Published in final edited form as:

J Mol Cell Cardiol. 2014 July ; 72: 281–291. doi:10.1016/j.yjmcc.2014.03.021.

Cardiomyocyte-Specific Expression of CRNK, the C-terminal Domain of PYK2, Maintains Ventricular Function and Slows Ventricular Remodeling in a Mouse Model of Dilated Cardiomyopathy

Yevgeniya E. Koshman¹, Miensheng Chu², Taehoon Kim¹, Olivia Kalmanson², Mariam Farjah³, Mohit Kumar², William Lewis⁴, David L. Geenen³, Pieter de Tombe², Paul H. Goldspink⁵, R. John Solaro³, and Allen M. Samarel^{1,2}

¹The Department of Medicine, Loyola University Chicago Stritch School of Medicine, Maywood, IL 60153

²The Department of Cell and Molecular Physiology, Loyola University Chicago Stritch School of Medicine, Maywood, IL 60153

³Department of Physiology and Biophysics, University of Illinois – Chicago, Chicago, IL 60612

⁴Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322

⁵Department of Physiology, Medical College of Wisconsin, Milwaukee, WI 53226

Abstract

Rationale—Up-regulation and activation of PYK2, a member of the FAK family of protein tyrosine kinases, is involved in the pathogenesis of left ventricular (LV) remodeling and heart failure (HF). PYK2 activation can be prevented by CRNK, the C-terminal domain of PYK2. We previously demonstrated that adenoviral-mediated CRNK gene transfer improved survival and LV function, and slowed LV remodeling in a rat model of coronary artery ligation-induced HF.

Objective—We now interrogate whether cardiomyocyte-specific, transgenic CRNK expression prevents LV remodeling and HF in a mouse model of dilated cardiomyopathy (DCM) caused by constitutively active Protein Kinase C ϵ (caPKC ϵ).

Methods and Results—Transgenic (TG; FVB/N background) mice were engineered to express rat CRNK under control of the α -myosin heavy chain promoter, and crossed with FVB/N mice with cardiomyocyte-specific expression of caPKC ϵ to create double TG mice. LV structure,

© 2014 Elsevier Ltd. All rights reserved.

Proofs and Correspondences to: Allen M. Samarel, M.D., The Cardiovascular Institute, Building 110, Rm 5222, 2160 South First Avenue, Maywood, IL 60153, V: 708-327-2829, F: 708-327-2849, asamare@lumc.edu.

DISCLOSURES

none

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

function, and gene expression was evaluated in all 4 groups (nonTG FVB/N; caPKC ϵ (+/-); CRNK(+/-); and caPKC ϵ x CRNK (PXC) double TG mice) at 1, 3, 6, 9 and 12mo of age. CRNK expression followed a Mendelian distribution, and CRNK mice developed and survived normally through 12mo. Cardiac structure, function and selected gene expression of CRNK mice were similar to nonTG littermates. CRNK had no effect on caPKC ϵ expression and vice versa. PYK2 was up-regulated ~6-fold in caPKC ϵ mice, who developed a non-hypertrophic, progressive DCM with reduced systolic (Contractility Index=151 $\pm$ 5 vs. 90 $\pm$ 4 sec⁻¹) and diastolic (Tau=7.5 $\pm$ 0.5 vs. 14.7 $\pm$ 1.3 msec) function, and LV dilatation (LV Remodeling Index (LVRI)=4.2 $\pm$ 0.1 vs. 6.0 $\pm$ 0.3 for FVB/N vs. caPKC ϵ mice, respectively; P <0.05 for each at 12mo). In double TG PXC mice, CRNK expression significantly prolonged survival, improved contractile function (Contractile Index=115 $\pm$ 8 sec⁻¹; Tau=9.5 $\pm$ 1.0 msec), and reduced LV remodeling (LVRI=4.9 $\pm$ 0.1).

Conclusions—Cardiomyocyte-specific expression of CRNK improves contractile function and slows LV remodeling in a mouse model of DCM.

Keywords

Heart failure; gene expression; protein kinase C; echocardiography; focal adhesion kinase

1. INTRODUCTION

Left ventricular (LV) dysfunction, whether due to myocardial infarction (MI), valvular heart disease resulting in chronic volume overload, or a genetic mutation in a specific cytoskeletal protein, activates mechanosensitive signal transduction pathways that ultimately lead to LV dilatation and pathological LV remodeling. The remodeling process is characterized by altered gene expression, subcellular changes in the cardiomyocyte cell population (including thinning and elongation of individual muscle cells), and alterations in the composition and orientation of the cardiac extracellular matrix. LV remodeling contributes to the progressive decline in contractile performance, and may ultimately lead to the clinical syndrome of heart failure (HF). Interventions to block the maladaptive cell signaling that leads to LV remodeling may be useful in preventing or attenuating the loss of ventricular performance in HF.

The Ca²⁺-dependent, nonreceptor protein tyrosine kinase (PTK) PYK2 has been implicated in cardiomyocyte cell signaling pathways leading to LV remodeling and HF [1,2]. PYK2 is a member of the focal adhesion kinase (FAK) family of nonreceptor PTKs. Like FAK, PYK2 is a component of the costameric mechanosensory apparatus of muscle cells [3,4], and coordinates Ca²⁺, integrin-, and protein kinase C (PKC)-dependent signal transduction in a number of tissues. In cardiomyocytes, PYK2 expression and phosphorylation are regulated by intracellular Ca²⁺ and the novel PKC isoenzyme PKC ϵ [3,5]. PYK2 serves as an “activatable” scaffolding protein, and its activation is dependent upon Ca²⁺-calmodulin binding at the FERM F2 subdomain within PYK2’s N-terminal region. The complex formation of PYK2 with Ca²⁺-calmodulin results in its activation by forming a homodimer, and stimulating transautophosphorylation of PYK2 at Y₄₀₂ [6]. This intermolecular autophosphorylation creates a docking site for Src to then bind via its SH2 domain. Once bound, Src then phosphorylates PYK2 at multiple sites, creating additional docking sites for Grb2, p130^{Cas} and other adaptor proteins [7]. Thus, PYK2 transduces signals from Ca²⁺,

integrins and G-protein coupled receptors to the mitogen-activated protein kinases (MAPK) and the phosphoinositol-3-kinase-PDK1-Akt signaling pathway depending upon which adaptor proteins bind to the phosphorylated PTK [8-11]. As recently reported by Lang and co-workers [12], PYK2 is expressed in the human heart, and its activation is markedly increased in LV tissue of patients with nonischemic, dilated cardiomyopathy (DCM).

The molecular mechanisms responsible for up-regulation and activation of PYK2 in experimental and human HF remain unclear. In previous reports, we described a distinct signaling pathway leading to contraction- and agonist-induced PYK2 activation in cardiomyocytes [5,13]. We also showed that both PKC ϵ [14,15] and PYK2 [16] were components of a signaling pathway that may regulate SERCA2 gene transcription in cardiomyocytes, and proposed that a PKC ϵ /PYK2/MAPK-dependent signaling cascade may have a role in abnormal Ca²⁺ handling, LV dysfunction, and HF. However, studies using highly specific inhibitors of PYK2 autophosphorylation, or cell type-specific “knockdown/knockout” strategies were required to fully define the role of PYK2 in LV remodeling.

Like FAK, the function of PYK2 is regulated by an endogenously expressed inhibitor known as PYK2-Related Non-Kinase [17], also known as Cell Adhesion Kinase- β -Related Non-Kinase (CRNK) [18]. CRNK consists of the C-terminal portion of PYK2, containing its focal adhesion targeting sequence, paxillin binding site, and proline-rich region, but lacking its N-terminal autoinhibitory domain, Ca²⁺-calmodulin binding site, autophosphorylation site, and kinase domain. CRNK is structurally analogous to FAK-Related Non-Kinase (FRNK), the autonomously expressed C-terminal domain of FAK. CRNK is expressed at relatively high levels in the brain, spleen, and lung, but not in the heart [17]. However, when ectopically expressed, CRNK can inhibit PYK2 (but not FAK) tyrosine autophosphorylation, presumably by displacing PYK2 from its cytoskeletal binding sites [18]. Thus CRNK, like its structurally homologous polypeptide FRNK, has been used as a tool to specifically inhibit PYK2-dependent signal transduction in cultured cardiomyocytes and other cells [18-22].

In a previous study [23], the effect of PYK2 inhibition was evaluated using adenovirus (Adv)-mediated expression of CRNK in cultured cardiomyocytes, and *in vivo* in an animal model of post-MI ventricular remodeling. Our data indicated that CRNK was a potent and specific inhibitor of PYK2-dependent signal transduction. Furthermore, endovascular Adv-CRNK gene transfer into the rat myocardium improved survival and LV function, and slowed the progression of LV remodeling [23]. The present study examines if cardiomyocyte-specific transgenic (TG) expression of CRNK prevents (or protects against) deleterious changes in gene expression, pathological LV remodeling and HF in a genetically engineered mouse model of DCM due to expression of constitutively active (ca) PKC ϵ [24].

2. METHODS

2.1. Materials and reagents

A detailed description of the materials and reagents used in these experiments is provided in the On-line Data Supplement.

2.2. Generation of CRNK, caPKC ϵ , and PXC double transgenic mice

All mice used in these experiments were handled in accordance with the *Guiding Principles in the Care and Use of Laboratory Animals*, published by the US National Institutes of Health and approved by the American Physiological Society. A detailed description of these animals is provided in the Online Data Supplement.

2.3. M-mode and 2-D echocardiography

A detailed description of this method is provided in the On-line Data Supplement.

2.4. LV catheterization

A detailed description of this method is provided in the On-line Data Supplement.

2.5. Tissue homogenization and Western blotting

Frozen LV tissue was homogenized in lysis buffer [25], centrifuged at 100,000 $\times$ g for 20min, and extracted proteins were subjected to SDS-PAGE and Western blotting on 10% polyacrylamide gels. Following electrophoretic transfer to nitrocellulose, primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies, and visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Developed X-ray films were then scanned on a HP Deskjet 4890 Scanner, and band intensity was quantified using UN-SCAN-IT Gel Software, Ver. 6.1 (Silk Scientific; Orem, UT).

2.6. RNA isolation and real-time RT-PCR

Total cellular RNA was isolated from frozen LV tissue using TRIzol Reagent (Invitrogen), and further purified using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). All samples were treated with DNase to eliminate contaminating genomic DNA. RNA was quantified by absorbance at 260 nm. α MHC, β MHC, ANF, SERCA2, COL1A1 and COL3A1 mRNAs were then analyzed by real-time RT-PCR, as previously described [15,16]. Further details are provided in the On-line Data Supplement.

2.7. Skinned cardiomyocyte preparations and steady-state force measurements

Cardiomyocytes were harvested from frozen LV tissue by mechanical homogenization, and used in measurements of steady state force and Ca²⁺ sensitivity of the contractile apparatus as described in detail in the On-line Data Supplement.

2.8. Protein-bound tissue hydroxyproline concentration

Protein-bound hydroxyproline concentration (μ g/mg total protein) was analyzed in LV tissue homogenates as previously described [26]. Further details of the method are provided in the On-line Data Supplement.

2.9. Statistical analysis

A detailed description of this method is provided in the On-line Data Supplement.

3. RESULTS

3.1. Effects of CRNK and α PKC ϵ on survival

The PYK2 inhibitor CRNK was targeted to the myocardium by driving expression with the murine α MHC promoter (Supplemental Figure 1A). Two founder lines that transmitted the CRNK transgene were established (CRNK-A and CRNK-B). The CRNK-A line exhibited greater CRNK mRNA and polypeptide expression as shown by Northern and Western blotting, respectively. We chose heterozygous CRNK (CRNK^(+/-)) animals from the A line for use in all described studies. At 3mo of age, CRNK polypeptide (appearing as a doublet of 35-38kDa) was readily detected by Western blot analysis of LV tissue extracts from the CRNK-A line (Supplemental Figure 1B).

The CRNK mice were normal in size, appearance, and behavior as compared to nonTG, FVB/N littermates, and genetic expression of the CRNK transgene followed a Mendelian pattern of inheritance. Survival was followed for 12mo and was similar to nonTG, control mice (mean survival time=349 $\pm$ 7 and 358 $\pm$ 10 days for FVB/N and CRNK mice, respectively; $P=0.47$; Log-Rank Test; Figure 1A). In contrast, heterozygous α PKC ϵ TG mice exhibited substantially reduced survival over the same observation period (Figure 1B). Mean survival time was 307 $\pm$ 18 days ($P<0.003$ vs. FVB/N mice) with most deaths occurring suddenly and unexpectedly. In double TG, α PKC ϵ x CRNK (PXC) mice, concomitant CRNK expression substantially reduced α PKC ϵ -induced mortality, as indicated by Kaplan-Meier analysis. Mean survival time for PXC mice was 352 $\pm$ 13 days, which was similar to the survival of wildtype FVB/N mice ($P=0.58$; Log-Rank Test). However, the reduction in mortality in PXC mice was not due to a reduction in α PKC ϵ expression, nor did α PKC ϵ appear to affect CRNK expression (Figure 1C).

3.2. Effects of α PKC ϵ and CRNK on PYK2 and FAK phosphorylation and expression

In ways that resemble expression patterns in other animal models of LV remodeling and heart failure [1,2,23,27], PYK2 was expressed at very low levels in nonTG adult hearts, but was up-regulated ~6-fold in LV tissue extracts from α PKC ϵ mice (Figure 2A and 2C). α PKC ϵ expression also markedly increased the amount of PYK2 phosphorylated at Y₄₀₂ (Figure 2A and 2B), resulting in a ~4-fold increase in the ratio of phosphorylated to total PYK2 (Figure 2A and 2D). However, concomitant expression of CRNK in PXC mice substantially reduced both PYK2 expression and autophosphorylation as compared to mice expressing only α PKC ϵ .

In contradistinction to the effects of α PKC ϵ and CRNK on PYK2, LV FAK phosphorylation and expression were relatively unaffected. As seen in Supplemental Figure 2, FAK expression was similar in whole tissue extracts of all 4 groups of mice. Furthermore, FAK was similarly phosphorylated at Y₃₉₇, which is the autophosphorylation site homologous to the Y₄₀₂ site in PYK2. Notably, expression of α PKC ϵ had no significant effect on the ratio of phosphorylated to total FAK, and CRNK expression, either alone or in combination with α PKC ϵ , did not significantly affect this ratio.

3.3. CRNK slows the progressive deterioration of LV structure and function in caPKC ϵ mice

Serial echocardiography was used to ascertain potential mechanisms for the salutary effects of CRNK on survival. As seen in Figure 3, LV structure and function were similar in FVB/N and CRNK mice postnatally. Echo-derived LV mass (Figure 3A), and LV/body weight ratio (Figure 3B) were not significantly affected by CRNK expression. Similarly, LV mass and LV/body weight ratio were not significantly increased by caPKC ϵ expression, suggesting that this level of caPKC ϵ expression alone was insufficient to induce LV hypertrophy. However, caPKC ϵ expression significantly reduced LV fractional shortening (FS) (Figure 3C) and ejection fraction (EF) (Figure 3D), beginning at ~3mo of age ($P<0.05$, 1-way ANOVA). The reduced FS and EF were initially the result of increased LV end-systolic dimension and LV end-systolic volume (LVESV) (Figure 3E), suggesting a primary defect in LV contractility in caPKC ϵ mice. Thereafter, LV end-diastolic dimension and LV end-diastolic volume (LVEDV) increased (Figure 3F), along with a progressive decrease in LV stroke volume (Figure 3G). As LV contractile function deteriorated, LV remodeling progressed. By 12mo of age, surviving caPKC ϵ mice demonstrated a profoundly increased LV Remodeling Index (LVRI) (Figure 3H), which resulted from both a large increase in LV end-diastolic dimension and a small reduction in LV posterior wall thickness.

Two-way ANOVA revealed that both the age of the animals and their genotype were significant factors in determining the mean FS, EF, and LVRI among the 4 groups. However, there was a significant interaction between these 2 factors. For instance, there was no significant difference in the mean FS, EF, or LVRI among the 4 animal groups at 1mo of age. Furthermore, there was no significant difference in FS, EF, or LVRI between FVB/N and CRNK mice at any age examined. In contrast, caPKC ϵ mice had a significantly lower FS and EF than either FVB/N or CRNK mice, beginning at 3mo of age, and the LV dysfunction worsened during the observation period. Similarly, LVRI in caPKC ϵ mice was significantly greater as compared to either FVB/N or CRNK mice beginning at 6mo age. More importantly, PXC animals had significantly higher FS and EF, and lower LVRI at 6-12mo of age as compared to caPKC ϵ mice. Overall, these echocardiographic results indicate that CRNK slowed the progressive reduction in ventricular performance and LV remodeling observed in aging caPKC ϵ mice.

LV catheterization corroborated the salutary effects of CRNK expression on LV performance. As seen in Table 1, both systolic and diastolic function were significantly impaired in caPKC ϵ mice, beginning at 6mo of age, and these parameters progressively declined over the next 6 months. In contrast, PXC mice had substantially better Contractility Index, $-dp/dt$, and Tau as compared to caPKC ϵ mice, with significantly improved end-diastolic pressure and volume. Thus confirming the echocardiographic results (Figure 3), concomitant expression of CRNK maintained systolic and diastolic function, and slowed ventricular remodeling in aging caPKC ϵ mice.

3.4. CRNK prevents the reduction in maximal force generation of skinned $\text{caPKC}\epsilon$ cardiomyocytes

Mechanically dissociated, skinned cardiomyocytes from each group of animals were then used to ascertain whether CRNK improved sarcomere function in response to varying Ca^{2+} concentrations at both short (1.9 μm) and long (2.3 μm) sarcomere lengths. As seen in Figure 4A, maximal force generation in FVB/N cardiomyocytes was 78.8 ± 3.0 and 94.5 ± 2.7 mN/mm^2 at 1.9 and 2.3 μm , respectively. As previously demonstrated using skinned trabeculae [24], $\text{caPKC}\epsilon$ cardiomyocytes demonstrated significantly reduced maximal force generation, now shown at both short and long sarcomere lengths. Moreover, the change in maximal force generation between the 2 sarcomere lengths was also significantly reduced (Figure 4B). Surprisingly, CRNK mice demonstrated a similar reduction in maximal force generation, and a smaller, length-dependent increase in maximal force. Nevertheless, concomitant expression of CRNK in $\text{caPKC}\epsilon$ mice maintained both maximal force generation and the length-dependent increase in maximal force at the level observed in nonTG littermates.

3.5. CRNK does not prevent the reduced length-dependent increase in Ca^{2+} sensitivity of skinned $\text{caPKC}\epsilon$ cardiomyocytes

As seen in Figure 4C, there was no significant difference in Ca^{2+} sensitivity of the contractile apparatus at either short or long sarcomere lengths amongst the 4 groups. These results confirm our previous findings indicating that 9-12mo $\text{caPKC}\epsilon$ mice have similar myofilament Ca^{2+} sensitivity as their nonTG littermates [24]. Furthermore, each group demonstrated a significant increase in Ca^{2+} sensitivity of the contractile apparatus at long vs. short sarcomere lengths. However, the length-dependent increase in Ca^{2+} sensitivity was significantly greater in FVB/N mice than in either $\text{caPKC}\epsilon$ or CRNK cardiomyocytes, and concomitant CRNK expression in $\text{caPKC}\epsilon$ mice did not restore length-dependency of Ca^{2+} sensitivity to that of wildtype, FVB/N littermates (Figure 4D).

3.6. Cardiac troponin I phosphorylation in FVB/N, $\text{caPKC}\epsilon$, CRNK and PXC mice

Differences in maximal force generation and length-dependent Ca^{2+} sensitivity may be related to differences in cardiac troponin I (cTnI) phosphorylation [28]. Using a back-phosphorylation protocol [24] and a novel one-dimensional, non-equilibrium isoelectric focusing technique [29], Goldspink and coworkers previously demonstrated an increase in phosphorylation of cTnI (and cTnT) in cardiac myofilaments of aging $\text{caPKC}\epsilon$ mice as compared to nonTG FVB/N controls. However, Western blot analysis of cTnI phosphorylation at Ser23/Ser24 (a putative PKA phosphorylation site) revealed no major differences amongst the four groups (Figure 5A and 5B). In contrast, phosphorylation of cTnI at Thr143 (a putative PKC phosphorylation site) was increased 2.1 ± 0.4 -fold in $\text{caPKC}\epsilon$ mice as compared to nonTG controls, and Thr143 phosphorylation remained increased in PXC mice (1.7 ± 0.4 -fold), although there was a small but statistically significant reduction in cTnI-Thr143 phosphorylation comparing $\text{caPKC}\epsilon$ vs. PXC mice (Figure 5C and 5D).

3.7. CRNK prevents the MHC isoenzyme switch and SERCA2 down-regulation in caPKC ϵ mice

As seen in Figure 6, decreased contractile function of caPKC ϵ mice was accompanied by alterations in cardiomyocyte gene expression characteristic of pathological LV remodeling and HF. Twelve-month old caPKC ϵ mice had significant up-regulation of β MHC mRNA levels (3.8 ± 0.8 fold; Figure 6B), along with a small, statistically insignificant reduction in α MHC mRNA levels (0.8 ± 0.1 fold; Figure 6A). LV ANF mRNA levels were also significantly increased (7.3 ± 0.9 -fold; Figure 6C). Although LV α MHC, β MHC and ANF mRNA levels in CRNK mice were similar to their nonTG littermates, concomitant CRNK expression in caPKC ϵ mice reduced β MHC and ANF expression to that observed in control mice. Furthermore, LV SERCA2 mRNA levels in 12mo caPKC ϵ mice were reduced $40 \pm 5\%$ as compared to 12mo FVB/N mice, but were normalized in PXC mice (Figure 6D). The observed down-regulation of SERCA2 mRNA levels in caPKC ϵ mice led to a significant reduction in SERCA2 protein levels. As seen in Figure 6E and 6F, Western blot analysis of LV tissue extracts revealed a parallel reduction in SERCA2 protein, which was prevented by concomitant CRNK expression. The reduced SERCA2 expression in 12mo caPKC ϵ mice occurred in the absence of any change in phospholamban expression (Supplemental Figure 3).

To further assess the PYK2-dependence and functional significance of SERCA2 down-regulation in this model system, SERCA2 protein levels were also analyzed in 6mo animals. As seen in Supplemental Figure 4, SERCA2 levels were similar in all 4 groups, despite the fact that PYK2 expression and autophosphorylation were already increased (Figure 2), and contractile function in caPKC ϵ mice (Figure 4, and Table 1) was already significantly impaired at this age.

3.8. Effects of CRNK on procollagen gene expression and collagen accumulation in caPKC ϵ mice

Goldspink et al. [24] previously demonstrated that the noncollagenous extracellular matrix (ECM) protein osteopontin accumulated in the LV tissue of aging caPKC ϵ TG mice. Here, we examined whether Type I and Type III procollagens, the major fibrillar collagens produced by cardiac fibroblasts, also accumulate in this mouse model, and whether procollagen gene expression and collagen accumulation are affected by concomitant CRNK expression. COL1A1 and COL3A1 mRNA levels were assessed by qPCR, and fibrillar collagen accumulation was measured by analysis of protein-bound tissue hydroxyproline concentration. Of note, tissue hydroxyproline correlated reasonably well with the presence of histologically identified, interstitial fibrosis in human DCM, in which mRNA levels for COL1A1 and COL3A1 were also substantially up-regulated [26]. As seen in Figure 7A and 7B, 9-12mo caPKC ϵ mice had a ~3-fold increase in Type I and Type III procollagen mRNA levels as compared to age-matched FVB/N mice. Similarly, tissue-bound hydroxyproline concentration was also elevated ~2.3 fold (Figure 7C). Although concomitant CRNK expression had no effect on procollagen mRNA levels by cardiac fibroblasts, the increase in tissue-bound hydroxyproline concentration in caPKC ϵ mice was largely prevented by CRNK expression.

4. DISCUSSION

4.1. Beneficial effects of PYK2 inhibition by CRNK in LV remodeling

The findings from this study further elucidate the adverse effects of PYK2 expression and phosphorylation that accompany LV remodeling and HF [1,2,23,27]. In the present report, we used a cardiomyocyte-specific promoter to overexpress CRNK, the C-terminal domain of PYK2, in the intact mouse heart, and mated these mice to animals with cardiomyocyte-specific expression of α PKC ϵ . CRNK is a highly specific inhibitor of PYK2-dependent signaling [18-23], and PYK2 inhibition by CRNK was thus limited to the adult cardiomyocyte population *in vivo*. But as in our previous study of nonselective CRNK expression by adenoviral gene transfer in rats with myocardial infarction [23], the PYK2 inhibitor prevented LV dysfunction, slowed the progression of LV remodeling, and reversed some of the gene expression changes that occur during HF progression. Thus, the results of the present experiments help to further define the role of the costameric mechanosensory apparatus and PYK2 in adverse LV remodeling and HF. As a sidelight, the use of another species that is often considered an authentic model of HF further points to the relevance and impact of our studies.

4.2. LV structure and function in α PKC ϵ mice

The α PKC ϵ mouse model was specifically chosen for the present experiments because previous studies had demonstrated that PYK2 activation was regulated by upstream activation of novel PKCs in cultured cardiomyocytes [3,5,11], and cardiomyocyte α PKC ϵ formed a multiprotein signaling complex with PYK2 in the intact heart *in vivo* [30]. LV PYK2 expression and activation were indeed markedly increased in α PKC ϵ TG mice as compared to their nonTG littermates, suggesting a role for PYK2 in the pathogenesis of this and other forms of experimental and human LV remodeling and HF [1,12,31]. α PKC ϵ expression *in vivo* resulted in progressive LV dysfunction, LV dilatation and wall thinning which was apparent at 3mo of age, and which progressed to overt HF and sudden death in older mice. However, the initial decline in LV contractile function, and the subsequent structural remodeling and mortality were in large part prevented by concomitant CRNK expression.

As found here and in previous reports [24,29,32,33], we observed a significant reduction in maximal force development, along with a significant reduction in the length-dependent increase in myofilament Ca^{2+} sensitivity in aging α PKC ϵ mice. These changes were associated with a substantial increase in β MHC expression, along with a ~2-fold increase in the phosphorylation of cTnI at Thr143. Goldspink et al. [24] previously demonstrated that cTnI was hyper-phosphorylated at multiple sites in α PKC ϵ mice, and partial replacement of Ser43/45 with a nonphosphorylatable mutant of cTnI attenuated the contractile dysfunction in these animals [29]. Both Ser43/45 and Thr143 sites are considered PKC phosphorylation targets responsible for reduced maximal Ca^{2+} activated force and Ca^{2+} sensitivity of force [28]. Thus it seems likely that these PKC- and PYK2-dependent, transcriptional and post-translational changes in myofilament composition contributed to the progressive deterioration in contractile performance, but were partially prevented by CRNK. However, reduced maximal force development (and its prevention by concomitant CRNK expression)

cannot be explained solely by alterations in MHC isoenzyme composition, as the isoenzyme switch should contribute only to the slowed rate of force development and not to the depressed maximal Ca^{2+} -activated force [34-36]. Furthermore, it is conceivable that other transcriptional and post-translational modifications of the contractile apparatus occurred during the progression of LV remodeling in caPKC ϵ mice, and were responsible for the eventual decline in contractile performance.

4.3. SERCA2 gene expression in caPKC ϵ mice

In addition to increased β MHC expression, 12mo caPKC ϵ mice displayed elevated ANF mRNA levels, and reduced SERCA2 mRNA and protein levels as compared to aged-matched nonTG littermates. Reduced SERCA2 gene expression is frequently observed in experimental animal models and patients with end-stage HF [37], but its role in the initial stages of LV remodeling and HF progression remains unclear. For instance, Periasamy and colleagues [38,39] have shown that cardiomyocytes isolated from heterozygous SERCA2 knockout mice have a ~35% reduction in SERCA2 protein, a 30-40% reduction in the amplitude of the cytosolic Ca^{2+} transient, and a 40-60% reduction in SR Ca^{2+} load. Functional studies at the cardiomyocyte level as well as in the intact heart *in vivo* demonstrated a parallel reduction in the rates of isometric contraction and relaxation, which occurred in the absence of a switch in MHC isoenzymes. However, the reduction in Ca^{2+} sequestering activity of heterozygous SERCA2 knockout mice was not sufficient alone to cause LV remodeling or overt HF, probably because of other compensatory mechanisms that maintained Ca^{2+} handling and contractility.

Nevertheless, there are data to suggest that SERCA2 down-regulation may contribute more directly to the progressive LV dilatation that accompanies HF progression. For instance, increasing SERCA2 gene expression in the neonatal heart delayed the development of LV dysfunction and structural remodeling in a mouse model of familial hypertrophic cardiomyopathy [40]. Furthermore, Kawase et al. [41] reported that long-term expression of SERCA2a (by *in vivo* rAAV1-mediated intracoronary gene transfer) preserved systolic function, potentially prevented diastolic dysfunction, and reduced LV dilatation/remodeling in a pig model of volume-overload induced HF resulting from chronic mitral regurgitation. Thus, preventing SERCA2 down-regulation may have both structural and functional consequences in preserving cardiac performance during disease progression. Finally, although we observed a significant decrease in SERCA2 mRNA and protein levels in 12mo caPKC ϵ mice with HF, SERCA2 was not significantly down-regulated in younger animals, despite the fact that caPKC ϵ and PYK2 levels were increased, contractility was depressed, and LV remodeling had already commenced. These results suggest that the observed SERCA2 down-regulation in aged caPKC ϵ mice was primarily the result of, rather than the cause of the LV remodeling and HF.

4.4. Roles of PYK2 and CRNK in regulating cardiomyocyte gene expression

Although our results demonstrate that CRNK slows the progression of LV remodeling and HF in two very different model systems ([23] and this report), the responsible mechanisms remain unclear. PYK2 and CRNK contain identical focal adhesion targeting sequences that direct the proteins to cardiomyocyte focal adhesions and costameres [16]. Inhibition of

PYK2-dependent signaling by CRNK expression may thus interfere with mechanotransduction and downstream signaling to stress-activated MAPKs and Akt that occur within these structures. In high-density, spontaneously contracting neonatal rat ventricular myocytes (NRVM), adenovirus-mediated CRNK expression had no effect on NRVM growth or viability, but the PYK2 inhibitor significantly reduced basal ANF mRNA levels, suggesting a role for PYK2 in directly (or indirectly) mediating the PKC and Ca^{2+} dependence of ANF gene transcription [42, 43]. CRNK expression also affected MHC gene expression, and substantially increased SERCA2 mRNA levels [23]. In contrast, in vivo cardiomyocyte CRNK expression alone did not substantially affect mRNA abundance of these gene products, but this is consistent with the relatively low levels of PYK2 expression and phosphorylation in adult as compared to neonatal cardiomyocytes [3]. Rather, CRNK expression in PXC mice dramatically reduced the increased β MHC and ANF expression observed in caPKC ϵ mice. Our data also confirm the specificity of CRNK for PYK2 inhibition [18], as CRNK had no apparent effect on FAK activation or expression, but substantially reduced the ratio of phosphorylated to total PYK2 in LV cardiomyocytes of caPKC ϵ mice.

In addition to preventing the up-regulation of ANF and β MHC, CRNK-mediated PYK2 inhibition also prevented the down-regulation of SERCA2 mRNA and protein observed in 12 mo caPKC ϵ mice. Our previous studies suggested that PYK2 is a component of a PKC ϵ /PYK2/MAPK signaling pathway that may regulate SERCA2 gene transcription in cultured NRVM [14,15], and our present results suggest that CRNK-mediated inhibition of cardiomyocyte PYK2 in vivo prevents SERCA2 down-regulation in caPKC ϵ mice. In another cell culture study [16], we used adenoviruses to overexpress wildtype and mutant forms of PYK2, and found that PYK2 was sufficient to down-regulate SERCA2 gene transcription in NRVM. However, there were a number of peculiar aspects of the study that suggested that the effects of PYK2 on SERCA2 might be indirect. First, both kinase-dead and nonphosphorylatable PYK2 mutants also down-regulated SERCA2 mRNA levels, suggesting that it was the scaffolding function of PYK2 rather than its direct kinase activity that was important. Also, PYK2-dependent SERCA2 down-regulation required at least 48h to develop, and involved downstream JNK/p38^{MAPK} activation which often occurs indirectly in a variety of stress responses. Finally, we provided evidence for a PKC-dependent, *PYK2-independent* signaling pathway that was also operative in NRVM. With respect to our previous study of CRNK gene transfer [23], we found that CRNK expression for 48h had no effect on total protein/DNA ratio (a measure of cardiomyocyte hypertrophy) as compared to uninfected NRVM, or NRVM infected with a control adenovirus. CRNK also did not prevent the increase in total protein/DNA ratio elicited by treatment with phorbol myristate acetate. However, CRNK expression did increase SERCA2 mRNA levels over 48h, in keeping with PYK2's potential role in directly (or indirectly) regulating SERCA2 gene transcription in NRVM. Nevertheless, CRNK gene transfer in vivo was ineffective in up-regulating SERCA2 mRNA in the normal myocardium, or in remodeled myocardium following myocardial infarction [23]. These data, along with the present results suggest that the effects of PYK2 on SERCA2 are likely to be indirect. Furthermore, the beneficial effects of CRNK on SERCA2 primarily resulted from a slower rate of progression

of LV remodeling and HF, rather than a direct, or indirect effect of PYK2 on SERCA2 gene expression.

4.5. LV structure and function in CRNK mice

We also evaluated the effects of CRNK expression alone on LV structure and function. Survival of CRNK mice was similar to nonTG littermates, and CRNK had no significant effect on echocardiographic or invasive indices of cardiac structure and function in vivo. It should be pointed out, however, that isolated skinned myofilaments of CRNK mice also displayed reduced maximal force generation, and a reduced length-dependent increase in maximal force as compared to FVB/N mice. Furthermore, the length-dependent increase in Ca^{2+} sensitivity of the contractile apparatus was smaller than in FVB/N mice. These changes occurred without a significant increase in βMHC expression, or alterations in cTnI phosphorylation at either Ser23/24 Thr143. Indeed, the responsible mechanisms for these changes are at present unknown, but may be related to previously unrecognized effects of PYK2 (or CRNK) on myofilament properties and composition. It is also conceivable that the CRNK-induced reduction in myofilament Ca^{2+} sensitivity and maximal force generation were compensated for by alterations in Ca^{2+} handling that maintained cardiomyocyte contractile performance at normal or near normal levels. It is important to note that these changes were also not sufficient to reduce global contractile performance, induce LV remodeling, or significantly impact survival in CRNK mice as compared to wildtype FVB/N littermates.

4.6. Summary

In conclusion, our present results demonstrate that cardiomyocyte-specific expression of CRNK improves contractile function and slows LV remodeling in a mouse model of DCM. Although additional studies are required to elucidate the mechanisms responsible for CRNK's beneficial effects, the development of a small-molecule inhibitor of PYK2 may have considerable utility in future therapies designed to slow the progression of LV remodeling and HF in DCM patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources: Supported by NIH P01 HL62426, R01 DA030996, R01 HL75494, and F32 HL096143.

References

1. Melendez J, Welch S, Schaefer E, Moravec CS, Avraham S, Avraham H, et al. Activation of Pyk2/related focal adhesion tyrosine kinase and focal adhesion kinase in cardiac remodeling. *J Biol Chem.* 2002; 277:45203–45210. [PubMed: 12228222]
2. Bayer AL, Heidkamp MC, Patel N, Porter MJ, Engman SJ, Samarel AM. PYK2 expression and phosphorylation increases in pressure overload-induced left ventricular hypertrophy. *Am J Physiol Heart Circ Physiol.* 2002; 283:H695–H706. [PubMed: 12124218]
3. Bayer AL, Ferguson AG, Lucchesi PA, Samarel AM. Pyk2 expression and phosphorylation in neonatal and adult cardiomyocytes. *J Mol Cell Cardiol.* 2001; 33:1017–1030. [PubMed: 11343423]

4. Menashi EB, Loftus JC. Differential effects of Pyk2 and FAK on the hypertrophic response of cardiac myocytes. *Cell Tissue Res.* 2009; 337:243–255. [PubMed: 19484266]
5. Bayer AL, Heidkamp MC, Howes AL, Heller Brown J, Byron KL, Samarel AM. Protein kinase C ϵ -dependent activation of proline-rich tyrosine kinase 2 in neonatal rat ventricular myocytes. *J Mol Cell Cardiol.* 2003; 35:1121–1133. [PubMed: 12967635]
6. Kohno T, Matsuda E, Sasaki H, Sasaki T. Protein-tyrosine kinase CAK β /PYK2 is activated by binding Ca²⁺/calmodulin to FERM F2 α 2 helix and thus forming its dimer. *Biochem J.* 2008; 410:513–523. [PubMed: 18031286]
7. Park SY, Avraham HK, Avraham S. RAFTK/Pyk2 activation is mediated by trans-acting autophosphorylation in a Src-independent manner. *J Biol Chem.* 2004; 279:33315–33322. [PubMed: 15166227]
8. Blaukat A, Ivankovic-Dikic I, Gronroos E, Dolfi F, Tokiwa G, Vuori K, et al. Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J Biol Chem.* 1999; 274:14893–14901. [PubMed: 10329689]
9. Pandey P, Avraham S, Kumar S, Nakazawa A, Place A, Ghanem L, et al. Activation of p38 mitogen-activated protein kinase by PYK2/related adhesion focal tyrosine kinase-dependent mechanism. *J Biol Chem.* 1999; 274:10140–10144. [PubMed: 10187797]
10. Taniyama Y, Weber DS, Rocic P, Hilenski L, Akers ML, Park J, et al. Pyk2- and Src-dependent tyrosine phosphorylation of PDK1 regulates focal adhesions. *Mol Cell Biol.* 2003; 23:8019–8029. [PubMed: 14585963]
11. Guo J, Sabri A, Elouardighi H, Rybin V, Steinberg SF. α_1 -adrenergic receptors activate AKT via a Pyk2/PDK-1 pathway that is tonically inhibited by novel protein kinase C isoforms in cardiomyocytes. *Circ Res.* 2006; 99:1367–1375. [PubMed: 17110596]
12. Lang D, Glukhov AV, Efimova T, Efimov IR. Role of Pyk2 in cardiac arrhythmogenesis. *Am J Physiol Heart Circ Physiol.* 2011; 301:H975–H983. [PubMed: 21666110]
13. Chu M, Koshman Y, Iyengar R, Kim T, Russell B, Samarel AM. Contractile activity regulates inducible nitric oxide synthase expression and NO_i production in cardiomyocytes via a FAK-dependent signaling pathway. *J Signal Transduct.* 2012; 2012:473410. [PubMed: 22900166]
14. Qi M, Bassani JW, Bers DM, Samarel AM. Phorbol 12-myristate 13-acetate alters SR Ca²⁺-ATPase gene expression in cultured neonatal rat heart cells. *Am J Physiol.* 1996; 271:H1031–H1039. [PubMed: 8853338]
15. Porter MJ, Heidkamp MC, Scully BT, Patel N, Martin JL, Samarel AM. Isoenzyme-selective regulation of SERCA2 gene expression by protein kinase C in neonatal rat ventricular myocytes. *Am J Physiol Cell Physiol.* 2003; 285:C39–C47. [PubMed: 12606313]
16. Heidkamp MC, Scully BT, Vijayan K, Engman SJ, Szotek EL, Samarel AM. PYK2 regulates SERCA2 gene expression in neonatal rat ventricular myocytes. *Am J Physiol Cell Physiol.* 2005; 289:C471–C482. [PubMed: 15829561]
17. Xiong WC, Macklem M, Parsons JT. Expression and characterization of splice variants of PYK2, a focal adhesion kinase-related protein. *J Cell Sci.* 1998; 111:1981–1991. [PubMed: 9645946]
18. Li X, Dy RC, Cance WG, Graves LM, Earp HS. Interactions between two cytoskeleton-associated tyrosine kinases: calcium-dependent tyrosine kinase and focal adhesion tyrosine kinase. *J Biol Chem.* 1999; 274:8917–8924. [PubMed: 10085136]
19. Park JG, Bose A, Leszyk J, Czech MP. PYK2 as a mediator of endothelin-1/G α_{11} signaling to GLUT4 glucose transporters. *J Biol Chem.* 2001; 276:47751–47754. [PubMed: 11602570]
20. Sorokin A, Kozlowski P, Graves L, Philip A. Protein-tyrosine kinase Pyk2 mediates endothelin-induced p38^{MAPK} activation in glomerular mesangial cells. *J Biol Chem.* 2001; 276:21521–21528. [PubMed: 11278444]
21. Watson JM, Harding TW, Golubovskaya V, Morris JS, Hunter D, Li X, et al. Inhibition of the calcium-dependent tyrosine kinase (CADTK) blocks monocyte spreading and motility. *J Biol Chem.* 2001; 276:3536–3542. [PubMed: 11062241]
22. van Buul JD, Anthony EC, Fernandez-Borja M, Burrige K, Hordijk PL. Proline-rich tyrosine kinase 2 (Pyk2) mediates vascular endothelial-cadherin-based cell-cell adhesion by regulating beta-catenin tyrosine phosphorylation. *J Biol Chem.* 2005; 280:21129–21136. [PubMed: 15778498]

23. Hart DL, Heidkamp MC, Iyengar R, Vijayan K, Szotek EL, Barakat JA, et al. CRNK gene transfer improves function and reverses the myosin heavy chain isoenzyme switch during post-myocardial infarction left ventricular remodeling. *J Mol Cell Cardiol.* 2008; 45:93–105. [PubMed: 18495152]
24. Goldspink PH, Montgomery DE, Walker LA, Urboniene D, McKinney RD, Geenen DL, et al. Protein kinase C ϵ overexpression alters myofilament properties and composition during the progression of heart failure. *Circ Res.* 2004; 95:424–432. [PubMed: 15242976]
25. Schlaepfer DD, Hunter T. Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol.* 1996; 16:5623–5633. [PubMed: 8816475]
26. Koshman YE, Patel N, Chu M, Iyengar R, Kim T, Ersahin C, et al. Regulation of connective tissue growth factor gene expression and fibrosis in human heart failure. *J Card Fail.* 2013; 19:283–294. [PubMed: 23582094]
27. Melendez J, Turner C, Avraham H, Steinberg SF, Schaefer E, Sussman MA. Cardiomyocyte apoptosis triggered by RAFTK/Pyk2 via Src kinase is antagonized by paxillin. *J Biol Chem.* 2004; 279:53516–53523. [PubMed: 15322113]
28. Hinken AC, Hanft LM, Scruggs SB, Sadayappan S, Robbins J, Solaro RJ, et al. Protein kinase C depresses cardiac myocyte power output and attenuates myofilament responses induced by protein kinase A. *J Muscle Res Cell Motil.* 2012; 33:439–448. [PubMed: 22527640]
29. Scruggs SB, Walker LA, Lyu T, Geenen DL, Solaro RJ, Buttrick PM, et al. Partial replacement of cardiac troponin I with a non-phosphorylatable mutant at serines 43/45 attenuates the contractile dysfunction associated with PKC ϵ phosphorylation. *J Mol Cell Cardiol.* 2006; 40:465–473. [PubMed: 16445938]
30. Ping P, Zhang J, Pierce WM Jr, Bolli R. Functional proteomic analysis of protein kinase C ϵ signaling complexes in the normal heart and during cardioprotection. *Circ Res.* 2001; 88:59–62. [PubMed: 11139474]
31. Bayer AL, Heidkamp MC, Patel N, Porter M, Engman S, Samarel AM. Alterations in protein kinase C isoenzyme expression and autophosphorylation during the progression of pressure overload-induced left ventricular hypertrophy. *Mol Cell Biochem.* 2003; 242:145–152. [PubMed: 12619877]
32. Montgomery DE, Rundell VL, Goldspink PH, Urboniene D, Geenen DL, de Tombe PP, et al. Protein kinase C ϵ induces systolic cardiac failure marked by exhausted inotropic reserve and intact Frank-Starling mechanism. *Am J Physiol Heart Circ Physiol.* 2005; 289:H1881–H1888. [PubMed: 15951344]
33. Hankiewicz JH, Goldspink PH, Buttrick PM, Lewandowski ED. Principal strain changes precede ventricular wall thinning during transition to heart failure in a mouse model of dilated cardiomyopathy. *Am J Physiol Heart Circ Physiol.* 2008; 294:H330–H336. [PubMed: 17965277]
34. Metzger JM, Wahr PA, Michele DE, Albayya F, Westfall MV. Effects of myosin heavy chain isoform switching on Ca²⁺-activated tension development in single adult cardiac myocytes. *Circ Res.* 1999; 84:1310–1317. [PubMed: 10364569]
35. Perez NG, Hashimoto K, McCune S, Altschuld RA, Marban E. Origin of contractile dysfunction in heart failure: calcium cycling versus myofilaments. *Circulation.* 1999; 99:1077–1083. [PubMed: 10051303]
36. Rundell VL, Manaves V, Martin AF, de Tombe PP. Impact of β -myosin heavy chain isoform expression on cross-bridge cycling kinetics. *Am J Physiol Heart Circ Physiol.* 2005; 288:H896–H903. [PubMed: 15471982]
37. Zarain-Herzberg A, Estrada-Aviles R, Fragoso-Medina J. Regulation of sarco(endo)plasmic reticulum Ca²⁺-ATPase and calsequestrin gene expression in the heart. *Can J Physiol Pharmacol.* 2012; 90:1017–1028. [PubMed: 22784385]
38. Ji Y, Lalli MJ, Babu GJ, Xu Y, Kirkpatrick DL, Liu LH, et al. Disruption of a single copy of the SERCA2 gene results in altered Ca²⁺ homeostasis and cardiomyocyte function. *J Biol Chem.* 2000; 275:38073–38080. [PubMed: 10970890]
39. Periasamy M, Reed TD, Liu LH, Ji Y, Loukianov E, Paul RJ, et al. Impaired cardiac performance in heterozygous mice with a null mutation in the sarco(endo)plasmic reticulum Ca²⁺-ATPase isoform 2 (SERCA2) gene. *J Biol Chem.* 1999; 274:2556–2562. [PubMed: 9891028]

40. Pena JR, Szkudlarek AC, Warren CM, Heinrich LS, Gaffin RD, Jagatheesan G, et al. Neonatal gene transfer of SERCA2a delays onset of hypertrophic remodeling and improves function in familial hypertrophic cardiomyopathy. *J Mol Cell Cardiol.* 2010; 49:993–1002. [PubMed: 20854827]
41. Kawase Y, Ly HQ, Prunier F, Lebeche D, Shi Y, Jin H, et al. Reversal of cardiac dysfunction after long-term expression of SERCA2a by gene transfer in a pre-clinical model of heart failure. *J Am Coll Cardiol.* 2008; 51:1112–1119. [PubMed: 18342232]
42. McDonough PM, Stella SL, Glembotski CC. Involvement of cytoplasmic calcium and protein kinases in the regulation of atrial natriuretic factor secretion by contraction rate and endothelin. *J Biol Chem.* 1994; 269:9466–9472. [PubMed: 7511588]
43. Hirotsani S, Higuchi Y, Nishida K, Nakayama H, Yamaguchi O, Hikoso S, et al. Ca^{2+} -sensitive tyrosine kinase Pyk2/CAK β -dependent signaling is essential for G-protein-coupled receptor agonist-induced hypertrophy. *J Mol Cell Cardiol.* 2004; 36:799–807. [PubMed: 15158121]

Highlights

- PYK2 is involved in left ventricular (LV) remodeling and heart failure (HF)
- PYK2 activation is prevented by CRNK, the C-terminal domain of PYK2
- Constitutively active PKC ϵ (caPKC ϵ) mice have a dilated cardiomyopathy and HF
- CRNK mice were crossed with caPKC ϵ mice to create double transgenic mice
- CRNK improved contractile function and slowed LV remodeling

Figure 1. Survival and characteristics of FVB/N, CRNK, caPKCε, and PXC mice
 (A) Kaplan-Meier survival curves for FVB/N (n=133) and CRNK (n=71) TG mice. Mean survival times were 349 ± 7 and 358 ± 10 days for FVB/N and CRNK mice, respectively. (B) Survival curves for caPKCε^(+/-) (n=46) and PXC (n=38) mice. Mean survival times were 307 ± 18 and 352 ± 13 days, respectively. (C) Western blot analysis of LV tissue extracts from 6mo FVB/N, caPKCε, CRNK and PXC mice (3 animals in each group). Apparent mol wt (kDa) of protein standards is depicted at the left of each blot.

Figure 2. Effects of caPKCε and CRNK on PYK2 phosphorylation and expression

(A) Representative Western blots of LV tissue extracts from 12mo FVB/N, caPKCε, CRNK and PXC mice (3 animals in each group). Apparent mol wt (kDa) of protein standards is depicted at the left of each blot. (B) Quantitative analysis of total PYK2/GAPDH ratio, (C) phosphorylated PYK2 (pPYK2)/GAPDH ratio, and (D) pPYK2/total PYK2 ratio. Data are means±SEM from n=8-9 mice in each group (represented by numbers within the bars), in which results for each animal were normalized and expressed as fold difference of the average of 3 FVB/N mice on each blot. * $P<0.05$ vs.FVB/N.

Figure 3. CRNK slows the progressive deterioration of LV structure and function in caPKCε mice

Serial 2-D guided, M-mode echocardiography was performed on 1, 3, 6, 9, and 12mo FVB/N, caPKCε, CRNK and PXC mice. (A) Echo-derived LV mass (mg); (B) LV/Body Wt ratio (mg/g); (C) LV fractional shortening (FS, %); (D) LV ejection fraction (EF, %); (E) LV end-systolic volume (LVESV, μ L); (F) LV end-diastolic volume (LVEDV, μ L); (G) LV

stroke volume (SV, μL); (H) LV remodeling index (LVRI, mm/mm). Data are means $\pm$ SEM for n=8-54 mice in each group. The number of animals in each group is depicted within the bars in (A). Data were analyzed by 2- and 1-way ANOVA. * $P<0.05$, FVB/N vs. caPKC ϵ ; $^+P<0.05$, PKC ϵ vs. PXC at each age group. Additional statistical analyses are described in the text.

Figure 4. Maximal force generation and Ca²⁺ sensitivity of myofilaments

Mechanically dissociated, skinned cardiomyocytes from 9-12 mo animals in each group were used to measure sarcomere function in response to varying Ca²⁺ concentrations at both short (1.9μm) and long (2.3μm) sarcomere lengths. (A) Maximal force generation and (B) the change in maximal force generation between the 2 sarcomere lengths are depicted. Data are means±SEM for each parameter derived from 6-11 cells in each group. **P*<0.05 vs. FVB/N at 1.9mm; +*P*<0.05 vs. FVB/N at 2.3mm; #*P*<0.05 for each genotype at 1.9 vs. 2.3mm. (C) Ca²⁺ sensitivity of the contractile apparatus at short and long sarcomere lengths; and (D) the length-dependent increase in Ca²⁺ sensitivity. Data are means±SEM for each parameter derived from 6-11 cells in each group. The number of animals in each group is depicted within the bars of each graph. **P*<0.05 vs. FVB/N; +*P*<0.05 vs. PXC.

Figure 5. Cardiac troponin I phosphorylation in FVB/N, caPKCε, CRNK and PXC mice
 (A) Representative Western blots of LV tissue extracts from 12mo FVB/N, caPKCε, CRNK and PXC mice (3 animals in each group). Apparent mol wt (kDa) of protein standards is depicted at the left of each blot. (B) Quantitative analysis of the ratio of cTnI phosphorylation at Ser23/Ser24 and Total cTnI. Data are means ± SEM from 12 9-12mo mice in each group, in which results for each animal were normalized and expressed as fold difference of the average of 3 FVB/N mice on each blot. The number of animals in each group is depicted within the bars of the graph. (C) Representative Western blots of LV tissue extracts from 9mo FVB/N, caPKCε, CRNK and PXC mice (3 animals in each group). Apparent mol wt (kDa) of protein standards is depicted at the left of each blot. (D) Quantitative analysis of the ratio of cTnI phosphorylation at Thr143 and Total cTnI. Data are means ± SEM from 12 9-12mo mice in each group, in which results for each animal were normalized and expressed as fold difference of the average of 3 FVB/N mice. The number of animals in each group is depicted within the bars of each graph.

Figure 6. CRNK prevents the myosin heavy chain (MHC) isoenzyme switch and SERCA2 down-regulation in caPKCε mice

Total RNA was isolated from LV tissue of 12mo FVB/N, caPKCε, CRNK and PXC mice (n=8 animals in each group). RNA was reverse-transcribed and subjected to qPCR with primers and probes specific for (A) αMHC; (B) βMHC; (C) ANF; and (D) SERCA2 mRNAs. Levels for each mRNA were normalized to the level of 18S rRNA in each sample, and expressed relative to the levels observed in FVB/N mice. Data are means±SEM; The number of animals in each group is depicted within the bars of each graph. **P*<0.05 vs. FVB/N. (E) Representative Western blots of LV tissue extracts from 12mo FVB/N, caPKCε, CRNK and PXC mice (2 animals in each group). Apparent mol wt (kDa) of protein standards is depicted at the left of each blot. (F) Quantitative analysis of SERCA2/GAPDH protein ratio. Data are means±SEM from 8-9 mice in each group, in which results for each animal were normalized and expressed as the fold-difference of the average of 2-3 FVB/N mice on each blot. The number of animals in each group is depicted within the bars of each graph. **P*<0.05 vs. FVB/N.

Figure 7. CRNK prevents fibrillar collagen deposition in caPKCε mice

Total RNA was isolated from LV tissue of 9-12mo FVB/N, caPKCε, CRNK and PXC mice (n=12-16 animals in each group). RNA was reverse-transcribed and subjected to qPCR with primers and probes specific for (A) COL1A1 mRNA and (B) COL3A1 mRNA. mRNA levels for each mRNA were normalized to the level of 18S rRNA in each sample, and expressed relative to the levels observed in FVB/N mice. Data are means±SEM; the number of animals in each group is depicted within the bars of each graph. * $P<0.05$ vs. FVB/N. (C) Protein-bound hydroxyproline in LV tissue hydrolysates of 12mo FVB/N, caPKCε, CRNK and PXC mice was measured by colorimetric assay, and expressed as μg/mg total protein. Data are the means±SEM for 9-14 animals in each group. The number of animals in each group is depicted within the bars of each graph. * $P<0.05$ vs. FVB/N.

Table 1

LV Catheterization Data of FVB/N, caPKCε, CRNK and PXC mice.

Parameter	6 mo				12 mo			
	FVB/N	caPKCε	CRNK	PXC	FVB/N	caPKCε	CRNK	PXC
Heart Rate (bpm)	516±8	488±10	529±25	490±21	525±22	438±19 [*]	464±26	501±14
Contractility Index (sec ⁻¹)	147±4	101±3 [*]	153±17	109±7	151±5	90±4 [*]	150±13	115±8 ⁺
-dP/dt (mmHg × sec ⁻¹)	-8477±636	-5452±299 [*]	-6992±615	-6488±499	-7360±610	-4180±441 [*]	-6669±950	-7196±874 ⁺
Tau (sec ⁻¹)	7.2±0.8	11.5±0.7 [*]	7.3±0.4	9.3±0.7 ⁺	7.5±0.5	14.7±1.3 [*]	9.8±1.5	9.5±1.0 ⁺
Maximum Systolic Pressure (mmHg)	129±8	98±4 [*]	115±5	104±7	108±5	89±7	131±10	109±5
End-diastolic Pressure (mmHg)	3±2	9±1	4±1	4±1	4±1	11±2 [*]	6±1	4±2 ⁺
End-diastolic volume (μl)	54±4	68±3 [*]	44±4	62±6	57±3	79±4 [*]	51±6	60±4 ⁺
No. of animals in each group	17	36	7	9	14	18	7	8

LV catheterization was performed on FVB/N, caPKCε, CRNK and PXC mice at 6 and 12 months of age. Data are means±SEM for 7-36 mice in each group.

^{*} $P<0.05$, FVB/N vs. caPKCε

⁺ $P<0.05$ caPKCε vs. PXC.