Molecular Cardiology

Cardiac CaM Kinase II Genes δ and γ Contribute to Adverse Remodeling but Redundantly Inhibit Calcineurin-Induced Myocardial Hypertrophy

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Background—Ca²⁺-dependent signaling through CaM Kinase II (CaMKII) and calcineurin was suggested to contribute to adverse cardiac remodeling. However, the relative importance of CaMKII versus calcineurin for adverse cardiac remodeling remained unclear.

Methods and Results—We generated double-knockout mice (DKO) lacking the 2 cardiac CaMKII genes δ and γ specifically in cardiomyocytes. We show that both CaMKII isoforms contribute redundantly to phosphorylation not only of phospholamban, ryanodine receptor 2, and histone deacetylase 4, but also calcineurin. Under baseline conditions, DKO mice are viable and display neither abnormal Ca²⁺ handling nor functional and structural changes. On pathological pressure overload and β-adrenergic stimulation, DKO mice are protected against cardiac dysfunction and interstitial fibrosis. But surprisingly and paradoxically, DKO mice develop cardiac hypertrophy driven by excessive activation of endogenous calcineurin, which is associated with a lack of phosphorylation at the auto-inhibitory calcineurin A site Ser411. Likewise, calcineurin inhibition prevents cardiac hypertrophy in DKO. On exercise performance, DKO mice show an exaggeration of cardiac hypertrophy with increased expression of the calcineurin target gene RCAN1-4 but no signs of adverse cardiac remodeling.

Conclusions—We established a mouse model in which CaMKII’s activity is specifically and completely abolished. By the use of this model we show that CaMKII induces maladaptive cardiac remodeling while it inhibits calcineurin-dependent hypertrophy. These data suggest inhibition of CaMKII but not calcineurin as a promising approach to attenuate the progression of heart failure. 

Key Words: calcineurin ■ CaMKII ■ cardiac hypertrophy ■ heart failure ■ signal transduction

Heart failure is the leading cause of death in developed countries. It results from adverse cardiac remodeling on pathological stress situations such as arterial hypertension, ischemic injuries, or genetic causes. Adverse cardiac remodeling is usually described by a combined appearance of myocardial hypertrophy, activation of a fetal gene program, cell death, and interstitial fibrosis. Ca²⁺-dependent signaling pathways including CaMKII and calcineurin were both proposed to play pivotal roles in adverse cardiac remodeling.3

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The protein kinase CaMKII consists of 4 different isoforms with distinct expression patterns. CaMKIIδ and CaMKIIβ are enriched in the brain, while CaMKIIδ and CaMKIIγ are expressed ubiquitously. CaMKIIδ is the predominant cardiac...
CaMKII isoform, but CaMKIIγ is also expressed in the heart. In human and experimental heart failure, CaMKII expression and activity is enhanced. Phosphorylation of the ryanodine receptor RyR2 by CaMKII has been reported to cause sarcoplasmic reticulum (SR) Ca²⁺ leak, which in turn seems to drive heart failure development. At the epigenetic level, CaMKII inactivates the negative regulator of adverse cardiac remodeling histone deacetylase 4 (HDAC4), leading to transcriptional activation of the myocyte enhancer factor 2 (MEF2), and phosphorylates histone 3. Transgenic overexpression of the splice variants CaMKIIδ (localizes to the nucleus) and CaMKIIδc (localizes to the cytosol) promote cardiac hypertrophy and dilated cardiomyopathy, respectively. CaMKII inhibitory peptides prevent structural heart disease. Mice with a global deletion of CaMKIIδ were protected against adverse cardiac remodeling. However, in all of these models, substantial phosphorylation of the target phospholamban (PLB) was still detectable, indicating an incomplete loss-of-function.

The protein phosphatase calcineurin consists of 2 subunits: calcineurin A (CnA), which contains the catalytic site, and calcineurin B (CnB), the small regulatory Ca²⁺-binding subunit. Transgenic CnA overexpression in mice induces massive cardiac hypertrophy by dephosphorylation-dependent translocation of the transcription factor NFAT from the cytosol to the nucleus. The CnA-α and CnA-β isoforms are expressed in the myocardium, but only CnAβ was demonstrated to be stress responsive. Moreover, mice lacking CnAβ were protected against cardiac hypertrophy. Inhibition of CnA has been suggested as a treatment option for cardiac hypertrophy because studies with the calcineurin inhibitory agent cyclosporine A (CyA) have been successful in rodents. On the other hand, the splicing variant CnA-β1 seems to activate Akt-dependent cardioprotective pathways without inducing NFAT-dependent maladaptive hypertrophy.

We developed a cardiomyocyte-specific double knockout model, in which the 2 cardiac isoforms, CaMKIIα and CaMKIIγ, were both deleted. This model identifies CaMKII as the critical regulator of maladaptive cardiac remodeling and reveals that CaMKII serves as a negative regulator of calcineurin activity in vivo. Unexpectedly, we provide evidence that cardiac hypertrophy attributable to overactivation of endogenous calcineurin does not cause systolic cardiac dysfunction.

Method

Mouse Experiments

We generated conditional knockout mice containing cardiomyocyte-specific deletions of CaMKIIδ (δ-CKO), CaMKIIγ (γ-CKO), or both isoforms (double knockout mice [DKO]) by crossing previously described conditional knockout mice with transgenic mice expressing Cre recombinase under the control of the α-myosin heavy chain promoter (αMHC-Cre) or by using the mER-Cre-mER fusion protein under the control of a cardiomyocyte-specific MHC-promoter. As controls, littermates homozygous for the conditional CaMKIIδ and CaMKIIγ alleles without αMHC-Cre transgene (δ-FF, γ-FF, and FFFF) were used. For some experiments, C57BL/6 mice were obtained from Charles River (wild-type mice). A detailed description of the surgical methods, the exercise protocol, and in vivo imaging as well as hemodynamic measurement techniques can be found in Methods in the online-only Data Supplement. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Regierungspräsidium Karlsruhe, Germany.

Results

Histology

The protocols for hematoxylin and eosin (H&E), Masson trichrome staining, and CD31 immunostaining can be found in Methods in the online-only Data Supplement.

Western Blotting, GST Pulldown, and Immunocytochemistry

The immunoblotting, GST-pulldown, and immunocytochemistry protocols as well as the antibodies used in this study can be found in Methods in the online-only Data Supplement.

CaMKII Kinase Activity

CaMKII kinase activity was measured by detecting the amount of endogenous CaMKII that associates to GST-HDAC4 419-670 or by radioactive kinase assay using β-P-ATP. A detailed description can be found in Methods in the online-only Data Supplement.

Gene Expression Analysis

The RNA isolation and quantitative RT-PCR as well as the primer sequences used in this study can be found in Methods in the online-only Data Supplement.

Culture of Primary Cardiomyocytes

Adenoviruses for gene transfer into cardiomyocytes were produced as described in Methods in the online-only Data Supplement. Neonatal mouse ventricular myocytes (NMVMs) and adult mouse ventricular myocytes were isolated from wild-type and mutant mice, and neonatal rat ventricular myocytes were isolated from Wistar rats (Charles River). A detailed description is included in Methods in the online-only Data Supplement.

Epifluorescence Microscopy

Freshly isolated adult mouse ventricular myocytes were incubated with Fura-2 AM or Indo-1 AM and Ca²⁺ transients as well as SR Ca²⁺ load were measured. Adult mouse ventricular myocytes were transilluminated by red light (>650 nm) to visualize sarcomeres, and fractional shortening was measured. A detailed description is included in Methods in the online-only Data Supplement.

Statistical Analysis

Data are summarized as mean±SEM. Statistical analysis was performed with the Graph-Pad Prism Software Package Version 5.0 (GraphPad Inc). When 2 groups were compared, we used Wilcoxon–Mann–Whitney U tests. When >2 groups were compared, a Kruskal–Wallis test was applied. When we obtained a significant P value we continued with pair-wise comparisons using Wilcoxon–Mann–Whitney U tests according to the closed testing principle. A value of P<0.05 was considered statistically significant.
models that we developed previously, and generated cardiomyocyte-specific conditional CaMKIIδ and γ single knockout (δ-CKO and γ-CKO) and CaMKIIδ/CaMKIIγ double knockout mice (DKO), CaMKIIδloxP-exon1/2-loxP (δ-FF), CaMKIIγloxP-exon1/2-loxP (γ-FF), and CaMKIIδloxP-exon1/2-loxP, CaMKIIγloxP-exon1/2-loxP (FFFF) mice were crossed to transgenic mice harboring Cre-recombinase under the control of the αMHC promoter. Western blot analysis using antibodies directed against CaMKII, CaMKIIγ, total PLB, phospho-PLB-Thr17, total RyR2, phospho-RyR2-Ser2814, phospho-RyR2-Ser2808, total HDAC4, phospho-HDAC4-Ser632, and GAPDH (as loading control). Left ventricular extracts from δ-CKO, γ-CKO and DKO mice and their Cre-negative littermates (δ-FF, γ-FF, and FFFF) were analyzed (n=4 per group). Quantitative analysis of the expression and phosphorylation of the proteins is shown at right. All values are presented as mean±SEM. *P<0.05. n.s. indicates not significant.

CaMKII DKO Mice Are Viable and Do Not Display Cardiac Abnormalities

DKO mice developed normally and showed no increase in mortality or apparent morphological abnormalities. Cardiac...
size as indicated by heart weight/body weight ratios, ventricular chamber dimensions, or fractional shortening assessed by echocardiography were unchanged in mice at age of 12 weeks (Figure 1B in the online-only Data Supplement). To study functional parameters in more detail we used a working heart preparation to standardize pre- and afterload (Figure IC in the online-only Data Supplement). We detected slight improvements in ESPVR (which was not significant in all experimental groups used in this study, see also Figure 3A) as a measure for maximal pressure that can be developed by the ventricle at any given LV volume. Based on the observation that the CaMKII sites on PLB and RyR2 were hypophosphorylated, we measured global parameters of Ca\(^{2+}\) handling (Figure ID and IE in the online-only Data Supplement). However, we did not detect any change in Ca\(^{2+}\) transients, SR Ca\(^{2+}\) content, diastolic Ca\(^{2+}\) concentration, and single-cell fractional shortening, surprisingly indicating that—at least under basal conditions—CaMKII activity is dispensable for intracellular Ca\(^{2+}\) handling and cardiomyocyte contractility.

Calcineurin Precedes CaMKII Activation on Pathological Pressure Overload

We analyzed the time course of CaMKII and calcineurin activation after TAC (Figure 2A and 2B). Starting at 24 hours after TAC, CaMKII expression (CaMKII\(_{\delta C} >\) CaMKII\(_{\delta B}\)) and CaMKII activity, as judged by its activity-dependent binding affinity to HDAC4,\(^{10}\) increased (≈2-fold; Figure 2A). We did not use the usual antibodies recognizing CaMKII

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**Figure 2.** Calcineurin precedes CaMKII activation on pathological pressure overload. Western blot analyses were performed in cardiac extracts from wild-type mice. Organs were harvested at different time points after transverse aortic constriction (TAC) surgery as indicated. A, Western blot analysis after GST-HDAC4 pulldown using GST-HDAC4 419 to 670, which contains a CaMKII-activity dependent binding site. GST-HDAC4 2 to 250, which lacks a CaMKII binding domain, served as a negative control. As an additional negative control, protein lysates from global CaMKII\(_{\delta}\) mice vs wild-type mice were used. GST-HDAC4 input was visualized by GST-immunoblotting, associated endogenous CaMKII is visualized by CaMKII-immunoblotting. The degree of CaMKII binding to GST-HDAC4 419-670 is a specific measure for CaMKII activity.\(^{10}\) A quantification of CaMKII binding normalized to the input of GST-HDAC4 is shown. B, Western blot analysis using antibodies directed against CaMKII, HDAC4, phospho-HDAC4-Ser632, RCAN1-4, and GAPDH. Quantitative analysis of the expression and phosphorylation of the proteins is shown. For all experiments n≥3 per group were used. All values are presented as mean±SEM. *P<0.05. n.s. indicates not significant.
Figure 3. Dissociation of cardiac dysfunction and interstitial fibrosis from myocardial hypertrophy. A, Double knockout (DKO) and CaMKIiδloxP-exon1/2-loxP, CaMKIiγloxP-exon1/2-loxP (FFFF) mice were randomized to either transverse aortic constriction (TAC) or sham (SH) surgery and euthanized after 3 weeks. Representative images of the total hearts, H&E, Masson trichrome, and CD31 staining, echocardiographic M-modes, and quantification of heart weight/body weight ratios, myocyte size, fibrosis area, capillary density, and fractional shortening are shown (n≥8 per group). Values of left ventricular ejection fraction, isovolumetric relaxation time constant (Tau), end systolic pressure volume relation (ESPVR), and stroke volume measured in a working heart preparation are shown (n≥7 per group). B, Fold-changes in mRNA levels of the hypertrophic markers ANP, BNP, αMHC, and βMHC and fold-changes in collagen expression (n≥5 per group). C, Heart weight/body weight ratios of SH- and TAC-operated mice with the indicated genotypes (n≥4 per group). All values are presented as mean±SEM. *P<0.05. n.s. indicates not significant.
autophosphorylation to determine CaMKII activity because in DKO 1 of these antibodies still showed a signal although CaMKII protein was gone (Figures IA and IIA in the online-only Data Supplement). As another indication for CaMKII activation, phosphorylation of HDAC4 at its CaMKII phosphorylation site Ser632 also started to increase 3 days after TAC. In contrast, expression of RCAN1-4, a specific target gene of the calcineurin-NFAT pathway, which is considered as an endogenous calcineurin reporter,\(^{29}\) was dramatically overexpressed (≈17-fold) 1 day after TAC (Figure 2B). These data indicate that CaMKII activation and HDAC4 phosphorylation depend on upstream signals distinct from the upstream signals of calcineurin activation.

**Dissociation of Cardiac Dysfunction and Interstitial Fibrosis From Myocardial Hypertrophy**

Next, we performed TAC surgeries in DKO and FFFF mice. Strikingly, DKO were protected against cardiac fibrosis, increased caspase 3/7 activity as marker for apoptosis, and systolic dysfunction and displayed an attenuated rarefication of capillarization (Figure 3A, see also Figure IIIA in the online-only Data Supplement). This protective effect was not associated with changes in intracellular Ca\(^+\) handling or cellular contractility (Figure IIC and IID in the online-only Data Supplement). Surprisingly, TAC still induced cardiac and cardiomyocyte hypertrophy in DKO. Moreover, the gene expression changes of typical fetal genes (ANP, BNP, βMHC) and remodeling marker (αMHC) were similar in DKO and FFFF (Figure 3B). These data indicate a dissociation of cardiac hypertrophy and reactivation of the fetal gene program on one hand from maladaptive cardiac remodeling including interstitial fibrosis, apoptosis, reduced capillarization, and cardiac dysfunction on the other hand. To confirm these data in an independent model, we applied neurohormonal stress to DKO. We injected isoproterenol (Iso) to DKO and FFFF mice. Iso led to cardiac hypertrophy (Figure IVA in the online-only Data Supplement) and fetal gene activation in DKO as observed in FFFF mice (Figure IVB in the online-only Data Supplement). But again and consistent with the TAC data, we observed less cardiac fibrosis, apoptotic markers, and collagen expression in DKO (Figure IVA and IVB in the online-only Data Supplement, see also Figure IIIA in the online-only Data Supplement). These observations were especially surprising in view of our previous finding that global deletion of 1 isoform, CaMKII\(\delta\), attenuated cardiac hypertrophy and fetal gene activation.\(^{30}\) However, in our previous model we could still detect substantial phosphorylation of the typical CaMKII target PLB-Thr17 in the myocardium because CaMKII\(\gamma\) was not deleted (see also Figure 1C). Thus, we hypothesized that complete CaMKII inhibition might exert dual effects on anti- and prohypertrophic pathways. To substantiate this hypothesis we performed a gene dosage experiment (Figure 3C). Consistent with our previous findings, the homozygous deletion of either CaMKII\(\delta\) or CaMKII\(\gamma\) resulted in a reduction of cardiac hypertrophy on TAC. Paradoxically, the additional heterozygous deletion of the other CaMKII isoform resulted in a less pronounced reduction of cardiac hypertrophy on TAC. Furthermore, the complete cardiac-specific deletion of all 4 CaMKII\(\delta\) and CaMKII\(\gamma\) gene copies (DKO) did not prevent cardiac hypertrophy. We also analyzed the effects of the single deletion of CaMKII\(\delta\) and CaMKII\(\gamma\) in isolated adult cardiomyocytes and could confirm the antihypertrophic effects of CaMKII\(\delta\) and CaMKII\(\gamma\) (Figure IIIC in the online-only Data Supplement). We were also interested whether CaMKII\(\delta\) or CaMKII\(\gamma\) play a specific role for apoptosis because our recent data suggested a specific role of CaMKII\(\gamma\) for apoptosis at least in macrophages.\(^{30}\) However, TUNEL assays indicated that CaMKII\(\delta\) deletion more than CaMKII\(\gamma\) deletion attenuates cardiomyocyte apoptosis and that the combined deletion is even more effective (Figure IVB in the online-only Data Supplement).

**CaMKII Controls the Calcineurin-NFAT Pathway**

Thus, we hypothesized that complete CaMKII deletion may result in an activation of a prohypertrophic pathway. Therefore, we searched for candidate signaling molecules that are activated by TAC or Iso. Whereas protein kinase D was not activated in DKO after TAC (Figure IIA in the online-only Data Supplement), we found the calcineurin reporter gene RCAN1-4 to be strongly upregulated in DKO mice after TAC and Iso (Figure 4A, Figure IIIE in the online-only Data Supplement). This could not be observed after deletion of only 1 CaMKII gene, in particular CaMKII\(\delta\) (Figure IIIE in the online-only Data Supplement), indicating that complete CaMKII deletion in the heart results in calcineurin activation. Consistently, Iso induced a dramatic increase in nuclear NFAT translocation and NFAT activity in NMVMs from DKO when compared with NMVMs from FFFF (Figure 4B and 4C). In this regard, it was reported that CaMKII directly phosphorylates CnA-Ser411 in vitro but it remained unclear whether this mechanism was of biological significance in vivo.\(^{31,32}\) Thus, using a newly synthesized specific antibody against CnA-Ser411 (Figure 4D) we aimed to detect endogenous CnA phosphorylation. Indeed, overexpression of active CaMKII\(\delta\) (T287D) in neonatal rat ventricular myocytes resulted in hyper-phosphorylation of CnA-Ser411 (Figure 4E). In accordance, we found that CnA was strongly hypophosphorylated in cardiac lysates from sham- and TAC-operated DKO hearts, which was associated with RCAN1-4 upregulation (Figure 4F). Moreover, as compared with DKO, CnA-Ser411 hypophosphorylation was less pronounced in cardiac extracts of δ-CKO, and not found in γ-CKO, explaining why calcineurin activity was only strongly increased in DKO but not in the single knockouts (Figure 4G).

**Cardiac Hypertrophy, but Not Dysfunction, Is Controlled by Calcineurin During Pathological and Physiological Hypertrophy in DKO**

To test whether cardiac hypertrophy after TAC in DKO depended on calcineurin activation, we performed CnA knockdown experiments in isolated NMVMs of mutant mice. CnA knockdown, which resulted in ≈50% decrease of CnA protein (Figure VA in the online-only Data Supplement), prevented Iso-induced cellular hypertrophy and activation of fetal genes in myocytes derived from DKO but not FFFF (Figure 5A and 5B). We then repeated the TAC experiment in DKO with the calcineurin inhibitor CyA. CyA was shown to inhibit cardiac hypertrophy after TAC in dosages...
Figure 4. CaMKII controls calcineurin A (CnA) activity. A, Real-time-PCR analysis of RCAN1-4 in hearts from animals with genotypes and treatments as indicated (≥5 per group). B and C, Neonatal mouse ventricular myocytes (NMVMs) from double knockout (DKO) and CaMKIIδloxP-exon1/2-loxP, CaMKIIγloxP-exon1/2-loxP (FFFF) mice were used for cell-based experiments. B, NFAT-GFP was adenovirally expressed in NMVMs. Cells were stained for α-actinin (shown in red) and with DAPI nuclear stain (shown in blue). The percentage of cells in which NFAT-GFP was localized to the nucleus is indicated (>100 cardiomyocytes per well; n≥3 per condition). C, NFAT-luciferase reporter assay in NMVMs (n≥3 per condition). D, Western blot analysis was performed using lysates from COS cells that were transfected with CnA in the absence and presence of constitutive active CaMKIIδ (CaMKII-T287D). A schematic diagram of CnA indicates the position of the CaMKII phosphorylation site at Ser411, which is located within the calmodulin-binding domain of CnA (AID indicates autoinhibitory domain; CalM, calmodulin-binding domain; CnB, calcineurin B-binding domain). E, Adenoviral overexpression of CaMKII-T287D (adCaMKII-T287D) in neonatal rat ventricular myocytes (NRVMs) results in hyper-phosphorylation of CnA-Ser411 compared with noninfected (control) NRVMs and NRVMs infected with GFP. F, Western blot analysis of total CaMKII, RCAN1-4, total CnA, and CnA-Ser411 in cardiac extracts from FFFF and DKO mice 3 weeks after transverse aortic constriction (TAC) or sham (SH) surgery (n≥3 per group). G, Western blot analysis of total CnA and CnA-Ser411 in δCKO, γCKO or DKO vs their Cre-negative littermates (n=4 per group). For all Western blot experiments, GAPDH was used as loading control. Quantitative analysis of the expression and phosphorylation of the proteins is shown at right (F and G). All values are presented as mean±SEM. *P<0.05. n.s. indicates not significant.
from 12.5 to 50 mg/kg body weight per day. Using a dosage of 4 mg/kg body weight per day, which did not affect TAC-induced cardiac hypertrophy in FFFF, we were able to inhibit RCAN1-4 expression as well as cardiac and cardiomyocyte hypertrophy in DKO but not FFFF (Figure 5C and Figure VB in the online-only Data Supplement), indicating that TAC-dependent hypertrophy in DKO depends on calcineurin. As CnA knockdown, CyA attenuated also the expression of fetal genes in DKO (Figure 5B and Figure VB in the online-only Data Supplement). In contrast to the...
antihypertrophic effects of CyA in DKO. CyA did not affect the protective effects of DKO on cardiac fibrosis or systolic function, providing evidence that—at least in this model—calcineurin-dependent cardiac hypertrophy is not maladaptive on the one hand but also not cardioprotective on the other hand. In line with the latter, we could not accumulate evidence that the potential cardioprotective calcineurin-dependent Akt pathway was activated (Figure VC in the online-only Data Supplement).

To clarify the role of the CaMKII-calcineurin pathway in physiological hypertrophy, we performed endurance exercise training by a swimming protocol in DKO and FFFF mice (Figure 5D and 5F). Interestingly, whereas CaMKII protein levels were not altered after swimming in control mice (FFF), CaMKII activity decreased about 30% (Figure 5D). Remarkably, we found exaggerated heart weight/body weight ratios and increased myocyte size in DKO mice after swimming, which was associated with increased expression of the calcineurin target gene RCAN1-4 (Figure 5E and 5F). Again, despite calcineurin activation and increased cardiac hypertrophy no signs for systolic cardiac dysfunction were detected, indicating that activation of endogenous calcineurin is not maladaptive.

Discussion

By generating mice lacking the 2 cardiac CaMKII isoforms δ and γ we introduce a new loss-of-function model with the deletion of both cardiac CaMKII isoforms in cardiomyocytes, and demonstrate that CaMKII—under unstressed conditions—is dispensable in cardiomyocytes with regard to integrity, growth, cardiomyocyte contractility, and Ca2+-handling. This is somewhat surprising in light of numerous reports about CaMKII functions. But from the drug developer’s point of view, this indicates that CaMKII inhibition does not exert major unwanted side effects on basal cardiac function. Furthermore, we show that—on pathological stress conditions—CaMKII plays a dual role in the regulation of adverse cardiac remodeling (Figure 6). On the one hand, CaMKII transduces maladaptive signaling. On the other hand, CaMKII negatively regulates calcineurin-dependent cardiac hypertrophy in vivo.

Redundancy of CaMKIIδ and CaMKIIγ

Because of a lack of loss-of-function models with the deletion of both cardiac CaMKII isoforms, it was so far not possible to study all essential roles of CaMKII in myocardial disease. Moreover, most of the reported CaMKII studies used nongenetic approaches such as overexpression of peptides or pharmacological compounds such as KN-93. However, off-target effects (as, eg, described on protein kinase D or ion channels [eg, L-type Ca2+ channels])34,35 could have complicated the conclusions. Therefore, we focused first on the development of a specific and complete genetic mouse model, in which the 2 CaMKII genes that are expressed in cardiomyocytes are deleted. We found that CaMKIIδ and CaMKIIγ compensate for each other and act redundantly on the phosphorylation of PLB-Thr17, RyR2-Ser2814, CnA-Ser411, and HDAC4-Ser632. In contrast to previously described approaches aiming at inhibiting CaMKII,7,8,17,18,37 the new DKO model results in profound hypophosphorylation of these typical CaMKII phosphorylation targets.

CaMKII Controls Calcineurin Signaling

Previous data from our laboratory showed that homozygous CaMKIIδ knockout mice are protected against cardiac hypertrophy and interstitial fibrosis.8 Here we demonstrate that the loss of >2 cardiac CaMKII gene copies unmasks a dual role of CaMKII in regulating cardiac hypertrophy. DKO mice appear to develop a similar hypertrophic response as FFFF littermates after TAC and Iso, but histological, gene expression, and biochemical analyses revealed that cardiac hypertrophy in DKO depends on activation of endogenous calcineurin. CaMKII phosphorylates CnA-Ser411, which was shown to lead to a decrease in its phosphatase activity.31,32 Here, we show that this phosphorylation event occurs in vivo and in a CaMKII-dependent manner. Moreover, we found CnA-Ser411 hypo-phosphorylation to be associated with cardiac hypertrophy after pathological and physiological stress. Importantly and in contrast to current paradigms, the data of this study suggest that antihypertrophic effects are not required for an improved cardiac function. Moreover, a vast amount of literature suggested that calcineurin induces pathological cardiac hypertrophy, ultimately leading to heart failure.20,21,37,38 However, the latter studies used forced overexpression of a truncated CnA construct lacking the regulatory domain that is phosphorylated by CaMKII, complicating the interpretation of these findings. A CnAδ loss-of-function study seemed to prove the idea that calcineurin mediates pathological cardiac hypertrophy, but this study focused mainly on cardiac hypertrophy and not on other aspects of pathological remodeling such as cardiac dysfunction.23 In fact, histological analyses did not show a protection against cardiac fibrosis in CnAδ null mice.23 In the present study we identify DKO mice as a model with activation of endogenous calcineurin. We
challenge current paradigms and conclude that—at least in the absence of CaMKII signals—activation of endogenous calcineurin is not maladaptive. Support comes from a recent study, demonstrating that calcineurin induces physiological hypertrophy in pregnant mice. Moreover, Heinke et al. suggested that calcineurin exerts cardioprotective functions under certain conditions: hearts of transgenic mice with mild overexpression of CnA were protected in a murine model of dilated cardiomyopathy. Thus it is tempting to speculate that calcineurin activation in DKO contributes to the observed cardioprotective effects on TAC. Because it was suggested that the splice variant CnAβ1 exerts cardioprotection via Akt, we measured Akt phosphorylation. But we found no changes. We cannot completely rule out that other calcineurin-dependent pathways contribute to the cardioprotective effects but our data show that CyA-treatment after TAC in DKO did not lead to cardiac dysfunction. Thus, we conclude that—in the setting of the current study—CaMKII-dependent but calcineurin-independent processes mediate pathological remodeling.

CaMKII Is Required for Pathological Remodeling but Not Cardiac Hypertrophy and Fetal Gene Expression

The question arises of how CaMKII regulates pathological remodeling, especially cardiac fibrosis. We have shown that HDAC4 transmits CaMKII signals to the cardiac genome via MEF2. Because gene deletion and overexpression studies revealed that MEF2D primarily drives cardiac fibrosis and dysfunction rather than cardiac hypertrophy, the CaMKII-HDAC4-MEF2 pathway is a likely pathway leading to adverse cardiac remodeling (see model in Figure 6). Likewise, HDAC4 hypophosphorylation was associated to the protective situation in DKO mice. But it was remarkable that DKO mice were not protected against reactivation of certain fetal genes including ANP, BNP, and βMHC. It is important to note that fetal gene activation is suggested to be driven not only by MEF2 but also NFAT. Thus, we speculate that overactivation of the calcineurin-NFAT pathway in DKO compensates for an attenuation of disturbed Ca2+ handling contributes to the improved cardiac function as observed in DKO after TAC in the present study.

CaMKII Isoforms and Calcineurin

CaMKII Is Dispensable for Intracellular Ca2+ Handling Under Unstressed Conditions

Another puzzling observation of this study was the lack of effect on Ca2+ handling, although profound changes on phosphorylation on RyR2 and PLB were detected. We speculate that the lack of CaMKII phosphorylation on Ca2+ handling proteins might be compensated by other mechanisms such as protein kinase A–mediated phosphorylation, although we could not detect significant increases in phosphorylation at the protein kinase A sites PLB-Ser16 or RyR2-Ser2808. Knock-in mouse models with a specific mutation of these sites would clarify the specific role of CaMKII phosphorylation at PLB-Thr17 and RyR2-Ser2814. Indeed, Respress et al. described RyR2-2814-S/A knock-in mice. Consistent with our data, they also observed an unaltered SR Ca2+ content, Ca2+ transient decay, and Ca2+ sparks at baseline, but they discovered that in the situation of heart failure, mutant mice were protected. Thus, further experiments are warranted to investigate DKO mice and Ca2+ handling in models of severe heart failure. However, it appears unlikely that an attenuation of disturbed Ca2+ handling contributes to the improved cardiac function as observed in DKO after TAC in the present study.

Summary

Taken together, we show that CaMKII is a key driver of maladaptive cardiac remodeling. This study describes maladaptive cardiac remodeling as a process of interstitial fibrosis and cardiac dysfunction but not cardiac hypertrophy and activation of commonly measured fetal genes such as ANP and BNP. Under certain conditions, CaMKII inhibits cardiac hypertrophy by a previously underestimated cross talk mechanism to calcineurin. In the absence of CaMKII signals, calcineurin does not seem to contribute to maladaptive cardiac remodeling, highlighting CaMKII and not calcineurin as a promising drug target to combat heart failure.

Acknowledgments

We thank Lonny Jürgensen, Jutta Krebs, Sylvia Katz, Claudia Heft, Michaela Oestriinger, and Claudia Liebetrut for their excellent technical assistance. We thank Derk Frank for RCAN PCR primer. We thank David Stanmore for editing the manuscript. We thank Lorenz Uhlmann for statistical advice.

Sources of Funding

Drs Backs, Katus, Dobrev, and Maier were supported by the DZHK (Deutsches Zentrum für Herz-Kreislauf-Forschung - German Center for Cardiovascular Research) and by the BMBF (German Ministry of Education and Research). Dr Backs was supported by the Deutsche Forschungsgemeinschaft (BA 2258/2-1, SFB 1118) and by the European Commission (FP7-Health-2010; MEDIA-261409). Dr Maier was supported by the Deutsche Forschungsgemeinschaft (SFB 1002, IRTG 1816) and the Fondation Leducq. Dr Lehman is recipient of a HRCMM (Heidelberg Research Center for Molecular Medicine) career development fellowship. Dr Kreusser was supported by a research grant from the Ernst-und-Berta-Grimmke foundation and by a Young Investigator grant of the University of Heidelberg. Dr Dobrev was supported by a grant from Foundation Leducq (07CVD03). Dr Maack was supported by the Deutsche Forschungsgemeinschaft (SFB 894 and Heisenberg Programme).

Disclosures

None.

References


Cardiac remodeling during heart failure is usually described by a combined appearance of myocardial hypertrophy, activation of a so-called fetal gene program, cell death, and interstitial fibrosis. Ca\textsuperscript{2+}-dependent signaling pathways including CaMKII and calcineurin were both proposed to play pivotal roles in adverse cardiac remodeling. By generating mice lacking the 2 cardiac CaMKII isoforms δ and γ in cardiomyocytes, we introduce a new specific and complete CaMKII loss-of-function model, and demonstrate that CaMKII—under unstressed conditions—is dispensable in cardiomyocytes with regard to integrity, growth, cardiomyocyte contractility, and Ca\textsuperscript{2+} handling. Furthermore, we show that—on pathological stress conditions—CaMKII plays a dual role in the regulation of cardiac remodeling. On the one hand, CaMKII mediates cell death, cardiac fibrosis, and left ventricular dysfunction. On the other hand, CaMKII inhibits cardiac hypertrophy and expression of certain fetal genes such as ANP and BNP. The latter is mediated through a previously underestimated cross talk to calcineurin. Importantly, in the absence of CaMKII, calcineurin does not contribute to adverse cardiac remodeling. Taken together, these data indicate that inhibition of CaMKII but not calcineurin is a promising therapeutic strategy to combat heart failure.

**CLINICAL PERSPECTIVE**

Cardiac remodeling during heart failure is usually described by a combined appearance of myocardial hypertrophy, activation of a so-called fetal gene program, cell death, and interstitial fibrosis. Ca\textsuperscript{2+}-dependent signaling pathways including CaMKII and calcineurin were both proposed to play pivotal roles in adverse cardiac remodeling. By generating mice lacking the 2 cardiac CaMKII isoforms δ and γ in cardiomyocytes, we introduce a new specific and complete CaMKII loss-of-function model, and demonstrate that CaMKII—under unstressed conditions—is dispensable in cardiomyocytes with regard to integrity, growth, cardiomyocyte contractility, and Ca\textsuperscript{2+} handling. Furthermore, we show that—on pathological stress conditions—CaMKII plays a dual role in the regulation of cardiac remodeling. On the one hand, CaMKII mediates cell death, cardiac fibrosis, and left ventricular dysfunction. On the other hand, CaMKII inhibits cardiac hypertrophy and expression of certain fetal genes such as ANP and BNP. The latter is mediated through a previously underestimated cross talk to calcineurin. Importantly, in the absence of CaMKII, calcineurin does not contribute to adverse cardiac remodeling. Taken together, these data indicate that inhibition of CaMKII but not calcineurin is a promising therapeutic strategy to combat heart failure.
Cardiac CaM Kinase II Genes δ and γ Contribute to Adverse Remodeling but Redundantly Inhibit Calcineurin-Induced Myocardial Hypertrophy

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_Circulation_. 2014;130:1262-1273; originally published online August 14, 2014;
doi: 10.1161/CIRCULATIONAHA.114.006185

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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The cardiac CaMKII genes δ and γ contribute redundantly to adverse remodeling but inhibit calcineurin-induced myocardial hypertrophy

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Supplemental Methods

**Generation of DKO mice.** All DKO animals (except the animals that were used for the endurance exercise experiment) were generated by crossing FFFF mice with transgenic mice expressing Cre-recombinase under the control of the α-MHC promoter (αMHC-Cre).\(^1\) DKO mice that were used for the endurance exercise experiment were generated by crossing FFFF mice with transgenic mice expressing the mER-Cre-mER fusion protein under control of a cardiomyocyte-specific αMHC-promoter.\(^2\) The latter animals were treated with tamoxifen to achieve CaMKII deletion. Therefore, 10 mg/ml tamoxifen (Sigma) was dissolved in a regular sunflower oil solution containing 6% ethanol. Tamoxifen was administered via gavage for 10 consecutive days once per day in a dose of 80 mg/Kg body weight per day. Efficient take-out of CaMKII was verified by Western blot. To avoid interferences with transient Cre expression, experiments were conducted at least 3 weeks after the last tamoxifen application.

**Transverse aortic constriction.** TAC to a 27 gauge stenosis was performed in 9-10 week-old male DKO, δ-KO, γ-KO, CaMKII δ\(^{-/-}\)/γ\(^{+/+}\), CaMKIIγ\(^{-/-}\)/δ\(^{+/+}\), FFFF and wild type mice as described previously.\(^3, 4\) A subgroup of the animals was treated twice daily by i.p. injection with cyclosporin A (CyA; Sandimmun, Novartis) with 24 mg, 12 mg or 4 mg/kg body weight per day for 7 days or 3 weeks, beginning at the day of surgery. CyA was diluted in 0.9% saline solution with 12% ethanol, and the vehicle alone served as control.

**Isoproterenol administration.** Isoproterenol (Iso; Sigma) diluted in 0.1% ascorbic acid was injected intraperitoneally (i.p.) once daily in 8-week-old male DKO mice and FFFF mice in a given dose of 10 mg/kg body weight per day over 14 days. 0.1% ascorbic acid alone (vehicle) served as control.
Swimming exercise protocol. Endurance exercise was carried out as described by others.\textsuperscript{5}\textsuperscript{,6} In brief, a ramp protocol was used starting at 10 minutes two times daily, with 10 minutes increase each day until 90 minutes, two times per day was reached. The protocol ended after 14 days. The animals were closely observed at all times to avoid relative hypoxia. With this protocol, there were no events of mice submerging under the water surface.

Transthoracic echocardiography and working heart preparation. Echocardiography and studies on isolated hearts were carried out as previously described.\textsuperscript{7,8}

Histology. Hematoxylin and eosin (H&E), Masson’s trichrome and CD31 stainings were performed as previously described.\textsuperscript{9,10} Cardiomyocyte size was assessed on H&E-stained sections by using Image J software (http://rsb.info.nih.gov/ij/). More than 200 randomly chosen cardiomyocytes from each group were analyzed to measure cross-sectional cardiomyocyte area. To quantify cardiac fibrosis, 20 trichrome-stained sections (magnification 20x) from the left ventricle were randomly selected, and morphometric analysis by using Image J was performed. Capillary density was determined as the ratio of capillaries to 100 cardiomyocytes in transversely sectioned left ventricular tissue immunostained with CD31 and counterstained with DAPI. Photographs were acquired with an Olympus SZH zoom stereo dissection scope with an Optronics DEI-750 CCD digital camera. All data were analyzed by a single observer blinded to the mouse genotypes.

Western Blotting and GST-pulldown. Proteins from heart tissue and cultured cardiomyocytes were isolated, and Western blot analysis was performed according to protocols described previously.\textsuperscript{11} GST-pulldown experiments were performed as described.\textsuperscript{11} Besides GST-HDAC4 419-670 (which contains a CaMKII activity-dependent binding domain) we used GST-HDAC4 2-250 (which does not contain a CaMKII binding domain) as a negative control. Primary antibodies used were anti-CaMKII total (BD Bioscience), anti-CaMKII\textgreek{y} (Santa Cruz), anti-p-CaMKII (Ser287) (Affinity Bioreagents), anti-PKD (Cell
signaling), anti-p-PKD (Ser744) (Cell signaling), anti-CnA (Abcam), anti-p-CnA (Ser411) (generated by Pineda antibodies), anti-GAPDH (Chemicon), anti-HDAC4 (Santa Cruz), anti-p-HDAC4 (Ser632) (Abcam), anti-phospholamban (PLB) (Upstate), anti-p-PLB (Thr17) (Santa Cruz), anti-p-PLB (Ser16) (Upstate), anti-RyR2 (Affinity BioReagents), anti-p-RyR2 (Ser2808) and anti-RyR2 (Ser2814) (kind gifts from Dr. Xander Wehrens, Houston, USA), anti-Akt (Cell signaling), anti-p-Akt (Ser473) (Cell signaling), anti-RCAN1-4 (kind gift from Dr. Timothy McKinsey, Denver, USA), and anti-α-actin (Sigma-Aldrich). Primary antibody incubation was followed by corresponding horseradish peroxidase (HRP)-conjugated secondary anti-mouse and anti-rabbit antibodies and ECL detection. Relative protein levels were detected by densitometry using the Image J program.

**CaMKII kinase activity.** CaMKII kinase activity was measured by radioactive kinase assays using GST-HDAC4 419-670 as a substrate and indirectly by detecting the amount of endogenous CaMKII that associates to GST-HDAC4 419-670 (see also above under GST pulldown). A detailed protocol has been described previously.3,11

**Caspase 3/7 activity measurements.** The Caspase-Glo 3/7 Assay (Promega Corporation) was used to measure caspase-3 and -7 activities according to the manufacturer’s instructions. Heart lysates were mixed with reaction solution at equal volumes in a 96-well plate. Luminescence was detected after 60 minutes of incubation at room temperature.

**RNA analysis.** Total RNA was isolated from ventricular tissue or from cultured cardiomyocytes using TRizol (Invitrogen). Total RNA was digested with DNase, and cDNA synthesis from 500 ng of RNA was carried out using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Quantitative real-time PCR (qPCR) was performed with Universal ProbeLibrary (Roche) by using TaqMan Universal PCR Mastermix (Applied Biosystems) and detection on a 7500 Fast Cycler (Applied Biosystems). Primers and probes were: For rat: ANP sense 5´-cccgaccagcatgg-3´ and antisense 5´-caactgcctttctgaaaggtg-
3′; For mouse: CaMKIIα sense 5′-gctgccaagattatcaacacc-3′ and antisense 5′-cacgctccagctttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg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in DMEM/199 medium (4:1) with 10% FBS, 2 mM l-glutamine, and penicillin-streptomycin. NRVMs were infected 24 h after plating, grown 12 h later in serum-free media for another 4 h.

**Culture of neonatal mouse ventricular cardiomyocytes (NMVMs).** Neonatal cardiomyocytes were isolated from 1- to 2-day-old DKO or FFFF mice according to previously published methods. Cells grew for 24 h in the fresh complete medium. Some of the cells were treated with CnA-siRNA or scrambled siRNA according to the manufacturer’s instructions (Sigma-Aldrich). Medium was then replaced with serum-free medium 24 h prior to adding isoproterenol (Sigma-Aldrich, 0.1 μM) for another 24 h. Cells were then harvested for immunocytochemical staining or luciferase assay. For some of the immunostainings and for luciferase assay, adenovirus harboring NFAT-GFP or NFAT luciferase reporter were added, respectively.

**Culture of adult mouse ventricular myocytes (AMVMs).** For AMVM isolation, δ-CKO, γ-CKO and DKO mice and their Cre-negative littermates (control), respectively, were anesthetized with isoflurane and hearts were excised. Explanted hearts were retrogradely perfused and digested as described before. Cells were plated onto regular cell culture plates or superfusion chambers, with the glass bottoms treated with laminin. Cells were immediately used for epifluorescence microscopy experiments or grew for 24 h in the fresh complete medium. Medium was then replaced with serum-free medium 24 h prior to adding isoproterenol (Sigma-Aldrich, 0.1 μM) for another 24 h.

**Immunocytochemistry.** NMVMs or AMVMs were fixed in 4 % paraformaldehyde (Sigma-Aldrich) in PBS, permeabilized with 0.3 % Triton X-100 (Sigma-Aldrich) for 10 min at room temperature and blocked for 30-60 min with 5 % goat serum (PAA) or BSA in PBS. Primary antibody for α-actinin (Sigma) was applied in PBS containing 5 % goat serum or BSA and 0.05 % Triton X-100 for 1 h. Cells were treated with secondary antibody (Texas Red-coupled
anti-mouse-antibody) in PBS containing 5 % goat serum and 0.1 % Triton X-100 for 1 h. Nuclei were labeled with DAPI (Invitrogen).

**Apoptosis assay and cell size in AMVMs.** TUNEL assays were performed using the in situ cell death detection kit (Roche) according to the manufacturer’s protocol. To quantify the number of apoptotic AMVMs, AMVMs were counterstained with sarcomeric α-actinin, and the total numbers of AMVMs and TUNEL-positive nuclei were counted in 10 low power fields. For each experimental condition, contiguous visual fields were counted to accumulate data on 200 AMVMs per condition. Stained cells were normalized to total cell count as judged by DAPI staining. More than 80% of cells were sarcomeric α-actinin positive. Cardiomyocyte size was assessed by using Image J software (http://rsb.info.nih.gov/ij/). More than 100 randomly chosen cardiomyocytes from each group were analyzed to measure cross-sectional cardiomyocyte area.

**Epifluorescence microscopy.** A Nikon Eclipse TE2000-U inverted microscope which was provided with an IonOptix fluorescence detection system for assessment of Ca^{2+} handling properties was used (IonOptix). For the studies on unstressed mice (was performed in the Maier lab), AMVMs were loaded with Fura-2AM and analysed as described previously. \(^{14}\) For the studies on sham and TAC-operated mice (was performed in the Maack lab), AMVMs were loaded with Indo-1AM and analysed as described previously. \(^{15, 16}\)
**Supplemental References:**


Fig. S1. Baseline characterization of DKO mice. (A) Western blot analysis of total CaMKII, CaMKIIγ, phospho-CaMKII-Thr287, and GAPDH as loading control in adult ventricular myocytes (AMVMs) from δ-CKO, γ-CKO and DKO mice and Cre-negative littermates (Control). (B) Representative images of whole hearts, sections and M-mode echocardiograms from DKO mice and floxed Cre-negative littermates (FFFF). Heart weight/body weight (HW/BW) ratios and fractional shortening for both groups are also indicated. Values are presented as mean±SEM (n=18). (C) Hemodynamic measurements in isolated Langendorff-perfused hearts (n≥10). Ejection fraction, end systolic pressure volume relations (ESPVR), stroke volume and isovolumetric relaxation time constant (tau) are shown. (D) Representative single cell measurements for Ca²⁺ transient amplitude, fractional Ca²⁺ release and sarcoplasmatic reticulum (SR) Ca²⁺ content in isolated ventricular cardiomyocytes from DKO and FFFF mice, loaded with fluo-4. (E) Ca²⁺ transient decay after field stimulation with 1, 2 and 3 Hz. Measurements for intracellular diastolic Ca²⁺ and fractional shortening (FS) given as % of resting cell length after stimulation with 1, 2 and 3 Hz. All measurements were conducted in n≥30 single cardiomyocytes from n≥3 mice. Values are reported as mean±SEM. *p<0.05. n.s., not significant.
A

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B

CaMKII activity

C

Fractional Ca\(^{2+}\) release

SR Ca\(^{2+}\) content

Tau (s)

D

Ca\(^{2+}\) transient amplitude

Fractional shortening

E

ANP

βMHC

Rcan1-4

Fig. S2
Fig. S2. Ca\textsuperscript{2+}-handling after TAC in DKO. (A) Western blot analysis of total CaMKII, phospho-CaMKII-Thr287, phospho-PLB-Thr17, phospho-PKD-Ser744, total PKD and GAPDH as loading control in cardiac extracts from FFFF and DKO mice three weeks after SH or TAC surgery. Quantitative analysis is shown (n≥3 per group). (B) Radioactive CaMKII kinase activity measured by \textit{in vitro} kinase assay using GST-HDAC4 419-670 as a substrate. Coomassie staining was used to demonstrate equivalent GST-HDAC4 input. Representative blots and quantification (n=4) are shown. (C+D) Intracellular Ca\textsuperscript{2+} handling and cell shortening after TAC. Measurements were conducted in adult mouse ventricular myocytes (AMVMs) from FFFF and DKO mice three weeks after SH or TAC surgery. AMVMs were loaded with indo-1. Averaged data for intracellular Ca\textsuperscript{2+} transient amplitudes and fractional shortening given as % of resting cell length after field stimulation with 0.5, 1, 2, 3, 4 and 5 Hz. Fractional Ca\textsuperscript{2+} release and averaged sarcoplasmatic (SR) Ca\textsuperscript{2+} content were estimated from the caffeine-induced peak in Ca\textsuperscript{2+} transients. Time constant (tau) of intracellular Ca\textsuperscript{2+} decline after field stimulation and under caffeine. All measurements were conducted in n≥25 single AMVMs from n≥3 mice. (E) Gene expression in wild type and δ-KO as well as DKO and FFFF mice after TAC or SH surgery. Animals were sacrificed after 3 weeks. Fold-changes in mRNA levels of ANP, βMHC and Rcan1-4 (n≥3 per group). All values are reported as mean±SEM. *p<0.05. n.s., not significant.
Figure S3
Fig. S3. Redundant roles for CaMKIIδ and γ for cardiomyocyte apoptosis and hypertrophy. (A) Quantification of cardiac caspase 3/7 activity in DKO and FFFF mice as measure for apoptosis induced by 3 weeks after TAC or 14 days of isoproterenol (Iso) treatment (n≥3 per group). (B-C) Experiments were conducted in adult ventricular myocytes (AMVMs) from δ-CKO, γ-CKO and DKO mice and their Cre-negative littermates (Control). Cells were stimulated with Iso or treated with vehicle. (B) Cells were stained for α-actinin (shown in red) and with DAPI nuclear stain (shown in blue). TUNEL-positive cells display green nuclei (more than 200 myocytes were analyzed per well). Shown are selected myocytes with TUNEL-positive and negative staining. The percentage TUNEL-positive cells is indicated (n=4 per condition). (C) Myocyte size was determined by measuring >100 cardiomyocytes per well (n=4 per condition). All values are presented as mean±SEM. *p<0.05. n.s., not significant.
Fig. S4
Fig. S4. CaMKII-dependent dissociation of cardiac fibrosis and dysfunction from cardiac hypertrophy and fetal gene activation after isoproterenol. (A) Representative images of whole hearts as well as H&E and Masson’s trichrome staining of left ventricular wall sections. Quantification of HW/BW ratios, myocyte size and fibrosis area (n>6 per group). FFFF and DKO mice were treated i.p. with isoproterenol (Iso) 10 mg/Kg/d or vehicle for 14 days, respectively (B) Fold-changes in mRNA levels of the hypertrophic markers ANP, BNP, α-MHC and β-MHC and fold-changes in collagen expression (n≥4 per group). All values are presented as mean±SEM. *p<0.05. n.s., not significant.
IB: CaMKII
IB: P-Ser-473-Akt
IB: Akt total

Figure S5
Fig. S5. Normalization of fetal gene program and unaltered Akt phosphorylation after CnA inhibition in DKO mice. (A) For data presented in Fig. 5A+B, neonatal mouse ventricular myocytes (NMVMs) were transfected with CnA-siRNA or scrambled siRNAs, or they were not transfected (non treated). Western blot analysis demonstrates efficient CnA knockdown. GAPDH shows equal loading. (B+C) Analog to the experiment shown in Fig. 5C, DKO and FFFF mice were randomized to either TAC or SH surgery and were treated with cyclosporine A (CyA; 4 mg/Kg/d i.p.) or vehicle (n≥3). Mice were sacrificed 7 days after TAC surgery. CyA-treated DKO mice were protected from TAC-induced hypertrophy (data not shown) as described in Fig. 5C (3 weeks after TAC). (B) Fold-changes in cardiac mRNA levels of the hypertrophic markers ANP, BNP, αMHC and βMHC and fold-changes of RCAN 1-4 expression (n≥3 per group). (C) Western blots for CaMKII, Akt total, phospho-Akt-Ser473, and GAPDH as loading control were performed in lysates from left ventricle. Quantitative analysis of the expression and phosphorylation of the proteins is shown (n=3). All values are presented as mean±SEM. *p<0.05. n.s., not significant.