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Combined cardiomyocyte PKC δ and PKC ϵ gene deletion uncovers their central role in restraining developmental and reactive heart growth

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Abstract

Cell growth is orchestrated by changes in gene expression that respond to developmental and environmental cues. Among the signaling pathways that direct growth are enzymes of the protein kinase C (PKC) family, which are ubiquitous proteins belonging to three distinct subclasses: conventional PKCs, novel PKCs, and atypical PKCs. Functional overlap makes determining the physiological actions of different PKC isoforms difficult. We showed that two novel PKC isoforms, PKC δ and PKC ϵ , redundantly govern stress-reactive and developmental heart growth by modulating the expression of cardiac genes central to stress-activated protein kinase and periostin signaling. Mice with combined postnatal cardiomyocyte-specific genetic ablation of PKC δ and germline deletion of PKC ϵ (DCKO) had normally sized hearts, but their hearts had transcriptional changes typical of pathological hypertrophy. Cardiac hypertrophy and dysfunction induced by hemodynamic overloading were greater in DCKO mice than in mice with a single deletion of either PKC δ or PKC ϵ . Furthermore, gene expression analysis of the hearts of DCKO mice

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SUPPLEMENTARY MATERIALS

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Fig. S1. Anti-phosphoserine PKC substrate immunoblotting validation.

Fig. S2. Time course of cardiac hypertrophy after TAC.

Fig. S3. Strategy for creation of mice lacking both PKC δ and PKC ϵ in cardiac myocytes.

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Fig. S6. Deletion of nPKC isoforms in hearts de-restrains hypertrophy gene expression.

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Fig. S8. Cardiac characteristics of emb DCKO “escaper” mice.

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Table S1. Viability of cardiac PKC δ /PKC ϵ DCKO mouse pups with postnatal PKC δ ablation using myh6-nuclear-directed Cre.

Table S2. Viability of cardiac PKC δ /PKC ϵ DCKO mouse pups with early embryonic PKC δ ablation using nkx2.5-Cre.

Table S3. Comparative transcriptomics data for genes encoding G $_q$, PKC, ERK, and p38 MAPK family members.

Table S4. Comparative transcriptomics data for Fig. 2E.

Table S5. Comparative transcriptomics data for Fig. 4A.

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Competing interests: The authors declare that they have no competing interests.

Data and materials availability: RNA-sequencing data describing unstressed and pressure-overloaded adult hearts, of all genotypes, have been uploaded to the NCBI GEO GSE62689 in both raw and processed forms. PKC δ ^{fl/fl} mice are available from G.W.D. through a material transfer agreement with Washington University in St. Louis.

revealed transcriptional derepression of the genes encoding the kinase ERK (extracellular signal-regulated kinase) and periostin. Mice with combined embryonic ablation of PKC δ and PKC ϵ showed enhanced growth and cardiomyocyte hyperplasia that induced pathological ventricular stiffening and early lethality, phenotypes absent in mice with a single deletion of PKC δ or PKC ϵ . Our results indicate that novel PKCs provide retrograde feedback inhibition of growth signaling pathways central to cardiac development and stress adaptation. These growth-suppressing effects of novel PKCs have implications for therapeutic inhibition of PKCs in cancer, heart, and other diseases.

INTRODUCTION

Parallelism and redundancy in cell signaling promote reactive plasticity and enhanced stress adaptation. If a given signaling factor is damaged by genetic mutation or environmental factors, one or more closely related factors are available as opportunistic substitutes to maintain overall pathway function. Accordingly, protein kinases duplicated by stochastic genetic events have not only been retained but also proliferated in the genomes of more complex organisms (1). Functional redundancy of duplicated factors is not absolute, however, because many evolve unique or niche functions over time. The most important role of one signaling factor within a larger family of related factors can therefore vary by cell type, developmental stage, and situational context.

The protein kinase C (PKC) family may be the prototype for a group of structurally similar protein isoforms with a common ancestral origin but divergent modern functions (2). PKCs are ubiquitous, highly abundant, and function as signaling effectors for many different pathways coupled to diacylglycerol production by phospholipases C and D (3, 4). PKCs transduce cell growth signals; phorbol esters that activate PKCs by substituting for diacylglycerol can therefore provoke tumor growth. PKCs have long been implicated in pathological reactive cardiac hypertrophy (5, 6).

Eleven PKC isoforms have been identified, encoded by 10 different genes. On the basis of structural characteristics and cofactor requirements, PKCs were grouped together into subclasses designated as conventional, novel, or atypical (4). The oldest and best-understood PKC isoforms belong to the conventional subclass (cPKC) distinguished by their activation requirement for both calcium and lipid: PKC α , alternately spliced β_I and β_{II} , and γ . Calcium responsiveness of cPKCs suggests that they are stress-responsive. Although it was considered likely that cPKC activation was central to reactive cardiac hypertrophy and subsequent heart failure (7, 8), concomitant genetic ablation of all three cPKC family members does not prevent cardiac hypertrophy (9). Instead, cPKC signaling in the heart is acknowledged as an important and drug-targetable modifier of adrenergic responsiveness and minute-by-minute contractility (10–13).

Less is known about the central functions of calcium-independent novel PKCs (nPKCs): PKC δ , ϵ , η , θ , and μ . For example, PKC ϵ is a tumor promoter (14), whereas related PKC δ has both proliferative and antiproliferative activities (15–17). In the heart, PKC δ and PKC ϵ (the two nPKC isoforms expressed at meaningful amounts) are best recognized for their opposing influences on the response to ischemia-reperfusion injury (18, 19), and for

apparently having similar hypertrophy-promoting effects (20–22). However, signaling crosstalk and cross-regulation between these two nPKC isoforms have complicated interpretation of phenotypes resulting from conventional gene ablation (23).

To deconvolve functional promiscuity and opportunistic compensation of cardiac PKC δ and PKC ϵ signaling, we created mice lacking both of these nPKC isoforms in cardiomyocytes, either from early in cardiac development or after birth. Whereas deficiency of either PKC δ or PKC ϵ alone had minimal consequences on heart development or function, their combined deficiency in cardiac myocytes disinhibited cardiomyocyte growth, evoking massive proliferation of fetal cardiomyocytes and exaggerated hypertrophy in pressure-overloaded adult hearts. These findings reveal unsuspected and overlapping growth-limiting effects of cardiac nPKC isoforms and warrant reexamination and reevaluation of current concepts of PKC δ and PKC ϵ signaling in the heart and other tissues and organs.

RESULTS

PKC signaling increases during pressure overload cardiac hypertrophy

Pressure overload cardiac hypertrophy is mediated in large part by G_q-coupled signaling pathways (24–26) coupled to PKC, the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK), and p38 stress-activated kinases (7, 8). We measured kinase activation periodically after surgical induction of cardiac pressure overload by transverse aortic constriction (TAC), a clinically relevant model of cardiac hypertrophy (27). p38 and Akt showed no consistent changes, and ERK abundance and phosphorylation increased nearly in parallel 1 week after pressure overloading (Fig. 1A). By contrast, PKC activation was markedly increased after 1 week (Fig. 1B) when measured by the usual method of assaying translocation to microsomal membranes (28). Of the three major PKC forms in the heart, PKC δ and PKC α showed the greatest microsomal localization, whereas the increase in microsomal content of PKC ϵ was not significant (Fig. 1B). PKC α has previously been shown to not be essential to the cardiac hypertrophy response (9).

PKC translocation is not necessarily synonymous with kinase activity (23). Therefore, we examined PKC-mediated phosphorylation of myocardial proteins after cardiac pressure overloading; the anti-phosphoserine PKC substrate immunoreactivity assay was validated in phorbol ester-treated human embryonic kidney (HEK) 293 cells (fig. S1). One higher-molecular weight PKC substrate, previously identified as myosin binding protein-C [MyBP-C, which is phosphorylated by PKC α (29)], showed increased serine phosphorylation within 2 hours of TAC (Fig. 1C). However, most PKC substrate proteins showed increased serine phosphorylation after 1 week, which gradually declined thereafter (Fig. 1C). Because 1 to 4 weeks after aortic banding is when the hearts were actively hypertrophying (fig. S2), we posited that nPKC isoforms might contribute to hypertrophy signaling.

Creation and baseline characterization of mice with cardiomyocyte-specific combined PKC δ /PKC ϵ deficiency

Forced cardiac expression or activation of PKC ϵ stimulates cardiac hypertrophy (19, 20, 30), but genetic ablation of PKC ϵ does not prevent reactive hypertrophy (31). We considered that

these seemingly disparate results could be explained by functional crosstalk and redundancies between PKC δ and PKC ϵ (23). To circumvent the problem of cardiac nPKC isoform redundancy, we introduced loxP sites flanking *prkcd* exons 10 and 11 (*prkcd^{f/f}*) and crossbred these mice germline PKC ϵ knockout (KO) mice (*prkce^{-/-}*) (32). Cre-mediated loxP recombination deletes amino acids 328 to 418 of PKC δ , eliminating its ATP (adenosine 5'-triphosphate)-binding domain (G³⁵⁹-Y³⁷²) and simultaneously introducing a frameshift (Fig. 2, A and B). By adding a cardiac-specific *myh6*-driven Cre allele, we generated viable *prkcd^{f/f};Myh6-Cre-prkce^{-/-}* mice in which PKC δ and PKC ϵ are both deficient in cardiac myocytes after birth, designated PKC δ /PKC ϵ double cardiac knockout (DCKO) mice (fig. S3, A and B, and table S1). Adult mouse hearts lacking either PKC δ , PKC ϵ , or both PKC δ and PKC ϵ (DCKO) (Fig. 2C) had normal mass, contractile performance, and histological characteristics (fig. S4). Using invasive in vivo hemodynamic measurements, we were, however, able to uncover a modest contractile defect in DCKO hearts upon high-dose intravenous infusion of the β -adrenergic agonist dobutamine (Fig. 2D). Nevertheless, the absence of any overt cardiac phenotype at baseline in hearts lacking both PKC δ and PKC ϵ was unexpected.

Dysregulation of cardiac genes can be a sensitive predictive marker of covert cardiac abnormalities. Because PKCs are potent modifiers of gene expression (33), we examined the consequences of nPKC isoform deficiency on the cardiac transcriptome. Deficiency of either PKC δ or PKC ϵ alone provoked changes in transcript abundance of only ~100 (of ~10,000) cardiac genes. Moreover, 31 of these mRNAs were similarly dysregulated by both nPKC isoforms (Fig. 2E). Strikingly, combined deficiency of both nPKC isoforms altered the expression of almost 1000 cardiac mRNAs, revealing ~90% functional redundancy of PKC δ and PKC ϵ at the level of regulated cardiac gene expression. Functional annotation showed a dominant effect of cardiac nPKC isoform deficiency on signaling pathways (Fig. 2F) and changes in individual gene expression reminiscent of pathological cardiac hypertrophy (Fig. 2G).

Pressure overload unmasks multiple hypertrophy-restraining actions of cardiac nPKCs

The above data showed that PKC δ and PKC ϵ are activated after induction of surgical pressure overload, concomitant with reactive hypertrophy, and that their combined deficiency alters the cardiac gene expression program in a manner that partially recapitulates pressure overload hypertrophy. We reasoned that the virtually normal physio-structural cardiac phenotype of DCKO mice could be explained if nPKCs function primarily as modulators of gene expression during cardiac stress. We therefore assessed the cardiac response to TAC-mediated pressure overload (34) in mice with individual and combined deficiencies of PKC δ and PKC ϵ in cardiomyocytes. Assays of myocardial PKC substrate phosphorylation before and 1 week after TAC in DCKO hearts revealed markedly diminished serine phosphorylation of numerous proteins whose phosphorylation normally increases after TAC, without any change in basal protein phosphorylation or TAC-induced phosphorylation of the PKC α substrate MyBP-C (Fig. 3A). Thus, simultaneously deleting both cardiac-expressed nPKCs markedly altered the phosphoprotein signature of pressure-overloaded hearts, but not that of unstressed hearts.

Strikingly, DCKO hearts responded to TAC with an accelerated and augmented increase in heart mass (Fig. 3, B to D). Moreover, pressure-overloaded DCKO mice exhibited progressive deterioration of contractile function (Fig. 3E and fig. S5A). Consistent with a previous report (31), absence of PKC ϵ alone did not change the typical TAC-induced cardiac hypertrophy response (Fig. 3, D and F). Likewise, cardiomyocyte-specific deletion of PKC δ did not change pressure overload hypertrophy (Fig. 3, D and F). Because transaortic gradient and peak LV pressure were similar between each of the four groups 1 week after TAC (fig. S5B), differences in the hemodynamic stimulus for hypertrophy do not explain exaggerated hypertrophy in DCKO mice. However, decreases in LV pressures and fractional shortening 4 weeks after TAC confirmed that early functional decompensation was specific to DCKO mice (fig. S5B).

Given that cardiac PKC δ /PKC ϵ deficiency partially recapitulated hypertrophy-associated changes in gene expression in normal hearts (Fig. 2G), we again performed deep RNA sequencing to discover genetic events that contribute to exaggerated pathological hypertrophy evoked by nPKC isoform deficiency. Just as absence of either one or the other nPKC isoform had little effect on physical pressure overload hypertrophy, deficiency of either PKC δ or PKC ϵ alone had little effect on hypertrophy-associated transcriptional reprogramming. A heat map of unsupervised misregulated mRNA clustering shows imperfect segregation of control, PKC δ KO, and PKC ϵ KO heart profiles 4 weeks after TAC (Fig. 4, A and B). By contrast, DCKO hearts perfectly segregated, exhibiting an exaggerated hypertrophy gene expression signature (Fig. 4, A and B). MetaCore analysis revealed nPKC-mediated orchestration of genes within, or impinging upon, stress-activated protein kinase pathways (especially ERK), periostin, and β -catenin abundance (Fig. 4C and fig. S6). [Except for ERK2 (Mapk1), nPKC isoform deficiency did not primarily affect the expression of canonical pressure overload, G_q-coupled signaling pathway genes including G_q itself, other PKC isoforms, ERK MAPKs, or p38 MAPKs; table S3.] Because these three factors modulate cardiac hypertrophy (35–38), we compared the effects of nPKCs and pressure overloading on their mRNA and protein abundance. ERK protein increased in both control and DCKO hearts 1 week after TAC. However, decreased ERK abundance at 4 weeks did not occur in DCKO hearts (Fig. 4D). Likewise, the typical late normalization of periostin, the abundance of which was markedly increased 1 week after TAC, was blunted in DCKO hearts (Fig. 4E). β -Catenin abundance was somewhat more variable in individual hearts and appeared only modestly decreased in DCKO hearts 4 weeks after TAC (fig. S7).

ERK signaling induces cardiomyocyte hypertrophy, and its failure to return toward normal in DCKO hearts subjected to TAC would be expected to increase cardiomyocyte size. Indeed, the TAC-induced increase in cardiomyocyte cross-sectional area (Fig. 5A), but not in phospho-histone H3 (pHH3) staining, was significantly greater in DCKO hearts (Fig. 5B). Periostin induced by cardiac stress has been linked to myocardial fibrosis and apoptosis (36, 39, 40). Persistently increased periostin would therefore be predicted to increase myocardial fibrosis and cardiomyocyte dropout, which is what we observed after TAC in DCKO hearts (Fig. 5, C and D). Together, these results indicate that a shared function of PKC δ and PKC ϵ is to decrease the expression of multiple genes within convergent signaling pathways that promote cardiac hypertrophy and chamber remodeling. Among the consequences of combined PKC δ and PKC ϵ inactivation was disinhibition of ERK and periostin pathways,

provoking exaggerated hypertrophy, adverse remodeling, and accelerated functional decompensation.

nPKCs restrain embryonic cardiomyocyte proliferation

Molecular reprogramming in adult pressure overload cardiac hypertrophy recapitulates gene expression during fetal heart development (41, 42). Accordingly, we reasoned that the growth-limiting effects of nPKC isoforms manifested as exaggerated reactive hypertrophy after pressure overload might also have a role during normal developmental cardiac growth. We examined this notion by replacing the *myh6*-Crs transgene with a *nkx2.5*-Cre knockin allele, which induces gene recombination in cardiomyocytes from early fetal cardiac development [*prkcd*^{f/f};Nkx2.5-Cre-*prkcd*^{-/-}; embryonic (emb) DCKO] (fig. S3, B and C). Multiple breedings of *prkcd*^{f/f};Nkx2.5-Cre-*prkcd*^{-/+} and *prkcd*^{f/f}-*prkcd*^{-/-} mice generated 203 pups, of which only 10 (4.9%) were emb PKCδ/PKCε DCKO mice [compared to 51 (25%) expected, $P < 0.0001$] (fig. S8A and table S2). Examination of three “escaper” emb DCKD mice that survived to 8 weeks of age revealed cardiac enlargement and thickened LV walls (fig. S8B). Markedly increased LV pressures at end-diastole reflected ventricular stiffening from myocardial hypertrophy (fig. S8C). Dobutamine-stimulated contraction and relaxation were also depressed (fig. S8, D and E).

Timed pregnancies revealed fewer than predicted emb DCKO embryos at E15.5 (fig. S8A and table S2). Examination of hearts of E15.5 embryos revealed abnormal thickening of the left and right ventricular walls (Fig. 6A), in most cases severely compromising or virtually abolishing the LV cavity. We measured histological cardiomyocyte size, but rather than the cardiomyocyte enlargement hypertrophy seen in pressure-overloaded adult hearts, we observed normal fetal cardiomyocyte size (Fig. 6B). Likewise, cardiomyocyte apoptosis was unaffected by nPKC deficiency (Fig. 6C). However, we determined that the rate of cardiomyocyte DNA synthesis measured as nuclear pHH3 labeling was twice normal in emb DCKO hearts (Fig. 6D). Thus, developmental cardiac enlargement in PKCδ/PKCε-deficient embryonic hearts is linked to increased cardiomyocyte proliferation, whereas “hyper-hypertrophy” in PKCδ/PKCε-deficient adult hearts is linked to cardiomyocyte hypertrophy. This difference reflects distinct mechanisms of cardiac growth stimulated by the same constellation of growth signaling pathways at these two stages of life (43). Together, these results uncover a broad modulatory function of nPKC isoforms on reactive and developmental myocardial growth.

DISCUSSION

Here, we demonstrate at the molecular, cellular, and whole-organ levels that deficiency of either PKCδ or PKCε has little effect on normal embryonic, normal adult, or pressure-overloaded adult hearts. However, combined deficiency of these two closely related nPKC isoforms provokes profound cardiac phenotypes linked to disinhibition of fetal developmental and adult stress-evoked cardiomyocyte growth. These results uncover redundant effects of these two nPKC isoforms that limit cardiac growth and myocardial remodeling by restraining cardiomyocyte and fibroblast growth signals from ERK, periostin, and other signaling pathways. The idea that these two PKCs might suppress cardiac

hypertrophy is contrary to the generally accepted notion that they are prohypertrophic signaling factors based on the following: (i) These and other PKCs are activated by hypertrophy-inducing G_q signaling, and are associated with pathological hypertrophy (7, 8); and (ii) genetic manipulations that increase cardiomyocyte-autonomous PKC δ or PKC ϵ activity, by forcing either increased expression or membrane translocation, can induce cardiac hypertrophy (although in some instances, the hypertrophy is “physiological” rather than “pathological”) (19–21, 30). Of course, increased nPKC signaling during cardiac hypertrophy could be either contributory as assumed or compensatory as demonstrated herein; forced PKC isoform expression obliterates natural regulatory mechanisms while increasing the chance for atypical promiscuous signaling. Thus, the data previously obtained by genetically manipulating nPKC isoforms in mouse hearts were valid but appear to have been interpreted incorrectly [except for cautionary statements by a few individuals who noted that these two PKC isoforms exhibit cross-regulation (44)].

The current findings reveal that the presence of one or the other cardiac-expressed nPKC isoform is sufficient both to maintain normal adult hearts and to sustain the normal stress response to hemo-dynamic overloading. Indeed, if defined by the extent of gene regulation in the double nPKC isoform-deficient hearts compared to the single isoform parental lines, PKC δ and PKC ϵ display ~90% functional redundancy. This has two intriguing implications: First, if the functional redundancies of these isoforms reflect their most important biological activities (because these are the functions that have been evolutionarily conserved), then we can conclude that a central purpose of nPKC signaling in the heart is to govern, that is, to restrain, cardiac growth; and second, the extensive functional overlap between these two PKC isoforms (at least in cardiomyocytes of the heart) suggests that the therapeutic window may be large for isoform-specific suppression of those functions that are not redundant, such as their opposing effects on post-ischemic cardiac dysfunction (45).

It is notable that de-restraint of cardiac growth through combined deficiency of both cardiac nPKCs largely reversed the direct and indirect genetic suppression of two potent mediators of pathological cardiac hypertrophy: ERK and periostin. PKCs can phosphorylate many different transcription factors, including SP1 (46), which is upstream of ERK and periostin gene expression (47, 48). Indeed, SP1 is also phosphorylated by ERKs (49, 50). These examples of parallelism and cross-signaling are typical, and demonstrate the limitations of one gene/one target/one effect paradigms. Accordingly, we have not identified a single effect or effector of nPKCs that mediates their shared growth-limiting effects. Instead, as illustrated in Fig. 4C, these largely functionally redundant PKC isoforms are acting systemically at multiple points within several distinct but interconnected signaling pathways. Nevertheless, the availability of floxed PKC δ alleles that can be combined with PKC ϵ (or other nPKC isoform) KO genes to generate viable mouse models of tissue-specific nPKC isoform deficiency provides an opportunity to define the role of nPKC signaling in organs other than the heart, and to identify common and isoform-specific direct and indirect effects of these factors on protein phosphorylation, gene expression, and cell/organ function in different pathophysiological contexts.

An important aspect of this work is the reminder that gene ablation does not necessarily produce loss of function. The ability to manipulate genomes in increasingly sophisticated

ways has produced an exponential increase in “knockout” and “knockin” models, widely regarded as the essential gold standard experiment to demonstrate critical functioning of the targeted gene product. The underlying principle for the standard reductionist approach is that a given gene product exerts a discrete number of unique effects. Ablating the gene is anticipated to erase these effects, thereby revealing its primary function. Functional redundancy between and within biological processes, especially signaling pathways, confounds this type of simplistic interpretation.

MATERIALS AND METHODS

Creation of PKC δ floxed allele mice and crossing to Cre lines

Exons 10 and 11 encoding the ATP-binding domain of mouse *prkcd* were flanked with *loxP* sites plus a *frt*-flanked neomycin phosphotransferase module to positively select for homologous recombinants in Sv129 embryonic stem cells, identified by Bam HI restriction digest and Southern blotting with a 5' external probe. Heterozygous *prkcd*-targeted mice were bred with *Flp* transgenic mice to delete the neomycin selection cassette. *Prkcd* floxed mice backcrossed onto C57/BL6 were bred to *Ella-Cre* transgenic mice to generate germline PKC δ null mice, or to cardiomyocyte-specific Cre lines for embryonic or postnatal cardiac-specific PKC δ deletion, alone or in combination with *prkce* null alleles.

Cardiac function testing

Echocardiography was performed on unsedated mice before and serially after TAC to determine the time course of hypertrophic (LV mass), morpho-metric (LV end-diastolic dimension), and functional remodeling (LV fractional shortening) in individual hearts. Terminal cardiac catheterization simultaneously measured peak LV systolic pressure and descending aortic pressure to determine the transaortic gradient (reflecting the degree of hemodynamic stress induced by TAC). LV end-diastolic pressure was measured as an index of diastolic function. Peak positive and negative LV dP/dt are afterload-independent measures of contractile function and relaxation, respectively. Mice were housed according to procedures approved by the Washington University in St. Louis Institutional Animal Care and Use Committee.

Histological studies

Formalin-fixed cardiac sections were analyzed for cardiomyocyte cross-sectional area after staining with fluorescein-tagged wheat germ agglutinin (Alexa Fluor 488 conjugate; Invitrogen, W11261), and for mitotic bodies by labeling with anti-pHH3 [anti-histone H3 (phospho S¹⁰) antibody; Abcam, ab47297], counterstained with nuclear DAPI (Vector Laboratories, cat. no. H-1200) and cardiomyocyte-specific anti-myosin heavy chain 6/7 [anti-heavy chain cardiac myosin antibody (3 to 48); Abcam, ab15]. TUNEL staining used the DeadEnd Fluorometric TUNEL System (Promega, G3250).

Embryonic studies

Embryos were harvested from timed pregnancies, frozen or fixed in 4% paraformaldehyde, and genotyped by PCR.

Cell signaling assays

ERK, p38, and JNK activities were measured as time-dependent phosphorylation after TAC using phospho-specific immunoblot analyses of myocardial homogenates. PKC activation in pressure-overloaded myocardium was assessed as isoform-specific subcellular translocation from cytosol to microsomes (PKC α and PKC ϵ). Antibodies were as follows: anti-p44/42 MAPK (Erk 1/2) antibody (Cell Signaling, #9102); anti-phospho-p44/42 MAPK (Erk 1/2) (Thr²⁰²/Tyr²⁰⁴) (Cell Signaling, #4370); anti-p38 MAPK (Cell Signaling, #9212); anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) (Cell Signaling, #9211, rabbit); anti-Akt (Cell Signaling, #4691); anti-phospho-Akt (Thr³⁰⁸) (Cell Signaling, #2965); anti-PKC α (C-20) (Santa Cruz Biotechnology, sc-208); anti-PKC δ (Cell Signaling, #2058, or Santa Cruz, SC-213); anti-PKC ϵ (Cell Signaling, #2683, or Santa Cruz, SC-214); anti-periostin (LSBio, LS-C150337); anti-pan cadherin (Abcam, Ab16505); anti-phosphoserine PKC substrate (Cell Signaling, #2261); anti-GAPDH (Abcam, Ab8245); anti-COX IV (Abcam, Ab14744).

Genome-wide transcriptional profiling by RNA sequencing

Total RNA was isolated from flash-frozen hearts using TRIzol (Invitrogen). Polyadenylated RNA selection on oligo(dT) Dynabeads, fragmentation, first- and second-strand complementary DNA cDNA synthesis, and ligation of indexed adapters for Illumina sequencing were carried out as previously described (21). Single-end, 50-nucleotide reads were obtained on an Illumina HiSeq 2500; total reads per individual heart sample averaged 23 million, with 59% alignment to the mouse transcriptome defined by the Illumina iGenomes annotation (Ensembl NCBIM37, mm9).

The number of reads pertaining to each known transcriptome feature (“gene”) was obtained from alignment (.bam/.sam) files produced by TopHat, using htseq-count. Genes with less than an average of 10 reads in any sample group were eliminated, leaving 12,753 cardiac-expressed genes for further comparisons. Using the R package DESeq 1.9, read counts were normalized for library depth and fold differences, uncorrected *P* values from the negative binomial distribution, and adjusted *P* values (false discovery rate or FDR) were derived. Significant comparisons were taken as those with a fold difference of at least 50% and FDR < 0.01. Normalized read counts from DESeq were subjected to unsupervised hierarchical clustering with Euclidean distance and average linkage using Partek Genomics Suite v6.6 (Partek). Standardized heat maps are shown, in which map colors represent the extent of deviation from the row average. Principal components analysis of depth-normalized expression values used Partek Genomics Suite v6.6. For clarity of presentation and to enable comparison with other studies, individual gene expression levels are rendered as FPKM. All RNA-sequencing data describing unstressed and pressure-overloaded adult hearts, of all genotypes, have been uploaded to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) GSE62689 in both raw and processed forms. Annotated lists of modified RNAs and their comparative expression values are in table S3 (G protein-coupled signaling genes), table S4 (data for Fig. 2E), and table S5 (data for Fig. 4A).

Gene Ontology (GO) annotations were downloaded from Open Biological and Biomedical Ontologies (OBO) release 2012/01 and visualized using OBO-Edit version 2.1.1. The

BiNGO plugin to Cytoscape was used to classify mRNAs and evaluate overrepresentation in GO categories. We evaluated overrepresentation in 32 GO “biological_process” terms at GO level 3 (the branch terms immediately downstream of the “biological_process” root); the 10 with overrepresentation at an FDR of <0.05 determined by Benjamini-Hochberg multiple adjustment testing are shown in Fig. 2F. Genes in the “signaling” category had substantial overlap with genes in the “developmental process,” “localization,” and “metabolic process” categories. The MetaCore gene and pathway annotation suite (Thomson Reuters) was used to explore protein function and pathway grouping for subsets of regulated genes. An “enrichment by protein function” analysis was used to assess over- and underrepresentation between ligands, receptors, kinases, phosphatases, proteases, and transcription factors. Fifteen dysregulated mRNAs in these pathways were used as input for a new network drawn by MetaCore, in which no more than two “indirect” or unregulated gene connections were required to link these mRNAs together using known activating or inhibitory events. A detailed description of the MetaCore symbols for the gene network diagram is in fig. S9.

Statistical analyses

Results are expressed as means $\pm$ SEM. Unpaired Student’s *t* test, and one- or two-way ANOVA with Tukey’s post hoc test were used as appropriate. $P < 0.05$ was considered statistically significant except for genome-wide RNA-sequencing analyses, in which an FDR of <0.01 was the threshold for significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Time course of cardiac kinase signaling evoked by acute left ventricular (LV) pressure overload

(A and B) Immunoblot analyses of myocardial kinases in sham-operated (24 hours) hearts and 2 hours (Immed), 24 hours, and 7 days (1 week) after TAC. Activation of hypertrophy signaling kinases (A) and cardiac protein kinase isoforms (B) after TAC. Kinase activation in (A) is indicated by increased phosphorylation (p-), and in (B) by translocation to microsomes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is cytosolic, cadherin is plasma membrane, and cytochrome oxidase IV (COX IV) is mitochondrial. $n = 4$ biological replicates in (A) and (B). t-, total. (C) Detailed time course of anti-phosphoserine PKC substrate immunoreactivity after TAC; $n = 3$ different hearts per time point. Quantifications for high-molecular weight early substrate (arrow; MyBP-C) and lower-molecular weight late substrates are to the right. * $P < 0.05$ compared to sham; # $P < 0.05$ compared to 1 day. AU, arbitrary unit.

Fig. 2. Creation and characterization of mice lacking both PKC δ and PKC ϵ in cardiac myocytes (A) Strategy for inserting flox sites into the mouse *prkcd* gene. (B) Southern blot (top) and polymerase chain reaction (PCR) validation (bottom) of *prkcd*-targeted mouse embryonic stem cells, $n = 2$ independent targeted clones. (C) (Left) PKC isoform immunoblot analysis of brains from floxed *prkcd* mice bred to Ella-Cre to achieve germline deletion, validating efficacy of PKC δ ablation for floxed allele compared to *prkce* germline mouse and C57/BL6 wild-type (WT) control. (Right) Immunoblots of isolated cardiomyocytes from cardiac-specific PKC δ null, PKC ϵ KO^{-/-}, and postnatal cardiac double PKC δ /PKC ϵ (DCKO) hearts. Brain is positive control. Representative of three biological replicates. (D) Cardiac contractility (+dP/dt) in DCKO and parent lines as a function of intravenous dobutamine dose. (E) Heat map showing unsupervised clustering of cardiac gene expression in DCKO and parent lines; each column is a different individual heart. Venn diagram depicts shared gene expression patterns, $n = 3$ to 4 biological replicates. (F) Functional categorization of DCKO misregulated genes. (G) Comparison of gene expression in DCKO and early pressure overload hypertrophy (Hyp). *Significantly different than WT. Genes are as follows: *Acta1*, α -skeletal actin; *Grk5*, heterotrimeric guanine nucleotide-binding protein (G protein) receptor kinase 5; *Myh6*, α -myosin heavy chain; *Myh7*, β -myosin heavy chain; *Pik3r1*, phosphoinositide 3-kinase regulatory subunit 1; *Ppargc1a*, peroxisome proliferator-activated receptor gamma coactivator 1; *Timp3*, metalloproteinase inhibitor 3; *Tubb2a*,

tubulin β 11a. mRNA expressed as FPKM (fragments per kilobase of exon per million mapped reads), $n = 3$ to 4 biological replicates. Right panel shows relative expression of 200 disease-associated cardiac genes in hypertrophied and DCKO mouse hearts.

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Fig. 3. Exaggerated reactive pressure overload hypertrophy in PKC δ /PKC ϵ -deficient mouse hearts

(A) Anti-phosphoserine PKC substrate immunoreactivity in sham and 1-week TAC mouse hearts. Quantitation excluding MyBP-C is to the right; $n = 3$ biological replicates. (B) Four-chamber heart sections in sham and 4-week TAC-operated WT C57/BL6 and DCKO hearts. Images are representative of four to five mice per genotype and treatment. (C) Representative serial M-mode echocardiograms of mice after TAC. (D and E) Group data from serial echocardiographic studies; each mouse was studied before (control) and serially after TAC. $n = 4$ to 8 mice per genotype and treatment, except 1 week wherein postsurgical technical issues precluded adequate visualization in two mice. (F) Time course of gravimetric cardiac hypertrophy after TAC. $*P < 0.05$ compared to all other groups at same time point [analysis of variance (ANOVA)].

Fig. 4. Deficiency of PKC δ and PKC ϵ disinhibits expression of cardiac growth signaling genes and their protein products

(A) Unsupervised hierarchical clustering of differentially expressed (FDR < 0.01, $\pm$ 50% change) cardiac mRNAs from deep RNA-sequencing studies at baseline and 4 weeks after TAC. Each column is a different heart; $n = 3$ to 4 biological replicates. (B) Principal components analysis of all ~12,000 cardiac-expressed mRNAs from the same data set. (C) Predicted nPKC-regulated cardiac gene network. Red indicates increased expression in sham DCKO compared to sham WT and/or in TAC DCKO compared to TAC WT; blue is decreased expression in same groups; purple is mixed regulation. (D and E) Regulation of ERK (D) and periostin (E) mRNA (left) and protein (right) after TAC. Quantitative protein amounts are to the right of immunoblots. $n = 3$ to 4 mice per genotype and treatment as indicated. For mRNA: * indicates FDR < 0.01 compared to Ctrl in same condition.

Fig. 5. Unrestrained growth in hemodynamically stressed PKC δ /PKC ϵ -deficient cardiomyocytes is histologically pathological

(A) Wheat germ agglutinin staining of cardiomyocytes in cross section after TAC; representative 4-week studies on the left; group data to the right, $n = 3$ to 5 mice per genotype and treatment. (B) Group data of the same hearts for anti-pHH3 staining, measuring DNA synthesis. (C) Anti-periostin staining (top) and Masson's trichrome staining (bottom). Scale bars, 50 μm . (D) Group data from terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays. * $P < 0.05$ compared to WT in same treatment group; #different from all other groups.

Fig. 6. Exaggerated heart growth caused by cardiomyocyte-specific nPKC δ /nPKC ϵ deficiency in embryonic hearts

(A) Frontal (left) and sagittal (right) hematoxylin and eosin–stained sections through hearts of WT control (top) and DCKO (bottom) E15.5 embryos. Hearts (in black squares) are enlarged to the right, with $\times 40$ magnification of frontal-sectioned LV wall below. RA, right atrium; LA left atrium. Smallest units on scale are 0.01 mm. Images are representative of five mice per genotype. (B) Cardiomyocyte cross-sectional area measured by wheat germ agglutinin (WGA; green) staining of LV sections counterstained with anti–myosin heavy chain (MYH6/7; red). Scale bar, 50 μm . (C) TUNEL assays of same hearts. Scale bar, 50 μm . (D) Cardiomyocyte proliferation assayed by anti-pHH3 labeling. (Left) Individually imaged $\times 60$ confocal sections for anti-pHH3 (red), nuclear 4',6-diamidino-2-phenylindole (DAPI) stain (blue), and merged panels; mitotic figures in DAPI-stained sections are selectively stained by anti-pHH3. Scale bar, 10 μm . (Right) Low-power image of pHH3-

stained LV apex of DCKO heart; arrows indicate pHH3-positive nuclei counterstained with blue DAPI. Scale bar, 50 μ m. Group data are to the right, $n = 4$ mice per genotype and treatment. * $P < 0.05$ compared to WT.

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