PKA and/or CaMKII and the functional impact on SR Ca leak in HF. The functional effects must also be evaluated at the cellular level. We previously showed enhanced diastolic SR Ca leak in HF myocytes,\(^3\) but whether these effects are kinase dependent was not previously assessed. Here we show that blocking CaMKII (but not PKA) inhibits SR Ca leak and significantly enhances SR Ca content in HF myocytes. Although this does not prove which CaMKII target (eg, RyR2-Ser2815, -2809, or other) is responsible for the enhanced leak, it raises CaMKII as an alternative or additional target in the treatment of HF.\(^42\)

Despite markedly enhanced \([\text{Ca}^{2+}]_{\text{SRT}}\), CaMKII inhibition increased Ca transient amplitude in HF cells only slightly. Why was there not more inotropic effect of CaMKII inhibition in HF myocytes? We suggest that this is partly because CaMKII also has positive inotropic effects that would be inhibited (\(I_{\text{Ca}}\) facilitation, PLB phosphorylation, and increased fractional SR Ca release).\(^9,22\) Thus, after CaMKII blockade, there may be higher \([\text{Ca}^{2+}]_{\text{SRT}}\), but a smaller fraction of that Ca is released during E–C coupling. This may be a direct manifestation of the notion that altered Ca-dependent RyR gating can only transiently alter twitch Ca transients.\(^32,43\) As for low caffeine concentration (which sensitizes RyR2 to Ca), abrupt RyR2 phosphorylation would increase fractional SR Ca release at the first beat, but that would drive more Ca extrusion from the cell and lower \([\text{Ca}^{2+}]_{\text{SRT}}\). Then, as the steady state is approached (where Ca influx and efflux are again matched), Ca transients are altered little (lower \([\text{Ca}^{2+}]_{\text{SRT}}\) with higher fractional release).

**Implications**

Increased CaMKII activity in HF can contribute to reduced SR Ca content and systolic function and also cause diastolic SR Ca leak and Ca current changes that may be arrhythmogenic. Apparently, the higher SR Ca leak in HF is mediated mainly by CaMKII (versus PKA). RyR2 phosphorylation may thus contribute to both reduced SR Ca content and triggered arrhythmias. However, these RyR2 effects do not appear to be major mediators of the reduced systolic function in HF (because decreased \([\text{Ca}^{2+}]_{\text{SRT}}\) is offset by greater fractional release). Other mechanisms that contribute to reduced \([\text{Ca}^{2+}]_{\text{SRT}}\), but do not sensitize the RyR2 to Ca (depressed SERCA function and enhanced NCX function), may therefore be more central to the depressed systolic function in HF.\(^1-5\) CaMKII inhibition may be important in HF treatment, especially in limiting arrhythmias and diastolic dysfunction but maybe less so in enhancing systolic function (however, see Maier et al.\(^23\) and Neumann et al.\(^42\)). Moreover, if CaMKII activation occurs downstream from \(\beta\)-adrenergic receptor (\(\beta\)-AR) stimulation, the addition of CaMKII inhibition to \(\beta\)-AR blockers may have a role in the treatment of patients with HF. Clearly further work is required in this relatively new area involving the relationship of CaMKII to \(\beta\)-AR stimulation in HF.
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References


Ca\textsuperscript{2+}/Calmodulin–Dependent Protein Kinase Modulates Cardiac Ryanodine Receptor Phosphorylation and Sarcoplasmic Reticulum Ca\textsuperscript{2+} Leak in Heart Failure

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Expended Materials and Methods

Arrhythmogenic rabbit nonischemic HF model

New Zealand White rabbits (~3.5 kg) underwent induction of HF by aortic insufficiency followed by thoracic aortic constriction. Progression of HF was assessed by LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD) and LV fractional shortening (FS) [FS (%) = (LVEDD-LVESD)/LVEDD] using 2-D echocardiography (under sedation with ketamine 0.35 mg/kg), as previously described.1,2 Animals were studied 9.5 ± 2 months later when severe heart failure developed (LVESD > 1.4 cm). HF and age-matched control rabbits were anesthetized with isoflurane. Hearts were rapidly excised and the LV free wall was flash-frozen in liquid nitrogen.

Northern and real-time PCR

Total RNA was isolated using TRIzol reagent (Gibco). Synthesis of RyR2 cRNA probe and Northern hybridization were performed as previously described.1 A 199 bps fragment (position 8524-8723) from a human RyR2 full length mRNA sequence (NCBI, NM001035) as the cRNA probe template was generated using PCR amplification with a sense primer (5’CAACCGGACTCGTATTTT 3’) and an antisense primer (5’GGATCCTAATACGACTCACTATTAGGGGTTTCCTCCTCCTTTGGAC 3’, 23-bp T7 promoter sequence appended at the 5’ end). Screening of the NCBI gene bank indicated that this probe does not have major homology with other published sequences of cardiac RyRs (RyR1, RyR3) and IP3Rs (IP3R1, 2, 3). 18S antisense control templates purchased from Ambion.

One-step hot-start real-time RT–PCR (Qiagen, Valencia, CA; Cepheid, Sunnyvale, CA) was performed using LightCycler RNA Master SYBR Green I kit (Roche) were based on methods of Fathallah-Shaykh et al with modifications.2 Triplicates of each samples were subjected to a standard PCR protocol using the following primers: FKBP12 forward 5’CTCCTCGTATGTGTGTTACCT3’ reverse
5’CTGTAGTG
TCCAGCATTGCAGT3’, FKBP12.6 forw ard 5’AAGGCAGGAAGGA CACTCAAGGT3’
reverse 5’GAGAGAA GTGACAGCAAAGGT3’, glyceraldehyde-3-phosphate dehydro-
genase (GAPDH) forw ard 5’GTCAGTGGTGACCTGACCT3’ reverse 5’GGTGGTCC
AGGGTTCTTACT3’.

PKA and CaMKII back-phosphorylation

LV tissue was homogenized in a buffer containing (in mmol/L) 50 Tris-HCl (pH 7.4), 200 NaCl, 20 NaF, 1.0 Na3VO4, 1.0 dithiothreitol, and protease inhibitors. Immunoprecipitation of RyR2 experiment was performed as previously described.3 Protein G-plus agarose beads with immunoprecipitated RyR2 protein were washed with 1x PKA phosphorylation buffer (8 mmol/L MgCl2, 10 mmol/L EGTA, 50 mmol/L Tris / PIPES, pH 6.8) or CaMKII phosphorylation buffer (10 mmol/L MgCl2, 0.1 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.5). The back phosphorylation of PKA and CaMKII was performed in a 1.5 x PKA phosphorylation buffer or 1 x CaMKII phosphorylation buffer with 33µM Mg-ATP contain 10% [γ-32P] (Amersham), and terminated by add 10ul loading buffer. RyR2 proteins were separated by 5% SDS-PAGE gel and the radioactivity was quantified by using a PhosphorImager and ImageQuaNT software (Amersham). The relative amounts of phosphorylated RyR2 in vivo were calculated as the inverse value of the CaMKII or PKA-dependent 32P incorporated signals normalized to immunoprecipitated RyR2.

SR Ca leak assay

All experiments were performed at room temperature using the general protocol as previously described.4 Resting [Ca], and [Ca]SRT were measured in intact myocytes loaded 40 min with 10 µM fluo-4 AM. Cells were stimulated at least 20 times at a particular frequency in 2 mmol/L Ca normal Tyrodes (NT) in order to bring the [Ca]SRT to steady state. When indicated the NT solution contained either 1 µM KN-93, a known CaMKII inhibitor, or 1 µM H-89, a PKA inhibitor. Na/Ca-exchange was blocked in 0 Na/0 Ca NT so that little or no Ca entered or left the cell following SR loading. In all
conditions [Ca], was monitored while 0/0 NT solution with tetracaine (1 µM) was perfused for 30 sec, followed by a rapid switch and 30 sec perfusion in 0/0 NT without tetracaine. Tetracaine effectively blocks the ryanodine receptor (the site of the leak), and the SR Ca-ATPase (SERCA) continues to function resulting in Ca transport into the SR from the cytosol. The transport continues until a new steady state has been achieved (<30 sec) in the absence of leak. The solution was then rapidly switched to 10 mmol/L caffeine NT solution for measurement of [Ca]SRT. The difference in [Ca]SRT (represented by the change in baseline) is indicative of the tetracaine-dependent shift of Ca from the cytosol to the SR and, therefore, of the SR Ca leak.

Results

Control experiments for KN-93 effects on SR Ca leak

Figure S1 shows results of experiments in control (not HF) myocytes using the same protocols, analysis and display as in Fig 7A-B. The SR Ca content was not driven very high here to limit the activation of endogenous CaMKII. Thus [Ca]SRT is not as high as in Fig 7. The pre-incubation with KN-93 did not alter the tetracaine-induced shift in [Ca]SRT or leak (Δ[Ca]SRT) for a given [Ca]SRT (top panel). Nor did KN-93 alter the load at which a given leak occurred (where average Δ[Ca]SRT was ~7 µmol/l cytosol (bottom panel). This provides information to support that KN-93 did not produce any non-specific effect that
by itself alters SR Ca leak, and that the effects of KN-93 in Fig 7 can be taken as the effects of CaMKII inhibition in the HF myocytes. These findings do not exclude the possibility that non-specific KN-93 effects are present at higher SR Ca load.
References


